Intracerebral Hemorrhage
Effects of Aging on Brain Edema and Neurological Deficits
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Background and Purpose—Intracerebral hemorrhage (ICH) is mostly a disease of the elderly, but current experimental ICH models have primarily used young animals. Age is an important factor in other forms of brain injury, affecting microglia and astrocyte reactions and plasticity. Therefore, the present study investigated the effects of aging on brain injury after ICH.

Methods—Young and aged (3 and 18 months old, respectively) male Sprague-Dawley rats received an intracerebral infusion of 100 μL autologous blood. Age-related changes in brain swelling, glial reaction, stress protein (heat shock proteins [HSPs] 27 and 32), and neurological deficits were examined.

Results—Brain swelling was more severe in old rats compared with young rats at 3 days after ICH (P<0.05). There were also more severe neurological deficits in the older rats at 1 day after ICH, which persisted for the 4 weeks of monitoring (P<0.05). The older rats also had stronger microglial activation and a greater perihematomal induction of HSP-27 and HSP-32 (P<0.05). In contrast, there was a weaker astrocytic reaction to the hematoma.

Conclusions—ICH causes more severe brain swelling and neurological deficits in old rats. Clarification of the mechanisms of brain injury after ICH in the aging brain should help develop new therapeutic strategies for hemorrhagic brain injury.

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Key Words: aging • brain edema • behavior • cerebral hemorrhage • heat shock proteins • microglia

Intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype. ICH is mostly a disease of the elderly, but current experimental ICH models have primarily used young animals. Age is an important factor affecting brain injury in ischemic stroke in animals and humans. Whether age affects brain injury after ICH has not been systematically studied in an animal model.

The study examines the effects of aging on neurological deficits, brain swelling, and the levels of heat shock proteins (HSPs) after ICH. The development of behavioral assays for rodent models of ICH has allowed assessment of potential drug therapies, but they also allow the assessment of the impact of age on ICH-induced brain injury. Brain swelling after ICH is an important component of brain injury after ICH, causing herniation-related deaths and long-term neurological deficits.

HSPs are brain injury markers, some of which are induced after ICH. HSP-32, also called hemeoxygenase-1 (HO-1), catalyzes the rate-limiting step in heme degradation in brain. HO-1 is upregulated primarily in microglia, and HO-1 upregulation may cause brain injury by increasing cellular iron content.

In addition, this study examines the perihematomal reactions of microglia and astrocytes. Microglia are cells within the brain that are activated in response to injury. In normal brain, microglia are quiescent, but they are activated by injuries such as ischemic and hemorrhagic stroke. They become highly phagocytic and clear debris from the damaged site. However, microglia secrete many toxic materials such as free radicals, and they may participate in brain injury. Indeed, an inhibitor of microglial activation, tuftsin fragment 1 to 3, significantly reduces ICH-induced brain injury in mice.

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 80 male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) were used in this study. They were either 3 or 18 months old (n=40 per group). These age groups are henceforth called young and aged rats, respectively.

Animals were anesthetized with pentobarbital (40 mg/kg IP). The right femoral artery was catheterized for continuous blood pressure monitoring and blood sampling. Blood was obtained from the catheter for analysis of blood pH, PaO₂, PaCO₂, hematocrit, and blood glucose. Core temperature was maintained at 37°C with use of a feedback-controlled heating pad. Rats were positioned in a stereotactic frame (Kopf Instruments), and a cranial burr hole (1 mm) was drilled on the right coronal suture 3.5 mm lateral to the midline. All rats received an injection of 100 μL autologous whole blood into the right caudate nucleus at a rate of 10 μL per minute through a 26-gauge needle (coordinates 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to bregma) with the use of a microinfusion pump (Harvard Apparatus). The needle was removed, and the skin incision was closed with suture after infusion.

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Experimental Groups
These experiments were divided into 4 parts. All rats had an intracerebral infusion of 100 μL autologous whole blood. In the first part, 4 groups of aged and young rats (n = 7) were killed 1 or 3 days after blood injection for brain water content measurements. In the second part, aged and young rats (n = 8) underwent behavioral testing the day before ICH and at days 1, 3, 5, 7, 14, and 28 after ICH. In the third part, rats were killed 1, 3, 7, and 28 days after ICH for HSP27, HSP32, glial fibrillary acidic protein (GFAP), and OX42 immunohistochemistry (n = 4 each time point). In the last part, brains were sampled 1, 3, 7, and 28 days after ICH for HSP analysis using Western blot analysis

Brain Swelling
Animals were decapitated under pentobarbital anesthesia (60 mg/kg). The brains were removed, and a coronal brain slice (~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 (henceforth called the basal ganglia sample). 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 basal ganglia, and cerebellum. Brain samples were immediately weighed on an electronic analytical balance (model AE 140, Mettler) to obtain the wet weight. Brain samples were then dried at 100°C for 24 hours to obtain the dry weight and the tissue water content as wet weight minus dry weight.

Typically, brain swelling after ICH has been examined by measuring percentage water content [(water content x 100)/wet weight]. Contralateral brain and cerebellar water content was found to be less in aging rats than in younger rats. Therefore, instead we calculated tissue swelling. For each sample, the ratio of wet weight to dry weight was determined and the percentage swelling was calculated as the percentage change in this ratio between the ipsilateral and contralateral sides.

Behavioral Tests
For behavioral tests, all animals were tested before and after surgery and scored by experimenters who were blind to neurological conditions. The following 3 types of tests were used.

Forelimb-Placing Test
Forelimb placing was scored using a vibrissae-elicited forelimb-placing test. Independent testing of each forelimb was induced by brushing the vibrissae ipsilateral to that forelimb on the edge of a tabletop once per trial for 10 trials. Intact animals placed the forelimb quickly onto the countertop. Percentage of successful placing responses were determined. There is a reduction in successful responses in the forelimb contralateral to the site of injection after ICH.

Forelimb Limb-Use Asymmetry Test
Forelimb use during explorative activity was analyzed by videotaping rats in a transparent cylinder for 3 to 10 minutes depending on the degree of activity during the trial. Behavior was quantified by determining the occasions when the nonimpaired (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 as a percentage of total number of limb use observations on the wall (B). A single overall limb use asymmetry score was calculated as: limb use asymmetry score = [(I/(I+C+B))− (C/(I+C+B))].

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

Immunohistochemistry
The immunohistochemistry method has been described previously. Briefly, the rats were reanesthetized and perfused with 4% paraformaldehyde in 0.1 mol/L PBS, pH 7.4. Brains were removed and kept in 4% paraformaldehyde for 6 hours, then immersed in 25% sucrose for 3 to 4 days at 4°C. After embedding in the mixture of 25% sucrose and optimal cutting temperature compound (SAKURA Finetek, Torrence, Calif), 18-μm sections were taken on a cryostat. The avidin–biotin complex technique was used in the staining, and hematoxylin was used for counterstaining. The primary antibodies were rabbit anti-rat HSP27 (1:200 dilution; StressGen Biotechnologies, San Diego, Calif), rabbit anti-rat HSP32 (1:200 dilution, StressGen Biotechnologies, San Diego, Calif), mouse anti-rat CD11b (MRC OX42; 1:200 dilution; Serotec, Oxford, UK), and mouse anti-rat GFAP (1:200 dilution; Chemicon, Temecula, Calif). Normal rabbit or mouse serum and the absence of primary antibody were used as negative controls.

Cell Counts
To assess possible aging effects on astrocyte and microglia reaction, we used 18-μm-thick coronal sections from 1-mm anterior and 1-mm posterior to the blood-injection site. Three high-power images (×40 magnification) were taken in the motor cortex, somatosensory cortex, and basal ganglia using a digital camera. GFAP and OX42-positive cells were counted by a blinded observer. Counts were performed on 3 areas from each of 3 young or aged rat brain sections.

Western Blot Analysis
Western blot analysis was performed at day 1, 3, 7, and 28 after ICH using methods described previously. In brief, rat brains (n = 3 each group) were perfused with saline for 1 minute, and a coronal brain slice was cut as described for brain water content measurements. The ipsilateral and contralateral basal ganglia were sampled and immersed in 0.5 mL of Western blot sample buffer and then sonicated for Western blot analysis. Protein concentration was determined by Bio-Rad protein assay kit. Samples (50 μg protein) were run on a polyacrylamide gel after being denatured by boiling at 95°C for 5 minutes, and then transferred to pure nitrocellulose membrane (Abersham). For HSP27 and HSP32 measurements, membranes were probed with 1:2500 dilution of rabbit anti-rat HSP27 or anti-rat HSP32 (StressGen Biotechnologies, San Diego, Calif), followed by a 1:2500 dilution of the secondary antibody (peroxidase-conjugated goat anti-rabbit antibody; Bio-Rad, Hercules, Calif). The antigen–antibody complexes were visualized with a chemiluminescence system (Abersham Pharmacia) and exposed to photosensitive film. Relative densities were analyzed using NIH Image software version 1.61.

Statistical Analysis
Mann–Whitney U tests and Student t test were used. Values are mean±SD. Statistical significance was set at P<0.05.

Results
Physiological parameters in all animal groups were recorded during intracerebral infusions. There was a significant difference between young and aged rats in body weight. However, the other physiological variables, including mean arterial blood pressure, blood pH, blood gases, hematocrit, and blood glucose, were not different between the groups (Table).

To assess behavioral deficits forelimb placing, forelimb use asymmetry and corner turn tests were used. Rats were tested from day 1 to day 28 after injection of autologous whole blood. The battery of tests indicated that there were marked neurological deficits by day 1 after ICH but that there...
was progressive recovery of function over 4 weeks. The initial (day 1) behavioral deficit, as assessed by the forelimb-placing score and the forelimb-use asymmetry scores, was greater in aged compared with young rats (Figure 1A and 1B). This difference with age was maintained for the 4 weeks after ICH (Figure 1). ICH has no effect on the ipsilateral (nonimpaired) forelimb placing. In contrast to the other 2 tests, there was little evidence of recovery in the corner test score after ICH, with the scores being close to 100% (maximum deficit). There were no significant differences between age groups in this score apart from day 14, during which aged rats had a greater deficit (Figure 1C).

The enhanced behavioral deficits in aged rats were associated with enhanced brain swelling. Perihematomal swelling was more severe in the aged compared with young rats at 3 days after ICH but not at 1 day (Figure 2).

Further evidence for enhanced injury in aged animals came from analysis of HSP27 and HSP32, 2 brain injury markers. In young and aged normal brains, HSP27 and HSP32 immunoreactivities were not detectable. After ICH, young rats had increased HSP27 and HSP32 levels by day 1. These levels peaked at days 3 to 7, and there was a decrease at day 28, particularly for HSP32 (Figure 3A). The time course for HSP32 in aged rats was similar, and HSP32 levels peaked at day 7 (an 11-fold increase compared with day 1). However, HSP27 upregulation was prolonged in aged rats (a 1.5-fold increase at day 28 compared with day 1; Figure 3A). Quantitatively, the upregulation of HSP27 and HSP32 at 3 days after ICH was greater in aged rats compared with young rats ($P<0.05$; Figure 3B).

Aged animals also had evidence of enhanced microglial activation 3 days after ICH compared with young rats (OX42 staining; Figure 4A). Numbers of microglia in the motor cortex, somatosensory cortex, and basal ganglia were determined. Aged rats had significantly more activated microglia in all 3 areas (Figure 4B). In contrast, there was a weak astrocytic reaction in aged rats on day 3 after ICH compared with that found in young rats (Figure 5).

### Discussion

In the present study, we found that ICH caused greater neurological deficits, more severe brain swelling, greater induction of HSPs, and enhanced microglial activation in aged rats compared with young rats. These results suggest that age is a significant factor in determining brain injury after ICH. In addition, our present behavioral data showed that the temporal profiles of recovery in aging and young rats are identical. This result suggests that differences in acute injury, rather than less plasticity, cause the greater brain swelling and neurological deficits in aged rats.
Accumulating evidence indicates that multiple mechanisms are involved in brain injury after ICH.4,10 These include coagulation cascade activation with thrombin production,11 red blood cell lysis with hemoglobin-induced toxicity,12 inflammation,13 and complement cascade activation in the brain parenchyma.14 The age-dependent injury may reflect differences in the number of these mechanisms.

Thrombin has been shown to play a major role in brain injury after ICH.11,15 Evidence indicates that coagulation rates in plasma are accelerated with age,16 which suggests the hematoma can produce much more thrombin in aging rats. It is important to know whether the effect of thrombin inhibition would also be age dependent.

Another factor that may contribute to severe injury in aged rats is erythrocyte fragility. Higher fragility of erythrocytes in old rats may result in easier hemolysis after ICH.17 Erythrocyte lysis and iron toxicity are associated with edema formation after ICH. Our recent studies have demonstrated that iron overload occurs in the brain after ICH, and iron chelation with deferoxamine reduces perihematomal brain edema.6,18 HSPs are very sensitive markers for brain injury.19 HSP27 and HSP32 were chosen as 2 brain injury markers for the current study. The upregulation of perihematomal HSP27 and HSP32 levels was found to be higher in the old rats. It should also be noted that HSP32 (also called HO-1) is a key enzyme for heme degradation. By increasing the release of iron from hemoglobin, HSP32 may participate in brain injury. Indeed, HO inhibitors reduce brain edema after ICH or the intracerebral injection of hemoglobin.20,21 Greater upregulation of HSP32 in aged rats may not only be a response to greater injury, but it may participate in that injury.

Evidence also suggests that inflammation is involved in injury after ICH. ICH causes an influx of leukocytes into the brain and activation of microglia around the hematoma.13 Whole body irradiation to deplete circulating leukocytes has been reported to reduce ICH-induced injury, whereas tuftsin fragment 1-3, an inhibitor of macrophage/microglia activation, reduces injury volume and improves neurological deficits in a collagenase-induced ICH model in mice.8 Microglial activation is a brain injury marker for many CNS diseases and is associated with neuronal death in ischemic penumbra.7,22 Microglia become progressively more activated with age in humans.23 The finding of greater microglia activation in the ipsilateral hemisphere in aged rats after ICH suggests that this may be a component of the enhanced brain injury. Further experiments are necessary to determine whether the effects of age on ICH-induced microglia activation are the cause of the enhanced injury or the result of that injury. A role for complement in ICH-induced brain injury has been identified.14 The receptor recognized by OX42 is the complement receptor type 3.

Astrocytes are essential for the survival of neurons.24 In the current study, the reaction of astrocytes in response to the ICH was weaker in the aged rats. It is known that aged rats have less astrocytic response to injury.25 However, whether astrocyte reaction is neuroprotective remains controversial. A recent study found that astrocytic reaction is stronger in older rats after cerebral ischemia.26
Neurological deficits after ICH result from the balance of injury and repair. Thus, the greater neurological deficits in aged rats could reflect alterations in either the initial injury or the ability to recover functions (for example, by unaffected areas of the brain adopting the functions of injured tissue or by neurogenesis). Figure 1B and our previous study have shown that there is time-dependent recovery after ICH that may result from plasticity. Studies indicate a general decline in the degree of plasticity with age. It is likely that the effects of ICH are exacerbated in aged rats by the combination of greater initial injury and reduced recovery of function.

There is an underlying assumption that enhancement of neurogenesis and delayed brain edema formation following intracerebral hemorrhage in rats. J Neurosurg. 1998;89:991–996.


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References


Figure 6. GFAP-positive cells in the ipsilateral hemisphere 3 days after ICH. A, Young rats. B, Aged rats. C, Cell count. Bar=20 μm. Values are mean±SD; n=4. *P<0.01 vs young rats.