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Brain Injury After Intracerebral Hemorrhage in Spontaneously Hypertensive Rats

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Abstract

Object—Hypertension is the main cause of spontaneous intracerebral hemorrhages (ICH), but the effects of hypertension on ICH-induced brain injury have not been well studied. In this study, we examined ICH-induced brain injury in spontaneously hypertensive rats (SHR).

Methods—This two-part study was performed on 12 weeks old male SHR and Wistar Kyoto (WKY) rats. First, rats received an intracaudate injection of 0.3 units collagenase and hematoma sizes were determined at 24 hours. Second, rats were injected with 100-μL autologous whole blood into the right basal ganglia. Brain edema, neuronal death, ferritin expression, microglia activation, and neurological deficits were examined.

Results—Hematoma sizes were the same in SHR and WKY rats 24 hours after collagenase injection. SHR had greater neuronal death and neurological deficits after blood injection. ICH also resulted in higher brain ferritin levels and stronger activation of microglia in SHR. However, perihematomal brain edema was same in the SHR and WKY rats.

Conclusion—Moderate chronic hypertension resulted in more severe ICH-induced neuronal death and neurological deficits, but did not exaggerate hematoma enlargement and perihematomal brain edema in the rat ICH models.

Keywords

brain edema; cerebral hemorrhage; ferritin; hypertension; microglia; neuronal death

Introduction

Spontaneous intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype. Studies have indicated a mortality of more than 40%, and many survivors are left with significant neurological deficits[17]. Hypertension is a major risk factor for ICH. High blood pressure is found in 90% of patients with ICH and persists for a few days[5]. In the emergency department, about 27% of ICH patients have a systolic blood pressure (SBP) of above 160 mmHg [23]. Transient or sustained high blood pressure has increased risk of continuous bleeding, subsequent rebleeding, or both within the first 24 h after the initial hemorrhage[4]. Several clinical trials of aggressive blood pressure lowering in acute ICH are ongoing[3,24]. However, the effects of hypertension on pathological changes in the brain after ICH are still not well understood.

Spontaneously hypertensive rats (SHR), derived from Wistar Kyoto (WKY) rats, have been used extensively to study the effects of chronic hypertension on ischemic stroke[10,15].

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Mortality after ICH is higher in SHR[8]. In the present study, we investigated ICH-induced brain injury in SHR and WKY rats using both collagenase and blood injection models.

Materials and Methods

Animal Preparation and Blood Pressure Measurement

Male SHR and WKY rats were purchased from Charles River Laboratories at 9 weeks of age and used for experiments at 12 weeks old. The protocols for these animal studies were approved by the University of Michigan Committee on the Use and Care of Animals. All animals were housed under standard 12:12-h light-dark conditions and allowed free access to food and water. Systolic and mean blood pressure measurements (in triplicate) were taken using a noninvasive tail cuff blood pressure system (IITC Life Science Inc., Model 229) once a week before surgery for three weeks. Blood pressure was also measured during surgery and at one day after surgery.

Intracerebral Injection

Animals were anesthetized with pentobarbital (45 mg/kg, i.p.). The right femoral artery was catheterized for continuous blood pressure monitoring and blood sampling. The rats were placed in a stereotactic head frame (Kopf Instruments) and a 1-mm cranial burr hole drilled on the right coronal suture 3.5 mm lateral to midline. A 26-gauge needle was inserted stereotaxically into the right basal ganglia (coordinates: 0.2 mm anterior, 3.5 mm lateral, and 5.5 mm ventral to the bregma). This needle was used for injections of autologous blood or collagenase[14]. Body temperature was maintained at 37.5°C with a feedback-controlled heating pad. Blood pH, PaO_2, PCO_2, hematocrit, and glucose levels were monitored. After infusion, the needle was removed, the skin was closed and the animals were allowed to recover.

Experimental Groups

There were two parts to this study which used a total of 96 rats.

Part 1. To determine whether there are differences in hematoma volume between SHR and WKY rats after intracaudate injection of collagenase. Rats (n=6 each) received a 1.5-μL injection (at 0.1-μL/min) of saline containing 0.3 U bacterial collagenase (type VIIs, Sigma). The syringe was left in place for 10 minutes after the infusion was completed. Animals were killed at 24 hours for brain hemoglobin content determination.

Part 2. There were three sets of experiments in this part. All rats received an infusion of 100-μL autologous blood into the right basal ganglia at a rate of 10-μL/min. In the first set of experiments, SHR and WKY rats were killed at days 1 and 3 (n=6 each time point) for brain water content measurement. In the second set, rats were killed at days 1, 3 and 14 (n=6 each time point) for immunohistochemistry. Behavioral test were performed at days 1, 7 and 14. In the third set, rats were killed at days 1 and 3 (n=6 each time point) for Western blotting.

Brain Water Content

Animals were reanesthetized with pentobarbital (60 mg/ kg, i.p.) and decapitated at one and three days after ICH[33]. Brains were removed and a 3-mm thick coronal brain slice cut 4 mm from the frontal pole, and dissected into ipsi-and contralateral cortex, and ipsi- and contralateral basal ganglia. The cerebellum was taken as a control. Brain samples were immediately weighed on an electronic analytical balance to obtain the wet weight, and then dried at 100°C for 24 hours to obtain the dry weight. Percent water content was determined as: ((wet weight- dry weight)/wet weight)*100%.

Hemoglobin Contents

Hematoma size was measured by determining hemoglobin content with a spectrophotometer[22]. Animals were perfused transcardially with 0.1mol/L phosphate-buffered saline under deep anesthesia until the outflow fluid from the right atrium was colorless. The brain was rapidly removed and dissected into left and right hemisphere. The brain tissue was then homogenized in 0.1mol/L phosphate-buffered saline followed by 30-minute centrifugation (13,000 g). Fifty micro-liter supernatant was mixed with 200-μL reagent (QuantiChrom Hemoglobin Assay Kit; BioAssay Systems). After 15 minutes, optical density was measured at 400 nm with a spectrophotometer (Ultrospec 3; Pharmacia LKB) and hemispheric hemoglobin content expressed as milligrams per hemisphere.

Immunohistochemistry and Fluoro-Jade C Staining

The immunohistochemistry method has been described previously [33]. Briefly, rats were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH7.4). Brains were removed and kept in 4% paraformaldehyde for 6 h, then immersed in 25% sucrose for 3–4 days at 4 °C. After embedding in a mixture of 25% sucrose and OCT (SAKURA Finetek, USA), 18-μm sections were taken on a cryostat. The avidin–biotin complex technique was used for staining with hematoxylin as counter stain. The primary antibodies were mouse anti-rat OX6 (1:400 dilution, AbD Serotec) and rabbit anti-human ferritin (1:400 dilution, Sigma). Normal rabbit or mouse serum and the absence of primary antibody were negative controls.

For Fluoro-Jade C Staining, brain sections were incubated with 0.0001% Fluoro-Jade C (Histo-Chem Inc.) in 0.1% acetic acid in the dark at room temperature for 10 min. Following incubation, sections were rinsed and allowed to dry in the dark. Slides were covered with coverslip and examined with an Olympus BX51 fluorescence microscope.

For cell counting, we used 18-μm thick coronal sections from both 1 mm anterior and 1 mm posterior to the blood injection site. Three high-power images (x40 magnification) were taken adjacent to the hematoma and the positive cells were manually counted.

Western Blot Analysis

Western blot analysis was performed as previously described[33]. Briefly, brain tissue was immersed in Western sample buffer and sonicated. Protein concentration was determined by Bio-Rad protein assay kit and 50-μg protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane (Amersham). Membranes were probed with primary antibodies: rabbit anti-ferritin heavy chain polyclonal antibody (1:2000, Cell Signaling Technology) and goat anti-ferritin light chain polyclonal antibody (1:2000, Abnova). The antigen-antibody complexes were visualized with the ECL chemiluminescence system (Amersham) and exposed to Kodak X-OMAT film. The relative densities of bands were analyzed with NIH ImageJ.

Behavioral Tests

Three behavioral tests, including the forelimb placing test, the forelimb use asymmetry test and the corner turn test were used in this study as described before[12]. All animals were scored by a behavioral tester who was blind to the neurological condition.

Statistical Analysis

Student t test was used. Values are mean±SD. Statistical significance was set at $p<0.05$.

Results

Blood Pressure

Systolic artery blood pressure (SBP) and mean artery blood pressure (MBP) of the rats were measured once a week from 9 weeks old until the end of study (Figure 1). SBP and MBP were significantly higher in the SHR (e.g., SBP/MBP at 11 weeks, 177±10/145±8 mmHg vs. 140±12/103±10mmHg in WKY rats, $p<0.01$). Other physiological parameters were also recorded during intracerebral injections and all were in the normal range except that hematocrit was higher in SHR strain (48±4.2% vs. 42.3±3.5 in WKY rats, $p<0.01$).

Collagenase Model

Brain hemoglobin content was measured 24 hours after collagenase injection to estimate the hematoma size. We found that hemoglobin content in the ipsilateral hemisphere was the same in SHR and WKY rats (13.6±3.2 mg vs. 13.2±2.1 mg, $p=0.817$, Figure 2).

Blood Injection Model

Brain injury after an ICH was examined using the blood injection model. Degenerating neurons were detected by Fluoro-Jade C staining in the perihematomal zone after both 1 and 3 days. ICH caused more neuronal death around the hematoma in SHR strain at the first day (666±86/mm^2 versus 478±129/mm^2 in WKY rats, $p<0.05$, Figure 3). There still was a trend for more neuronal death in SHR strain at day 3 (365±172/mm^2 versus 216±104/mm^2 in WKY rats, $p=0.09$, Figure 3).

ICH induced neurological deficits in both SHR and WKY rats. Both strains had forelimb placing recovery at day 7 and day 14 after ICH but the WKY rats had better forelimb placing scores than SHR strain at all the time points (Figure 4). Forelimb use asymmetry scores recovered at day 7 and 14 in WKY rats, but there was little recovery in the SHR strain which had more severe deficits at days 7 and 14 (Figure 4). There were no differences between the two groups for corner turn deficits which were at or close to maximal at days 1, 7 and 14 (Figure 4). There was also no difference in brain edema between SHR and WKY rats at day 1 (Figure 4) and day 3 (data not shown).

Activated microglia (OX-6 positive) were found around the hematoma in both strains. The SHR strain had significantly more OX-6 positive cells in the perihematomal area at day 1 ($p<0.05$, Figure 5) and day 3 (523±225 vs. 327±51 positive cells/mm^2 in WKY rats, $p<0.05$).

Protein levels of ferritin, a key iron storage protein in the brain, were examined in the ipsilateral basal ganglia of both strains at day 1 and 3 after ICH. Immunohistochemistry showed more ferritin positive cells present around the hematoma in SHR strain at day one (516±89 vs. 272±42 positive cells/mm^2 in WKY rats, $p<0.05$, Figure 5). Very weak ferritin immunoreactivity was present in the contralateral basal ganglia in both strains. Brain ferritin levels were also quantified by Western blots. Both ferritin light and heavy chain levels were higher in SHR compared with WKY rats one day after ICH (Figure 6) but not at day 3 (light chain: 21203±2666 vs. 22224±5623 pixels in WKY, $p>0.05$; heavy chain: 26081±2439 vs. 24976±5190 pixels in WKY, $p>0.05$).

Discussion

Our present study demonstrated that intracerebral hematomas caused more severe neuronal death, microglia activation, ferritin upregulation and neurological deficits in SHR compared to WKY rats and these results indicate that chronic hypertension has a role in brain damage

following ICH. Interestingly, though, hematoma size was not affected by hypertension in the collagenase model.

ICH resulted in more neuronal death in the brain. It is well known that thrombin and iron are two major factors causing brain injury after ICH[11]. Hypercoagulable state and severe iron overload may contribute to more severe neuronal death in SHR rats after ICH. However, brain injury after ICH is complicated with many potential factors affecting ICH-induced neuronal death. For example, studies showed there is an intrinsic vulnerability participating in the exacerbation of brain damage after cerebral insults in SHR[27,34]. In addition, intracerebral injection of AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) provoked greater damage and higher mortality in adult SHR[15].

The main factor which affects ICH patient outcome is hemorrhagic volume. Hematoma enlargement occurs in about a third of ICH patients[32] which may be related with hypertension. Recent studies showed that early intensive blood pressure-lowering treatment can reduce hematoma growth[4]. Therefore, we predicted larger hematomas in SHR rats. However, we found the hematoma sizes were the same in SHR and WKY rats. The reasons resulted in same hematoma sizes could be: 1) a moderate increase of systolic blood pressure (<200mmHg) may not enlarge the hematoma; 2) a hypercoagulable state occurs in SHR rats, [2] and the blood prothrombin time is much shorter in SHR rats compared with that in WKY rats [19], which may limit hematoma growth. A prothrombotic state has also been reported in human hypertension[26]. This may have multiple underlying causes [26] and might be an adaption to prevent hemorrhage; 3) the type of blood vessel disruption that occurs in the collagenase model differs from that occurring in spontaneous ICH in man with regards to the mechanism of disruption and the type of vessels that hemorrhages. It should also be noted that while the hypertensive state is well established by 12 weeks in the SHR rat, it is possible that the effects of hypertension on hematoma size may vary with the duration of hypertension. It is known, for example, that there continues to be changes in the vascular wall of the SHR rat with age, even when the blood pressure has ceased to increase [29]. Further experiments are needed to examine the effect duration of hypertension on hematoma size in the SHR rat.

Edema formation following ICH elevates intracranial pressure and may result in herniation [25]. There are several phases of edema formation after ICH, including a very early phase involving hydrostatic pressure and clot retraction with movement of serum from the clot into the surrounding tissue, and a second phase related to the coagulation cascade and thrombin production and a third phase related to erythrocyte lysis and iron toxicity[32]. We hypothesized that perihematomal edema would be more severe in SHR because of the hypertension, the hypercoagulable state, and higher hematocrit, but in this study we found that hypertension has no effects on perihematomal edema formation. This result is supported by a recent study indicating blood pressure lowering treatment has no effects on edema formation in ICH patients[3]. Whether or not edema resolution is enhanced in SHR should be examined. CSF is the primary pathway for edema clearance and the CSF turn-over rate is more than twice greater in SHR than that in WKY [1].

Enhanced activation of microglia was present in the SHR strain after ICH. It is well known that inflammation exacerbates hemorrhagic brain damage. An inflammatory response in the surrounding brain with microglial activation occurs shortly after ICH [6,13]and activated microglial cells persist for at least a month [7,13]. Inhibition of microglia activation with tuftsin fragment 1-3 and minocycline reduces brain damage after ICH in animals [28,31]. Whether enhanced microglia activation contributes to the worse behavioral outcome in SHR needs elucidation.

Ferritin is a major iron storage protein that can be produced in brain. Ferritin has two subunits, heavy and light chains. Ferritin protein synthesis is regulated by both iron mediated and non-iron mediated induction. The greater ferritin upregulation in the SHR strain in this study suggests more severe iron release. We have gathered substantial evidence that iron release from hemoglobin breakdown has a major role in ICH-induced brain injury. There is a build-up in non-heme brain tissue iron and the appearance of iron positive cells around the perihematomal zone as early as the first day [30]. Increased brain iron levels cause oxidative stress, neuronal death and neurological deficits following ICH [32]. Deferoxamine, an iron chelator, reduces hemorrhagic brain injury in aged rats and piglets [9,20]. Recent clinical studies also showed that high ferritin levels in serum are associated with severe perihematomal brain edema and poor outcome in ICH patients[16,21].

However, non-iron mediated ferritin upregulation may also occur in the brain after ICH, although hemoglobin degradation products such as iron and heme are strong ferritin inducers. For example, ICH causes a significant increase of tumor necrosis factor-α in the brain and this inflammatory mediator can induce ferritin synthesis[18]. Therefore, future studies need to determine the precise mechanisms underlying the greater ferritin upregulation in SHR including the greater hematocrit in that strain.

In summary, moderate chronic hypertension (< 200mmHg SBP) did not enlarge the hematoma or exacerbate brain edema after ICH. However, it did result in increased neuronal death and worse function outcome, which may be associated with microglia activation and iron toxicity.

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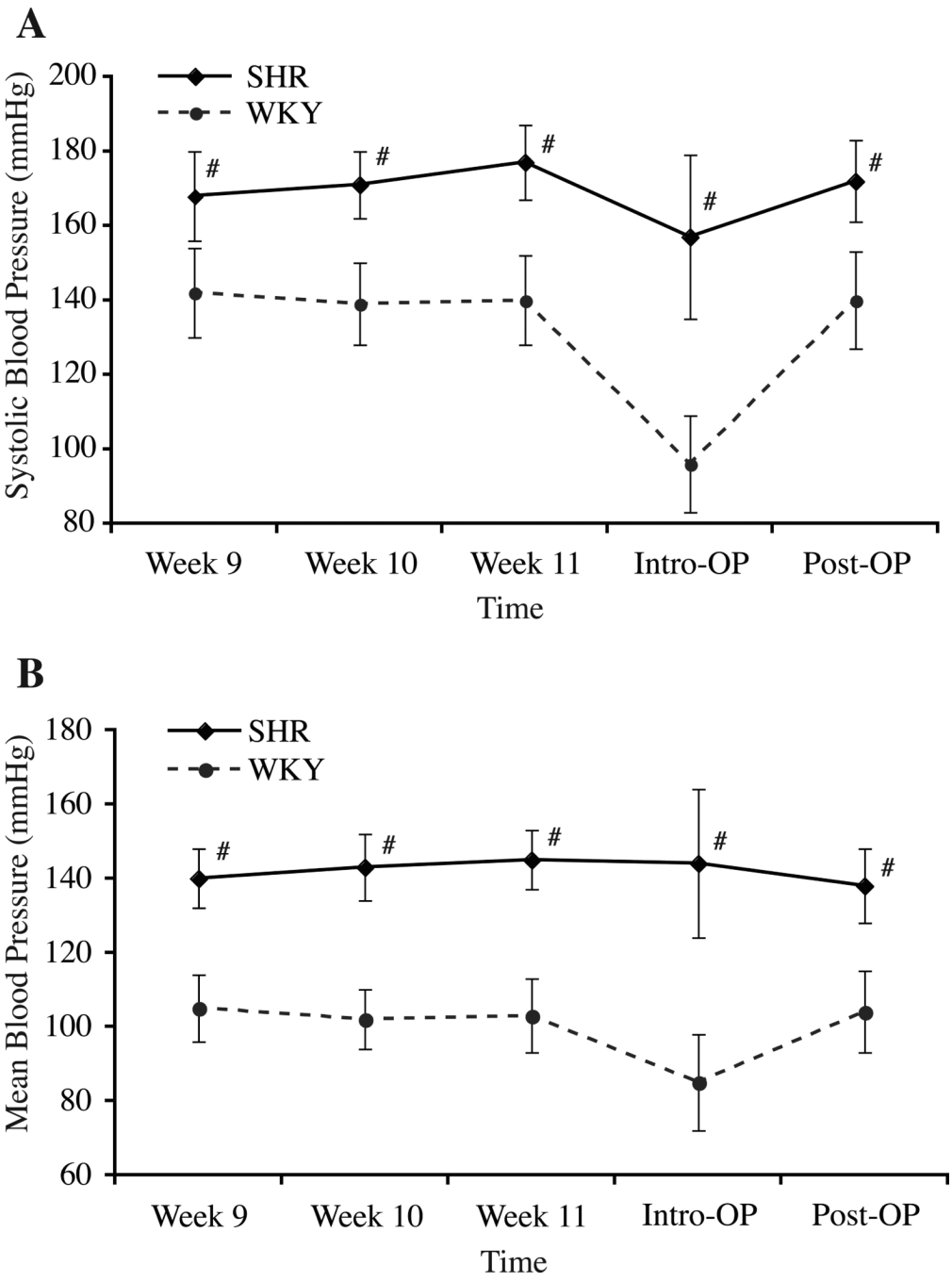

Figure 1.

Graphs showing systolic blood pressure (A) and mean blood pressures (B) in age-matched spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats since 9 weeks of age (Week 9) till the end of this study. Data are expressed as mean±SD. #$p<0.01$ vs. WKY.

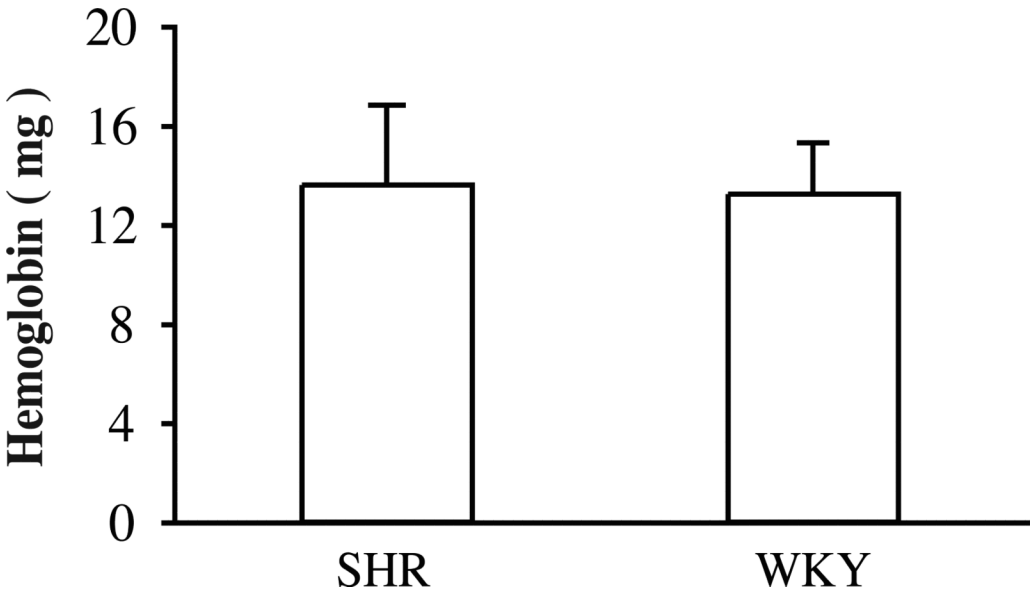

Figure 2.
Brain hemoglobin content (a measure of hematoma size) in the ipsilateral hemisphere one day after intracerebral injection of collagenase in SHR and WKY rats. Values are expressed as mean±SD.

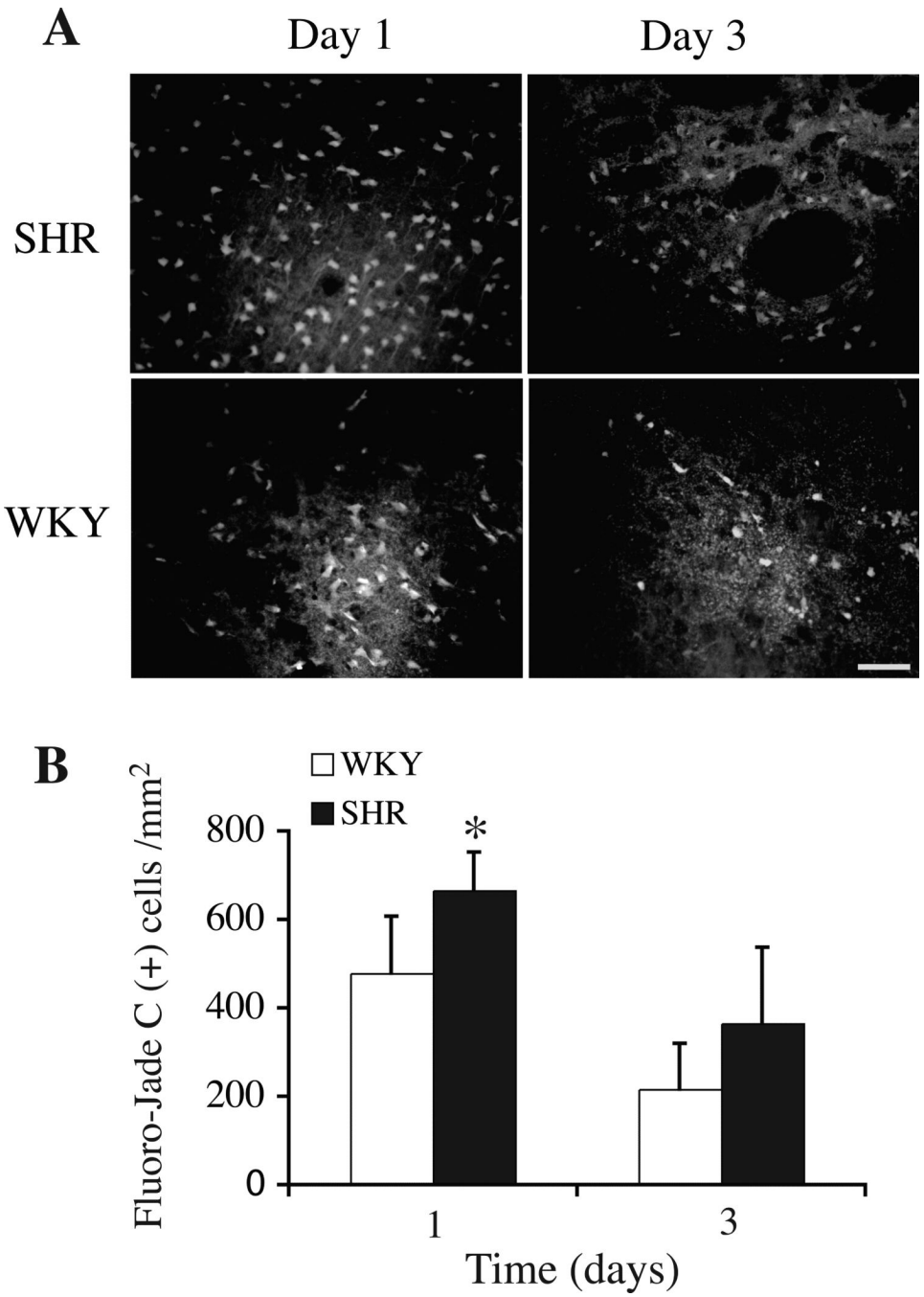

Figure 3.
(A) Fluoro-Jade C staining showing degenerating neurons in the ipsilateral basal ganglia of SHR and WKY rats one and three days after ICH. Bar=50 μm. (B) Bar graph showing numbers of Fluoro–Jade C staining positive cells around the hematoma. Values are expressed as mean±SD. *$p<0.05$ vs. WKY.

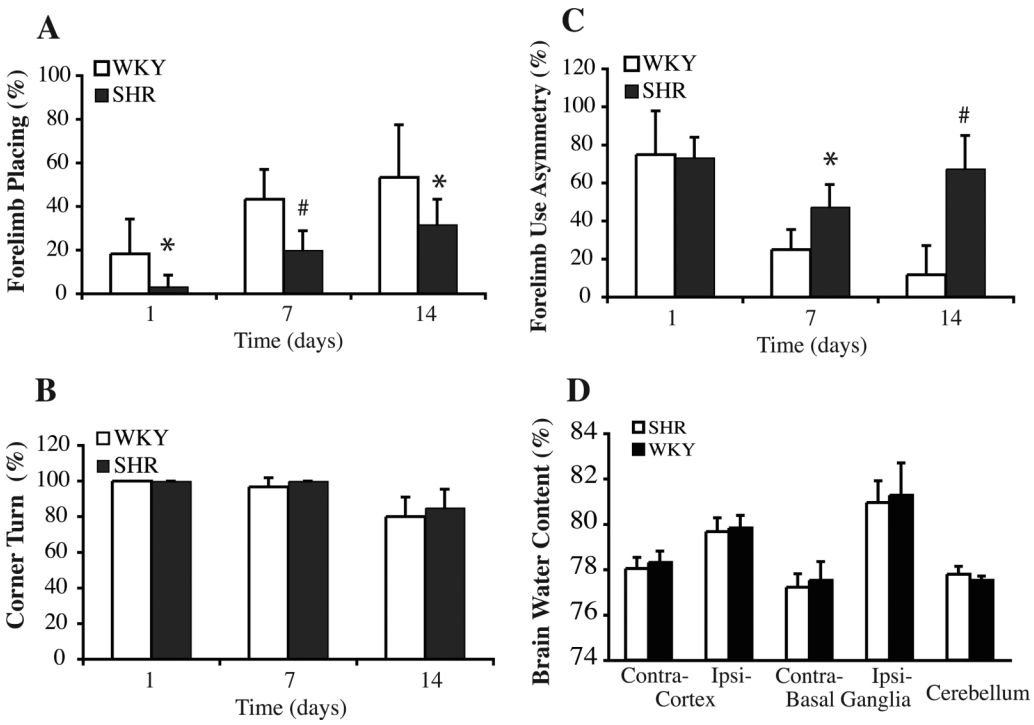

Figure 4.

Bar graphs showing the results of the forelimb placing (A, normal=100%), corner turn (B, normal=50%), and forelimb use asymmetry (C, normal=0%) tests in SHR and WKY rats after ICH. Values are mean±SD. *$p<0.05$, #$p<0.01$ vs. WKY. (D) Brain water content one day after ICH in SHR and WKY rats. Values are expressed as mean±SD. There were no differences in water content in the ipsilateral basal ganglia and cortex between the two strains.

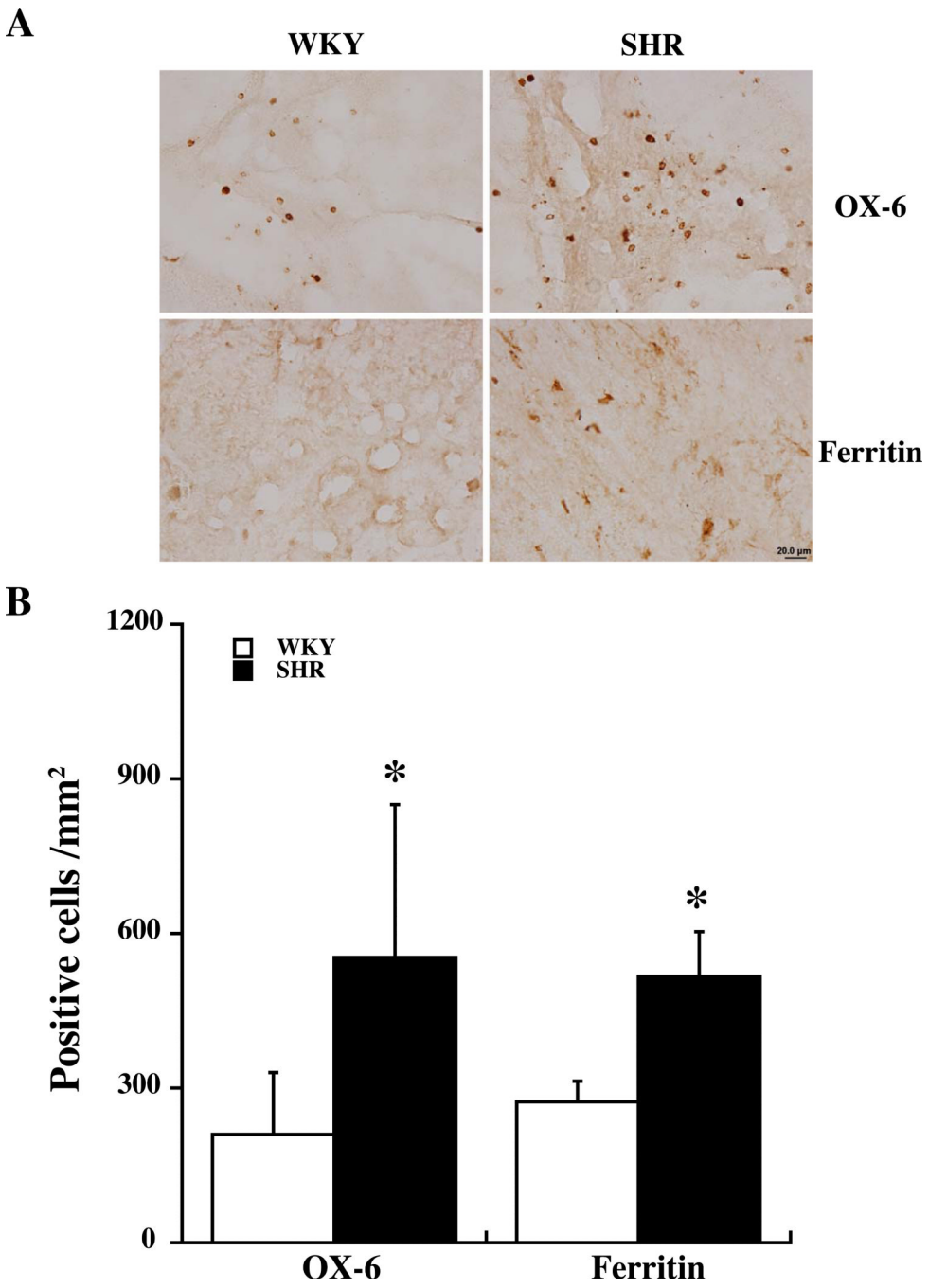

Figure 5.

OX-6 and ferritin positive cells in the ipsilateral basal ganglia of SHR and WKY rats one day after ICH. (A) OX-6 and ferritin positive cells. Scale bar=20 μm. (B) Cell count. Values are mean±SD. *p<0.05 vs. WKY.

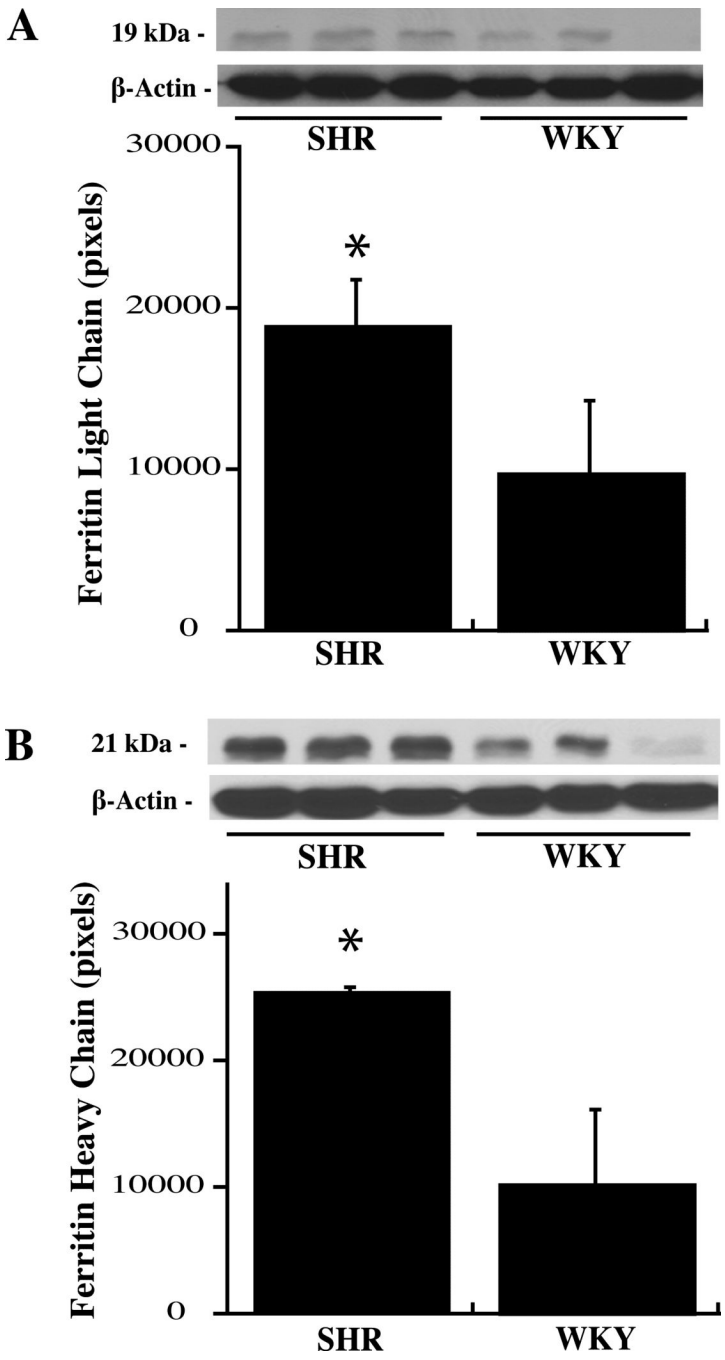

Figure 6.
Western blot analysis showing ferritin light chain (A) and ferritin heavy chain (B) levels in the ipsilateral basal ganglia of SHR and WKY rats one day after ICH. Values are mean±SD. *p<0.05 vs. WKY.