Role of Blood Clot Formation on Early Edema Development After Experimental Intracerebral Hemorrhage

Guohua Xi, MD; Kenneth R. Wagner, PhD; Richard F. Keep, PhD; Ya Hua, MD; Gabrielle M. de Courten-Myers, MD; Joseph P. Broderick, MD; Thomas G. Brott, MD; Julian T. Hoff, MD

Background and Purpose—Blood “toxicity” is hypothesized to induce edema and brain tissue injury following intracerebral hemorrhage (ICH). Lobar ICH in pigs produces rapidly developing, marked perihematomal edema (>10% increase in water content) associated with clot-derived plasma protein accumulation. Coagulation cascade activation and, specifically, thrombin itself contribute to edema development during the first 24 hours after gray matter ICH in rats. In the present study, we sought to determine whether blood clot formation is necessary for edema development by comparing intracerebral infusions of heparinized and unheparinized blood in pig (white matter) and in rat (gray matter). We also examined heparin’s effect on thrombin-induced gray matter edema.

Methods—In pigs, we infused autologous blood (with or without heparin) into the cerebral white matter to produce lobar hematomas and froze the brains in situ at 1, 4, or 24 hours after ICH. We determined hematamal and perihematomal edema volumes on coronal sections by computer-assisted morphometry. In rats, we infused either blood or thrombin (with or without heparin) into the basal ganglia and measured water, sodium, and potassium contents at 24 hours after ICH.

Results—In pigs, unheparinized blood induced rapid (at 1 hour) and prolonged (24 hours) perihematomal edema (average volume, 1.29±0.20 mL; n=6). No perihematomal edema was present following heparinized blood infusions (n=6). In rats, unheparinized blood produced significantly greater edema than heparinized blood infusions. As with whole blood, thrombin-induced gray matter edema at 24 hours was significantly reduced by coinjection of heparin.

Conclusions—After ICH, blood clot formation is required for rapid and prolonged edema development in perihematomal white and gray matter. Thrombin also contributes to prolonged edema in gray matter.

Key Words: brain edema ■ cerebral hemorrhage ■ heparinized blood ■ pigs ■ rats

Edema development after ICH can elevate intracranial pressure and cause herniation, brain stem compression, and death. Clinical studies demonstrate that the peak of ICH-induced deaths occurs within the first few days following ictus and is likely to be associated with progressive edema development. Thus, it is important to define the pathophysiological mechanisms underlying edema development so that therapies can be instituted to interrupt its formation and thereby prevent ICH-induced deaths and improve patient outcome.

The concept that intracerebral blood is itself “toxic” independent of its mass effect was first demonstrated by Suzuki and Ebina, who compared intracerebral injections of whole blood with similar infusions of an inert oil-and-wax mixture. They found that blood produced considerably greater histological changes indicative of edema over the first 24 hours compared with the inert substance. On the contrary, mass effect alone, as produced by microballoon inflation, fails to produce edema even though CBF is reduced to <20 mL/100 g/min.

Recently, new details of the pathophysiological mechanisms involved in edema formation after ICH and the contributions of individual components to the “toxicity” of blood have been demonstrated. Findings by the University of Cincinnati group in a pig ICH model provide strong support for several early events in perihematomal edema development. These include (1) clot retraction with decreasing clot volumes and increasing perihematomal edema volumes dur-

See Editorial Comment, page 2586
ing the first 4 hours after ICH; (2) plasma protein extravasa-
tion that acts oncotically to induce rapid (already at 1 hour) peri-
hematomal edema development; and (3) marked perihe-
matomal immunoreactivity for fibrinogen, suggesting the
presence of extravascular coagulation and fibrin deposition.
Studies of edema development in gray matter in the rat by the
University of Michigan group\textsuperscript{6-12} have defined the major role
of the coagulation cascade and thrombin itself in edema
development during the first 24 hours after ICH. Further-
more, the significance of thrombin in this early edema
formation is substantiated by the ability of thrombin inhibi-
tors to reduce edema. Other recent findings\textsuperscript{10,13} also demon-
strate that intracerebral infusions of packed red blood cells
fail to produce early perihematomal edema.

To further define the importance of blood clotting on early
edema development after ICH, we compared heparinized
with unheparinized whole-blood infusions in the 2 animal
models. Preliminary results from these studies have been
presented.\textsuperscript{14}

\section*{Materials and Methods}

\subsection*{Studies in Pigs}

The protocol for these animal studies was approved by The Cincin-
nati Veterans Affairs Medical Center and University of Cincinnati
Institutional Animal Care and Use Committees. Pigs in both the
heparinized and unheparinized blood infusion groups, although not
strictly randomized, were not studied sequentially. Rather, these
experiments were conducted over a several-month period, along with
other ongoing animal studies in the laboratory.

All surgical, blood infusion, and brain freezing methods in pigs
have been described previously in detail.\textsuperscript{6} Briefly, pigs (\textasciitilde 10 kg)
were anesthetized with ketamine (25 to 30 mg/kg IM). After
sedation, pentobarbital (35 mg/kg IV) was administered to achieve
deep surgical levels. Anesthesia was then maintained by continuous
pentobarbital infusion (10 mg/kg per hour). A tracheotomy was
performed, and the pigs were intubated and mechanically ventilated
with air (oxygen added at 0.5 L/min). Femoral vessels were
catheterized: arteries to record blood pressure and measure arterial
acid-base status, respiratory gases, and glucose; veins to infuse saline
and drugs. Core temperatures (38.5\(\pm\)0.5\(^\circ\)C) were monitored and
controlled. Hematomas were induced by slowly (over a 15-minute
period) infusing 2.5 mL autologous blood through a 20-gauge Teflon
catheter into the frontal cerebral white matter. Silastic tubing was
used to prevent blood clotting. For heparinized blood infusions,
autologous blood (3.5 mL) was withdrawn into a syringe that had
been coated with heparin (approximately 300 U), and 2.5 mL were
infused into the frontal white matter. To test blood-brain barrier
(BBB) permeability to albumin-bound Evans blue dye, we infused
Evans blue intravenously (1 mL/kg of a 2\% wt/vol solution)

\begin{itemize}
\item immediately after completion of the intracerebral blood infusions.\textsuperscript{5}
\end{itemize}

In pigs killed at 24 hours, the pentobarbital infusion was stopped,
the endotracheal tube and the femoral artery catheter were removed,
the femoral vein catheter was left in place, and the incisions were
closed with silk sutures after 4 hours following blood infusions. Pigs
were cared for in an animal intensive-care unit and were noted to
achieve sternal recumbency within a few hours after the pentobar-
bital infusion was stopped. All pigs were awake and ambulatory the
next morning. At 24 hours after blood infusion, the pigs were
reanesthetized as described above with ketamine (intramuscular) and
pentobarbital (through their indwelling femoral vein catheter). They
were prepared for in situ brain freezing as described below.

Brains were frozen in situ with liquid nitrogen at 1, 4, or 24 hours
after hematoma induction, as previously described.\textsuperscript{15} Coronal sec-
tions were cut with a band saw, and both sides of the coronal sections
containing hematomas and/or edema were photographed together
with a millimeter ruler. Prints (8\(\times\)10 inches) were prepared from
2\(\times\)2-inch photographic slides.

Hematomas and visible perihematomal edema volumes were
determined by 1 of the following 2 methods: (1) by outlining the
hematomas and visible perihematomal edema on the photographs
while directly viewing the frozen slices and then determining the
outlined areas by computer-assisted morphometry (Bioquant or NIH
Image) or (2) by importing the color images from 2\(\times\)2-inch color
slides (input via Nikon Slide Scanner) and determining hematoma
and edema areas by computer-assisted morphometry with use of
Image Tool, a freeware image analysis system developed at the
University of Texas Health Science Center at San Antonio. Areas
determined by Image Tool were identical to those determined with
NIH Image. For both methods, areas were corrected for image sizes
from the millimeter ruler, the hematoma and edema areas from the 2
sides of each slice were averaged, and the values were multiplied by
the slice thickness to calculate volumes and the volumes summed to
calculate the total hematoma and edema volumes for each brain. The
individual performing the hematoma and edema volume measure-
ments was blinded to the blood-infusion group of the animal being
studied.

\subsection*{Studies in Rats}

The protocol for these animal studies was approved by The Univer-
sity of Michigan Committee on the Use and Care of Animals. Thirty
adult male Sprague-Dawley rats (Charles River Laboratories, Por-
tage, Mich), weighing between 300 and 400 g, were used in this
study. They were anesthetized with an intraperitoneal injection of
pentobarbital (40 mg/kg). A polyethylene catheter (PE-50) was
inserted into the right femoral artery for continuous blood pressure
monitoring and blood sampling. Arterial blood was obtained for
analysis of blood pH, PO\textsubscript{2}, P CO\textsubscript{2}, hematocrit, and blood glucose and
as a source of blood for intracerebral infusion. Body temperature was
maintained at 37.5\(^\circ\)C using a feedback-controlled heating pad.

The rats were positioned in a stereotactic frame (Kopf Instrument),
and a cranial burr hole (1 mm) was drilled near the right coronal
suture, 4.0 mm lateral to the midline. A needle (26-gauge) was
inserted stereotaxically into the right basal ganglia (coordinates:
0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma).
Whole blood, heparinized blood and saline were infused at a rate of
10 \(\mu\)L/min into right basal ganglia with use of a microinfusion pump
(Harvard Apparatus Inc.). After infusion, the needle was removed
and the skin incisions were closed with sutures. Animals were
allowed to recover.

The experiments in rat were divided into 2 parts. Part 1: Three
groups of 5 rats each were investigated in this part. The first group
received a 50-\(\mu\)L saline infusion. The second group was infused with
50 \(\mu\)L autologous heparinized blood (2.5 U heparin). The third group
had 50 \(\mu\)L autologous whole blood. Part 2: Three groups with 5
animals each received a 60-\(\mu\)L infusion of either saline or thrombin
(5 U, Sigma Chemical Co) or thrombin (5 U) plus heparin (5 U) into
the right basal ganglia. All animals were decapitated at 24 hours.

The rats were decapitated after deep anesthesia (80 mg/kg IP
pentobarbital). The brains were removed. A coronal brain slice 4 mm
from the frontal pole was cut approximately 3 mm thick. Five tissue
samples (ipsilateral cortex and basal ganglia, contralateral cortex and
basal ganglia, and cerebellum) were weighed on an electronic
analytical balance (model AE 100, Mettler Instrument Co) to obtain
the wet weight (WW). The tissue was then dried in a gravity oven at
100\(^\circ\)C for 24 hours to obtain the dry weight (DW). Water contents
were calculated as follows: Water content (\%) \(=\) \((\text{WW-DW})/\text{WW}\) *
100.

The dehydrated brain samples were digested in 1 mL of 1 N nitric
acid for 1 week. The sodium and potassium ion contents were
measured by flame photometry. Ion contents were expressed in
milliequivalents per kilogram of dehydrated brain tissue.

\subsection*{Statistical Analysis}

All data in the figures and tables are presented as mean\(\pm\)SD. Data
from different animal groups were analyzed with ANOVA with a
TABLE 1. Physiological Parameters in Pigs Receiving Intracerebral Blood Infusions

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unheparinized Blood</th>
<th>Heparinized Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP, mm Hg</td>
<td>95±8</td>
<td>99±17</td>
</tr>
<tr>
<td>pH</td>
<td>7.49±0.05</td>
<td>7.35±0.03</td>
</tr>
<tr>
<td>$\text{Paco}_2$, mm Hg</td>
<td>129±11</td>
<td>149±17</td>
</tr>
<tr>
<td>$\text{PaO}_2$, mm Hg</td>
<td>40±3</td>
<td>41±1</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>29±3</td>
<td>31±4</td>
</tr>
<tr>
<td>Plasma glucose, mM</td>
<td>8.0±1.2</td>
<td>8.2±1.0</td>
</tr>
<tr>
<td>Core temperature, °C</td>
<td>38.6±0.2</td>
<td>38.4±0.4</td>
</tr>
</tbody>
</table>

Values are mean±SD; n=6 pigs in the unheparinized group and n=6 pigs in the heparinized group.

Scheffé F test or Student t test. The differences were considered significant at P<0.05.

Results

Pig ICH Studies: Unheparinized Versus Heparinized Blood Infusions
A total of 13 pigs were subjected to heparinized blood infusions, and a successful hematoma was obtained in 6 (n=1 at 1 hour; n=2 at 4 hours; n=3 at 24 hours). In the other 7 animals (n=1 at 1 hour; n=3 at 4 hours; n=3 at 24 hours), either no hematoma was present (n=2) or the hematoma volumes were markedly smaller than the unheparinized blood infusions (n=5), suggesting significant blood loss due to lack of clotting (average hematoma volume in these pigs was 0.25±0.12 mL). Because hematoma induction with heparinized blood infusions was successful in only 46% of the attempts and in this group none (0/6) of the brains developed perihematomal edema that lead to a change in the physical appearance in tissue (“translucency”), whereas in contrast all (6/6) of the unheparinized blood infused brains did so, we chose to average the volumes from the successful animals at the 3 time points (1, 4, and 24 hours). These results were compared with an identical number of pigs at these time points that received unheparinized blood infusions. The data presented in Table 1 demonstrate no differences in physiological variables between the experimental groups.

The photographs of the coronal brain sections in Figure 1 and the quantitation of the hematoma and edema volumes in Figure 2, demonstrate significantly (P=0.01) smaller (~60%) hematoma volumes in the unheparinized blood infusion group compared with brains that received heparin-

Figure 1. A and B, Representative coronal sections from pig brains frozen in situ at 4 hours (A) and 24 hours (B) after unheparinized blood infusions into the frontal white matter. These sections demonstrate areas of markedly edematous white matter (see asterisk) adjacent to hematomas that are visibly changed. BBB opening that develops during the first 24 hours after infusion is evidenced by Evans blue staining (B). C and D, Representative coronal sections at frontal pole and caudate levels from brains frozen in situ at 4 hours (C) and 24 hours (D) after heparinized blood infusions demonstrating hematomas and “layering” of plasma (see arrows) above the settled red blood cells. Marked edema is not present in white matter adjacent to the hematomas.
ized blood infusions. Furthermore, while perihematomal edema volume in the unheparinized–infused brains averaged 1.29 mL (Figure 2), no perihematomal edema was present in the brains of pigs receiving heparinized blood infusions (not detectable [ND] in Figure 2). Thus, unheparinized blood that was capable of clotting produced perihematomal edema and significantly smaller hematoma volumes compared with infusions of heparinized blood.

**Rat ICH Studies**

Table 2 presents the physiological parameters in 6 animal groups. All physiological variables were within normal ranges.

**Unheparinized Versus Heparinized Blood Infusions**

The infusions of heparinized blood produced slight brain edema, but the unheparinized blood induced marked water-content increase in the ipsilateral basal ganglia (Figure 3). The water content increase at 24 hours after blood infusion was associated with accumulation of sodium ions. There was no significant potassium loss at this time point (Figure 4).

**Thrombin Versus Thrombin Plus Heparin Infusions**

Figure 5 shows the brain water content after infusions of saline, thrombin (5 U), and thrombin (5 U) plus heparin (5 U). The thrombin-induced brain edema, sodium ion accumulation, and potassium loss in ipsilateral cortex and basal ganglia were significantly reduced by heparin (Figures 5 and 6).

### Table 2: Physiological Parameters in Rats

<table>
<thead>
<tr>
<th>Variable</th>
<th>Saline, 50 μL</th>
<th>H-Blood, 50 μL</th>
<th>Blood, 50 μL</th>
<th>Saline, 60 μL</th>
<th>Thrombin + Saline</th>
<th>Thrombin + Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP, mm Hg</td>
<td>115±13</td>
<td>113±4</td>
<td>106±11</td>
<td>109±10</td>
<td>110±6</td>
<td>98±3†</td>
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<tr>
<td>pH</td>
<td>7.43±0.02</td>
<td>7.41±0.02</td>
<td>7.44±0.03</td>
<td>7.42±0.01</td>
<td>7.44±0.03</td>
<td>7.45±0.03</td>
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<tr>
<td>PaO₂, mm Hg</td>
<td>85±3</td>
<td>82±3</td>
<td>84±6</td>
<td>81±2</td>
<td>84±5</td>
<td>84±4</td>
</tr>
<tr>
<td>PaCO₂, mm Hg</td>
<td>46±2</td>
<td>47±4</td>
<td>45±3</td>
<td>47±2</td>
<td>45±5</td>
<td>43±4</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>42±1</td>
<td>42±1</td>
<td>40±1*</td>
<td>40±1</td>
<td>39±1</td>
<td>40±1</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>8.1±0.1</td>
<td>7.1±0.1</td>
<td>6.9±0.1</td>
<td>7.0±0.1</td>
<td>7.2±0.1</td>
<td>7.2±0.1</td>
</tr>
</tbody>
</table>

Values are mean±SD; n=5. MABP indicates mean arterial blood pressure; H-Blood, heparinized blood.

*P<0.05 vs Saline 50 μL and H Blood; †P<0.05 vs Thrombin+Saline.

**Discussion**

Our present findings demonstrate that coagulation cascade activation, prothrombin conversion to thrombin, and resultant blood clot formation are fundamental steps in rapid and prolonged perihematomal edema development following ICH. This conclusion is supported by the finding that heparinized blood infusions into pig white matter fail to produce any visible perihematomal edema formation during the first 24 hours, which is in marked contrast to the extensive edema development following unheparinized blood infusions. Similarly, in rat gray matter, although water content was increased with heparinized blood infusions, these increases were significantly lower than with blood infusions without heparin. Thus, the failure of a hematoma containing heparinized blood to induce edema comparable to that with clottable blood, even when present for 24 hours, and the ability of heparin to reduce thrombin-mediated edema demonstrate the essential roles of blood clot formation and thrombin itself in perihematomal white and gray matter edema development during the first 24 hours after ICH.

The anticoagulant effect of heparin is due to its high-affinity binding with antithrombin III (AT III) and the ability of the heparin/AT III complex to inactivate thrombin and other coagulation factors. However, the fact that heparin also significantly reduced thrombin-induced edema in the absence of blood and AT III indicates that heparin may also have effects on edema formation not mediated by AT III. Tulinsky found that heparin can bind to the exosite, active
site, and fibrinogen recognition exosite of thrombin. In addition, heparin may potentiate the effect of serine protease inhibitors that naturally occur in brain. The ability of both protease nexin 1 and plasminogen activator inhibitor-1 to inhibit thrombin is increased in the presence of heparin. The findings of a blood-fluid level in pig brains with heparinized blood infusions confirm repeated findings on CT scans of ICH patients with coagulopathies that interfere with clot formation or cause lysis of the fibrin matrix. Similar blood-fluid levels on CT scans have also been described in patients receiving anticoagulation therapy. It is interesting that recent findings from a radiographic evaluation of symptomatic ICH complicating thrombolysis for acute myocardial infarction (GUSTO-I trial) demonstrate minimal perihematomal edema, particularly in patients with hematoma blood-fluid levels. In this trial, all patients received heparin in combination with the thrombolytic agent, which support the importance of blood clot formation in the evolution of early perihematomal edema.

Previously, we demonstrated that autologous blood infused into frontal cerebral white matter in pigs produces rapidly (at 1 hour), developing marked perihematomal edema (10% increases in water content). This edema colocalized with interstitial fibrin(ogen) and other plasma protein accumulations despite an intact BBB. The present findings support the conclusion that coagulation cascade activation leading to clot formation is necessary for this rapid edema development in perihematomal white matter after ICH.

The present results also further support the role for early surgical intervention for hematoma removal. The development of increased BBB permeability after a delay of several hours (Figure 1B) suggests that early intervention may also prevent this secondary pathophysiological event. In this regard, we have demonstrated that early clot aspiration following lysis with tissue plasminogen activator not only markedly reduced (>70%) edema development at 24 hours but also protected the BBB. The edema reduction and BBB protection with early clot removal using a fibrinolytic agent may be through "interstitial...

Figure 4. Brain sodium (A) and potassium ion (B) contents at 24 hours after infusion of 60 μL saline or heparinized blood or blood in rats. Values shown are mean±SD; n=5. *P<0.05 vs saline group; †P<0.01 vs saline group; ‡P<0.05 vs heparinized blood group.

Figure 5. Brain water content in rats 24 hours after infusion of 60 mL saline or thrombin−saline or thrombin−heparin in rats. Values shown are mean±SD. *P<0.01 vs saline group; †P<0.01 vs saline group; ‡P<0.05 vs thrombin−saline group; §P<0.05 vs saline and thrombin−heparin groups.

Figure 6. Brain sodium (A) and potassium ion (B) contents 24 hours after infusion of 60 μL saline or thrombin−saline or thrombin−heparin in rats. Values shown are mean±SD. *P<0.01 vs saline group; †P<0.01 vs saline group; ‡P<0.01 vs thrombin−saline group; §P<0.05 vs saline and thrombin−heparin groups.
fibrinolysis” and reduction of extracellular plasma proteins, including thrombin that may be retained within the fibrin mesh.26

The present paradigm of using intracerebral infusions of heparinized blood may also provide important information regarding the role of the coagulation cascade and thrombin on long-term neuropathological outcome. Thrombin infusion into rat caudate nucleus did cause inflammation, brain edema, reactive gliosis, and scar formation.9–12,27 We also demonstrated that at 3 days after lobar ICH, white matter edema is still present and associated with marked astrogliosis and reduced Luxol fast blue staining suggestive of demyelination.28 These results suggest that early and prolonged edema following ICH may contribute to the damage and the long-term morbidity that has been described in ICH patients. The role of early and prolonged edema in this white matter injury could be studied by examining the outcome after heparinized blood infusions.

In conclusion, blood clot formation is a mandatory step for rapid (at 1 hour) and prolonged (24 hours) edema in both white and gray matter after ICH. Furthermore, findings from these and other studies6–12 support the role of plasma proteins (especially thrombin), an activated coagulation cascade, clot retraction, and fibrin deposition in both rapid (at 1 hour) and prolonged (24 hours) edema development adjacent to clots in white and gray matter after ICH. Further studies are required to determine the relative contributions of these various factors and events in perihematomal edema formation following ICH.

Acknowledgments

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References

The experiments described in this study clearly show that blood clotting is necessary for edema formation with intracerebral hematomas. Considering that with intracerebral hematomas the edema may be more responsible for the poor outcome than the size of the hematoma itself, every bit of new insight into the pathophysiology of edema formation is welcome. Nevertheless, one wonders how we can use this information in practice, especially when clotting takes place so quickly. Moreover, I certainly do not have the impression that the prognosis is better for patients with preexistent spontaneous or iatrogenic disturbed clotting mechanism: there may indeed be less edema, but the average hematoma size is extraordinary (72 mL in the material of Gebel et al1). The role of ultra-early surgery remains controversial, but even in the NINDS t-PA study,2 in which only centers with emergency neurosurgical capabilities could participate, of the 22 patients who suffered in-hospital symptomatic intracerebral hemorrhage only 1 underwent surgical removal of the hematoma (and died). This does not bode well for patients who suffer their intracerebral hemorrhage outside a hospital with special interest in stroke!

J. Paul Muizelaar, MD, PhD, Guest Editor
Department of Neurosurgery
University of California, Davis
Sacramento, California

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