Behavioral Tests After Intracerebral Hemorrhage in the Rat

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Background and Purpose—In humans, intracerebral hemorrhage (ICH) causes marked perihematomal edema formation and neurological deficits. A rat ICH model, involving infusion of autologous blood into the caudate, has been used extensively to study mechanisms of edema formation, but an examination of behavioral outcome would improve its preclinical utility and provide a more rigorous assessment of the pathological cascade of events over time. The purpose of this study was to use a battery of sensorimotor function tests to examine the neurological effects of ICH in the rat and to examine which components of the hematoma are involved in generating those effects.

Methods—The behavioral tests used were forelimb placing, preference for forelimb use for weight shifts during vertical exploration of a cylindrical enclosure, and a corner turn test. Rats were tested from day 1 to day 28 after injection of autologous whole blood; injection of blood plus hirudin (thrombin inhibitor), packed red blood cells, thrombin, or saline; or needle placement only.

Results—The battery of tests indicated that there were marked neurological deficits by day 1 after ICH, with progressive recovery of function over 4 weeks. The forelimb placing score paralleled changes in edema. Injection of thrombin caused and injection of hirudin reduced the ICH-induced neurological deficits. Injection of packed red blood cells, which causes delayed edema formation, induced delayed neurological deficits.

Conclusions—These tests allow continuous monitoring of neurological deficits after rat ICH and assessment of therapeutic interventions. The time course of the neurological deficit closely matched the time course of cerebral edema for both ICH and injection of blood components. There was marked recovery of function after ICH, which may be amenable to therapeutic manipulation. (Stroke. 2002;33:2478-2484.)

Key Words: behavior, animal brain edema cerebral hemorrhage erythrocytes thrombin rats

Spontaneous intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype. If the patient survives the ictus, the resulting hematoma within brain parenchyma triggers a series of events leading to secondary insults and severe neurological deficits.1 To understand the underlying mechanisms of ICH-induced brain injury and to evaluate therapeutic interventions, a number of animal models of ICH have been developed.2–5 A reproducible rat ICH model, involving infusion of autologous blood into the caudate, has been used extensively to study mechanisms of brain injury and, in particular, edema formation.4,6–9 The extent to which behavioral deficits occur in this model has not been characterized. The inclusion of behavioral end points in experimental stroke studies represents an important step forward because in order to be optimal clinically, a potentially therapeutic compound should maintain or restore function after stroke.

Although the mechanisms of brain injury after ICH are not fully understood, several mechanisms appear to contribute to edema development. Our previous studies have demonstrated that the coagulation cascade, especially thrombin production, plays a major role in early edema formation, and erythrocyte lysis and hemoglobin toxicity contribute to delayed edema development.4,5,10–12 Whether these blood components are also responsible for inducing behavioral deficits is unknown.

A battery of tests for assessing acute and chronic changes in sensorimotor function and plasticity have been devised for rat models of unilateral brain injury such as cerebral ischemia.13 These tests have been useful in studies of recovery of function after injury to sensorimotor regions of the central nervous system and have been used to assess the efficacy of behavioral or pharmacological interventions.13–17

The purpose of this study was therefore to use these behavioral tests to examine the neurological effects of ICH in the rat, to determine the role of different blood components in eliciting such effects, and to examine the relationship among neurological outcome, brain edema, and histological changes in this model.

Materials and Methods

Animal Preparation and Intracerebral Infusion

Animal use protocols were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 83 male rats...
Experimental Groups
These experiments were divided into 5 parts. In part 1, rats received either needle insertion (sham, n = 10) or infusion of 100 μL autologous whole blood (n = 12). In part 2, rats received an infusion of either 50 μL saline (n = 6) or 5 μL thrombin in 50 μL saline (n = 6). In part 3, rats received an infusion of either 100 μL blood+5 μL saline (n = 5) or 100 μL blood+5 μL hirudin, a thrombin inhibitor (n = 5). In the first 3 parts, the rats were tested for behavior at days 1, 3, 7, 14, 21, and 28 and then were killed for histological examination.
In part 4, rats received either a needle insertion (n = 9) or 50 μL packed RBCs (n = 10). Behavioral tests were performed at days 1, 3, 5, 7, 14, and 28, and then the rats were killed for histological examination. In part 5, 4 groups of 5 rats were used. All rats had an infusion of 100 μL autologous whole blood and were killed at 1, 3, 7, and 14 days for water content measurements.

Behavioral Tests
Three behavioral tests were used: a forelimb placing test, a forelimb use asymmetry (cylinder) test, and a corner turn test. The advantages of these tests in cerebral ischemia have been reviewed by Schallert et al.13

Measurement of Forelimb Placing
The first behavioral test was a vibrissae-elicited forelimb placing test (Figure 1). Animals were held by their torsos, which allowed the forelimb to hang free. The animal was gently moved up and down before the placing testing to facilitate muscle relaxation and eliminate any struggling movement. Trials during which extreme muscle tension, struggling, or placing of any of the limbs onto the experiment’s hand occurred were not counted. Independent testing of each forelimb was induced by brushing the respective vibrissae on the corner edge of a countertop. Intact animals place the forelimb ipsilateral to the stimulated vibrissae quickly onto the countertop. Depending on the extent of injury, placing of the forelimb contralateral to the injury in response to contralateral vibrissae contact with the countertop may be impaired. In the experiments each rat was tested 10 times for each forelimb, and the percentage of trials in which the rat placed the appropriate forelimb on the edge of the countertop in response to the vibrissae stimulation was determined. Testers were highly experienced and blind to the condition of the animal.

Forelimb Use Asymmetry Test
Forelimb tests during exploratory activity was analyzed by videotaping rats in a transparent cylinder (20 cm in diameter and 30 cm in height) for 3 to 10 minutes depending on the degree of activity during the trial (Figure 1). A mirror was placed to the side of the cylinder at an angle to enable the recording of forelimb movements even when the animal was turned away from the camera. Scoring was done by an experimenter blinded to the condition of the animal using a video cassette recorder with slow-motion and clear stop-frame capabilities. The behavior was scored according to the following criteria: (1) independent use of the left or right forelimb for contacting the wall during a full rear to initiate a weight-shifting movement or to regain center of gravity while moving laterally in a vertical posture and (2) simultaneous use of both the left and right forelimbs for contacting the cylinder wall during a full rear and for alternating lateral stepping movements along the wall.

Behavior was quantified by determining the occasions when the unimpaired (ipsilateral) forelimb was used as a percentage of total number of limb use observations on the wall (I); the occasions when the impaired forelimb (contralateral to the blood injection site) was used as a percentage of total number of limb use observations on the wall (C); and the occasions when both forelimbs were used simultaneously (or nearly simultaneously during lateral side-stepping movements) as a percentage of total number of limb use observations on the wall (B). A single overall limb use asymmetry score was calculated as follows: Limb Use Asymmetry Score = [I/(I+C+B)]−[C/(1+C+B)].

Corner Turn Test
The rat was allowed to proceed into a corner, the angle of which was 30 degrees. To exit the corner, the rat could turn either to the left or to the right, and this was recorded. This was repeated 10 to 15 times, with at least 30 seconds between trials, and the percentage of right turns was calculated.14 Only turns involving full rearing along either wall were included (ie, ventral tucks or horizontal turns were excluded). The rats were not picked up immediately after each turn so that they did not develop an aversion for their prepotent turning response.

Brain Water Content
Animals were decapitated under pentobarbital anesthesia (60 mg/kg). The brains were removed, and a coronal brain slice (approximately 3 mm thick) 4 mm from the frontal pole was cut with a blade. The brain slice was divided into 2 hemispheres along the midline; each hemisphere was dissected into the cortex and the striatum. The cerebellum was also detached to serve as a control. Thus, a total of 5 samples from each brain were obtained: the ipsilateral and the contralateral cortex, the ipsilateral and the contralateral striatum, and the cerebellum. Brain samples were immediately weighed on an electronic analytical balance (model AE 100, Mettler Instrument Co) to obtain the wet weight. Brain samples were then dried at 100°C for 24 hours to obtain the dry weight. The formula for calculation was as follows: (Wet Weight−Dry Weight)/Wet Weight.

Histology
Four weeks after ICH, rats were anesthetized with pentobarbital (60 mg/kg IP) and perfused with 4% paraformaldehyde in 0.1 mol/L PBS, pH 7.4. Removed brains were kept in 4% paraformaldehyde for 6 hours, then immersed in 25% sucrose for 3 to 4 days at 4°C. The brains were embedded in O.C.T. compound (Sakura Finetek U.S.A.,
Inc) and sectioned on a cryostat (18 μm thick). Hematoxylin and eosin staining was used for histological examination.

Statistical Analysis
Mann-Whitney U tests were used to compare behavioral data. ANOVA was used to compare water content data. Linear correlation analysis was performed to examine the relationship between behavioral deficits and brain edema. Values are mean±SEM. Statistical significance was set at \( P<0.05 \).

Results
Physiological parameters, including mean blood pressure, blood pH, blood gases, hematocrit, and blood glucose, were recorded and were within normal ranges. Forelimb placing, forelimb use asymmetry, and corner turn were scored by investigators (Y.H. and T.S.) who were blind to both neurological and treatment conditions (Figure 1).

Behavioral Changes After ICH
Animals that received an intracerebral infusion of autologous whole blood (100 μL) had significant forelimb placing deficits compared with controls from days 1 through 28 (Figure 2). The blood-induced forelimb placing deficits were most severe on days 1 and 3, with some recovery from day 7 and >50% recovery at 4 weeks (Figure 2A).

Compared with sham controls, blood-infused rats had marked forelimb use asymmetry in the first 2 weeks. However, there was again recovery from this deficit with time. The forelimb use asymmetry score was not significantly different from shams by day 21 (Figure 2B). For the corner turn test, the ICH group showed a significant increase in the percentage of right (ipsiversive) turns compared with sham controls at 2 weeks (Figure 2C).

Hematoxylin and eosin staining was performed to examine brain histological changes 4 weeks after ICH. By that time, the hematoma was replaced by a fibroglial cavity. Hematoidin, which consists of bilirubin, was found in the cavity. As described above, neurological functions (forelimb placing, forelimb use asymmetry, and corner turn) in most rats were recovered substantially or completely by that time. In 1 rat, however, there was no recovery of neurological function even at day 28 (forelimb placing score 0, forelimb use asymmetry score 55%, corner turn score 100%). That rat was found to have hydropschrosis and a severe injury to the ipsilateral cortex (Figure 3).

There was a temporal relationship between the ICH-induced forelimb placing deficits and ICH-induced edema. Forelimb placing deficits peaked at day 3 after ICH and almost recovered by day 14. Perihematomal brain edema also peaked at day 3 and resolved by day 14 (Figure 4). There was a negative correlation between forelimb placing score and brain edema \((y = -9.6x + 817; R^2=0.96, P<0.05)\). Although there was a tendency toward a correlation between forelimb use asymmetry, corner turn score, and brain edema, it did not reach a significant level.

Role of Blood Components in ICH-Induced Behavioral Changes
Intracaudate infusion of thrombin caused severe forelimb placing deficits with only a slow recovery after 2 weeks. Injection of saline also caused a slight forelimb placing deficit on day 1, but there was full recovery by 3 days (Figure 5A).

Thrombin injection also resulted in significant forelimb use asymmetry; this was present on day 1 and lasted at least 4 weeks. There were significant differences between thrombin and saline groups in this parameter at all time.
points (Figure 5B). With the corner test, the thrombin-treated rats showed an increased percentage of right (ipsiversive) turns compared with saline-treated rats at all time points (Figure 5C).

Hirudin, a thrombin inhibitor, improved ICH-induced forelimb placing (Figure 6A). Forelimb use asymmetry was also improved in the hirudin-treated group at days 1 and 3 (Figure 6B). There were, however, no significant differences in the corner test between the hirudin- and vehicle-treated ICH groups (Figure 6C).

There was a tendency for rats that received an injection of 50 μL packed RBCs to show forelimb placing deficits on days 1 and 3, but this did not reach significance (Figure 7A). There were, however, significant forelimb placing deficits at days 5 (34% versus 100% in sham control; \( P<0.05 \)) and 7 (36% versus 100% in sham control; \( P<0.05 \)). The forelimb placing function was recovered within 4 weeks (Figure 7A). Compared with sham control, there was no significant difference between the packed RBC group and sham control on forelimb use asymmetry (Figure 7B). However, the injection of packed RBCs caused a significant increase in the percentage of turns to the right in the corner test on day 3 (95±5% versus 48±6% in sham; \( P<0.01 \)) and day 7 (95±5% versus 63±9% in sham; \( P<0.05 \)).
These sensorimotor behavioral tests were also useful for examining recovery of function after ICH. Both the forelimb placing score and the forelimb use asymmetry score showed marked improvements with time (the results of the corner test showed improvement to a lesser extent). Most attention in animal models of ICH has focused on acute brain injury and its prevention. Relatively little attention has focused on recovery of function after injury and whether it is a therapeutic target. Evidence from models of cerebral ischemia suggests that it is a potential target.13 The extent to which this recovery of function after ICH reflects the resumption of normal function by ipsilateral neurons or the assumption of new functions by ipsilateral or contralateral neurons or neurogenesis is unknown.

With a different set of tests (spontaneous circling, beam walking, and forelimb extension), Peeling et al20 also found a marked neurological deficit after 1 day in a collagenase model of ICH and a recovery over 2 to 3 weeks. That model differs from the model used in the present study in that the collagenase disrupts the vasculature to induce ICH rather than direct blood injection. Our present results indicate that behavioral deficits can be induced by the presence of blood in the brain parenchyma even in the absence of direct vascular rupture.

The present study demonstrates a close temporal relationship between brain edema and forelimb placing score after ICH. In animal models, perihematomal edema increases in several hours and peaks around the third or fourth day after the hemorrhage, then declines gradually, resolving within approximately 2 weeks.4,6,21,22 This was a pattern very similar to the change in forelimb placing deficit, although there was still some residual deficit after 2 weeks (Figure 6). This temporal relationship suggests that these behavioral tests are a good surrogate measure for other ICH-induced brain injury markers, and, unlike the brain edema measurement and a number of other injury markers (eg, terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling [TUNEL] staining), the behavioral tests can be used repetitively on 1 rat. The relationship between brain edema and forelimb placing also suggests that there is a causal link between the 2 or that they are both the result of a common cause. Thus, the swelling induced by the increased water content may physically disrupt neuronal communication, or the behavioral and water content changes may be independently driven by the same cause: a disruption in cellular ion gradients or intracranial pressure.

Although hematoma in humans gradually resolves within several months, restoration of function is graded and usually incomplete. The neurological deficits in ICH patients are permanent and disabling. Except for brain edema in the acute phase, it is difficult to quantify the extent of brain injury after ICH in the rat because only a small cavity can be seen after the clot is absorbed. Therefore, neurological deficits are useful end points for ICH study. A good behavioral test should be able to assess the degree of damage, the process of recovery, and the residual impairment.23 In our rat ICH model, the clot is absorbed in several weeks, but there is evidence of chronic impairment. The utility of this prolonged
neurological deficit as a therapeutic end point for ICH needs to be examined further.

**Blood Components in ICH-Induced Behavioral Changes**

Brain edema after ICH exacerbates brain injury. An important aspect of ICH management is how to prevent edema formation since brain swelling may have a greater influence on poor neurological outcome after ICH than the hematoma mass itself. Edema formation after ICH may involve several phases. These include a very early phase (first several hours) involving hydrostatic pressure, clot retraction, and transient ischemia around the clot, a second phase (first day) involving the clotting cascade and thrombin production, and a third phase (approximately day 3 in the rat) involving erythrocyte lysis and hemoglobin toxicity. Activation of the complement system in brain parenchyma also plays an important role in the second and third phases. The results from the present study suggest that thrombin formation and erythrocyte lysis contribute to neurological dysfunction in ICH, although other factors may also contribute. Thrombin, as part of the coagulation cascade, is produced immediately in the brain after ICH. Direct infusion of large doses of thrombin into brain causes inflammatory cell infiltration, mesenchymal cell proliferation, scar formation, and seizures.5,10,11,24,25 Thrombin, at high concentrations, also kills neurons and astrocytes in vitro.26–28 Our present results demonstrate that direct injection of thrombin into the brain also causes marked sensorimotor deficits.

Because thrombin can be injurious at high concentrations and protective at low concentrations, it is important to know what concentrations of thrombin may occur in the brain after ICH. The concentration of prothrombin in the plasma is high enough (1 to 5 μmol/L) to produce a substantial amount of thrombin in the brain parenchyma after a hemorrhage. Thus, 1 mL of whole blood can produce approximately 260 to 360 U of thrombin, and a 100-μL clot could be expected to produce up to approximately 30 U of thrombin. In the rat, the brain edema produced by intracerebral hematoma can be significantly inhibited by thrombin inhibitors, and an intracerebral infusion of 5 U of thrombin causes a similar degree of edema. Therefore, this dose of thrombin was used in the present experiments.

Hirudin, a specific thrombin inhibitor, was used to examine whether thrombin contributes to neurological deficits in ICH. Animals treated with hirudin showed significant improvement in the forelimb placing test and in forelimb use asymmetry in the cylinder test compared with the vehicle treatment in ICH, indicating that thrombin plays an important role in neurological dysfunction after ICH. This improvement may, at least in part, be related to reduced ICH-induced edema formation in hirudin-treated rats. However, hirudin also reduced the chronic neurological deficits measured at 4 weeks, suggesting that its protective effect is not related solely to reduced edema. Thrombin has been implicated in the induction of apoptosis and other forms of cell death. Although hirudin treatment improved forelimb placing and forelimb use asymmetry scores after ICH, hirudin did not significantly improve results from the corner test. This may reflect a difference in test sensitivity. In particular, the corner test may be more sensitive to injury because it reflects multiple asymmetries, including postural, vibrissae sensory, and forelimb and hindlimb use asymmetries, which all combine at nearly the same time to bias the turning. There is evidence for such differential sensitivity in our ICH data. Thus, for example, early after ICH, the corner test score was 100%, indicating the rats only turned in 1 direction (a “maximal” deficit), but there was still use of the impaired limb in the asymmetry test. Given that there appear to be multiple mechanisms by which ICH can induce behavioral deficits (this study demonstrates that both thrombin and erythrocyte lysate are at least potential mechanisms), it is possible that the increased sensitivity of the corner test will require multiple mechanisms to be inhibited before an improvement is shown. This raises an important question regarding the appropriate tests for any preclinical trials. At present, a combination of tests appears appropriate to determine whether there is any improvement.

Injection of packed RBCs into the brain does not cause edema in the first 2 days. However, there is delayed edema formation that appears to be related to erythrocyte lysis and the release of hemoglobin and its degradative products.4,12 Our present behavioral results suggest that there is also a delayed neurological deficit after RBC injection.

The mechanisms that might contribute to neurological deficits after erythrocyte lysis are disruption of blood-brain barrier and direct damage to neurons and astrocytes. Intracerebral infusion of RBC hemolysate induces marked brain edema and blood-brain barrier disruption without inducing ischemic blood flow.30 Intracerebral infusion of lysed blood also induces strong expressions of heat shock protein 70, a neuronal injury marker, and causes cytochrome C release and DNA fragmentation.31,32 Hemoglobin has deleterious effects on the brain. For example, an intracortical hemoglobin injection in rats produces chronic focal spike activity and gliosis.33 Hemoglobin inhibits Na+/K+-ATPase activity,34 activates lipid peroxidation,35 exacerbates excitotoxic injury in cortical cell culture,36 and induces depolarization in hippocampal CA1 neurons.37 In addition, exposure of cultured rat spinal cord cells to hemoglobin produces concentration-dependent cell toxicity.38

In conclusion, these behavioral tests allow continuous monitoring of neurological deficits after ICH in the rat. The time course of the neurological deficit closely matches the time course of cerebral edema for both ICH and injection of blood components. These and other sensorimotor behavioral tests appear to be useful for evaluating extent of brain injury and the potential efficacy of therapeutic interventions in ICH.

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**References**


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