

# Effect of Gender on Iron-induced Brain Injury in Low Aerobic Capacity Rats

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## Introduction

Spontaneous intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype [14]. Iron has a major role in brain damage following ICH [17, 20, 22]. We have shown a significant increase in brain non-heme iron after ICH in rats, and this remains high for at least 1 month [19]. Brain iron overload causes brain edema in the acute phase and brain atrophy later after ICH, with an iron chelator, deferoxamine, reducing ICH-induced brain edema, neuronal death, brain atrophy, and neurological deficits in young rats [5, 10, 16], aged rats [12, 13], and pigs [2]. Recent studies show that high levels of serum ferritin, an iron storage protein, are independently associated with poor outcome and severe brain edema in ICH patients [8, 15].

Our previous study showed that females have less brain edema and a faster recovery of behavioral deficits after ICH and 17 $\beta$ -estradiol treatment markedly reduced ICH-induced brain injury [1, 9, 11]. Estrogen pretreatment attenuated iron-induced brain edema and neuronal death [3, 7]. We have shown in a model that low aerobic capacity (low capacity runner rats; LCRs) had more severe ICH-induced brain injury than high capacity runners, including

worse brain edema, brain atrophy, and neurological deficits, and that females were protected against ICH-induced brain edema formation in both high capacity runners and LCRs [4].

The present study examines whether gender-specific differences in iron-induced brain injury in LCR rats might contribute to the gender differences found in ICH-induced injury.

## Materials and Methods

### *Animal Preparation and Intracerebral Infusion*

All animal procedures were approved by the University Committee on Use and Care of Animals, University of Michigan. A detailed description of the development of low aerobic capacity rats has been published previously [18]. Adult male ( $n=7$ ) and female LCR rats ( $n=6$ ) were anesthetized with pentobarbital (45 mg/kg, intraperitoneally (IP)) and the right femoral artery was catheterized to monitor arterial blood pressure, blood pH, PaO<sub>2</sub>, PaCO<sub>2</sub>, hematocrit, and glucose levels. Body temperature was maintained at 37.5 °C by a feedback-controlled heating pad. A polyethylene catheter (PE-50) was inserted into the right femoral artery to monitor arterial blood pressure and blood gases. Animals were then positioned in a stereotactic frame and injections administered into the right basal ganglia (coordinates at 0.2 mm anterior to bregma, 5.5 mm ventral, and 3.5 mm lateral to midline). All the animals had 50  $\mu$ l FeCl<sub>2</sub> (0.5 mM) or saline infused into the right caudate at 5  $\mu$ l/min using a microinfusion pump. After injection, the needle was removed and the skin incisions closed. T2 magnetic resonance imaging (MRI) was performed at 24 h after iron injection and the rats were then used for brain histology and Western blotting.

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## Magnetic Resonance Imaging and Volume Measurement

Imaging was carried out in a 7.0-T Varian MR scanner (183-mm horizontal bore; Varian, Palo Alto, CA, USA) at the Center for Molecular Imaging (CMI) of the University of Michigan. Rats were anesthetized with 2 % isoflurane/air mixture throughout MRI examination. The imaging protocol for all rats included a T2 fast spin-echo sequence (TR/TE=4000/60 ms). The images were preserved as 256×256 pixels images; the lesion volumes and hemisphere volumes were measured by a blinded observer with NIH ImageJ [23]. Lesion volume was calculated by multiplying the total lesion area across all sections by the distance between the sections. Brain swelling calculation was based on 7 every other section of which the center was the anterior commissure layer. The value was ((volume of right hemisphere – volume of left hemisphere)/volume of right hemisphere)×100 %.

## Immunohistochemistry

Immunohistochemistry was performed as described previously [6, 21]. Briefly, rats were anesthetized and subjected to intracardiac perfusion with 4 % paraformaldehyde in 0.1 mM phosphate-buffered saline (pH 7.4). Brains were removed and kept in 4 % paraformaldehyde for 6 h, then immersed in 30 % sucrose for 3–4 days at 4 °C. After embedding in a mixture of 30 % sucrose and OCT, 18 µm sections were cut on a cryostat. For immunohistochemistry, the primary antibodies were goat anti-albumin antibody (Bethyl Laboratories, Inc., 1:600 dilution, Montgomery, TX) and rabbit polyclonal HO-1 antibody (1:600 dilution; Assay Designs/Stressgen, Farmingdale, NY).

## Western Blot Analysis

Western blot analysis was performed as previously described [21]. 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 samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane. Membranes were probed with goat

anti-albumin antibody (Bethyl Laboratories, Inc., 1:10,000 dilution, Montgomery, TX), and rabbit polyclonal HO-1 antibody (1:2,000 dilution; Assay Designs/Stressgen, Farmingdale, NY). The antigen–antibody complexes were visualized with the ECL chemiluminescence system and exposed to Kodak X-OMAT film. The relative densities of bands were analyzed with NIH ImageJ.

## Statistical Analysis

All the data in this study are presented as mean±standard deviation (SD). Data were analyzed by Student's *t*-test or analysis of variance (ANOVA). Differences were considered significant at  $p < 0.05$ .

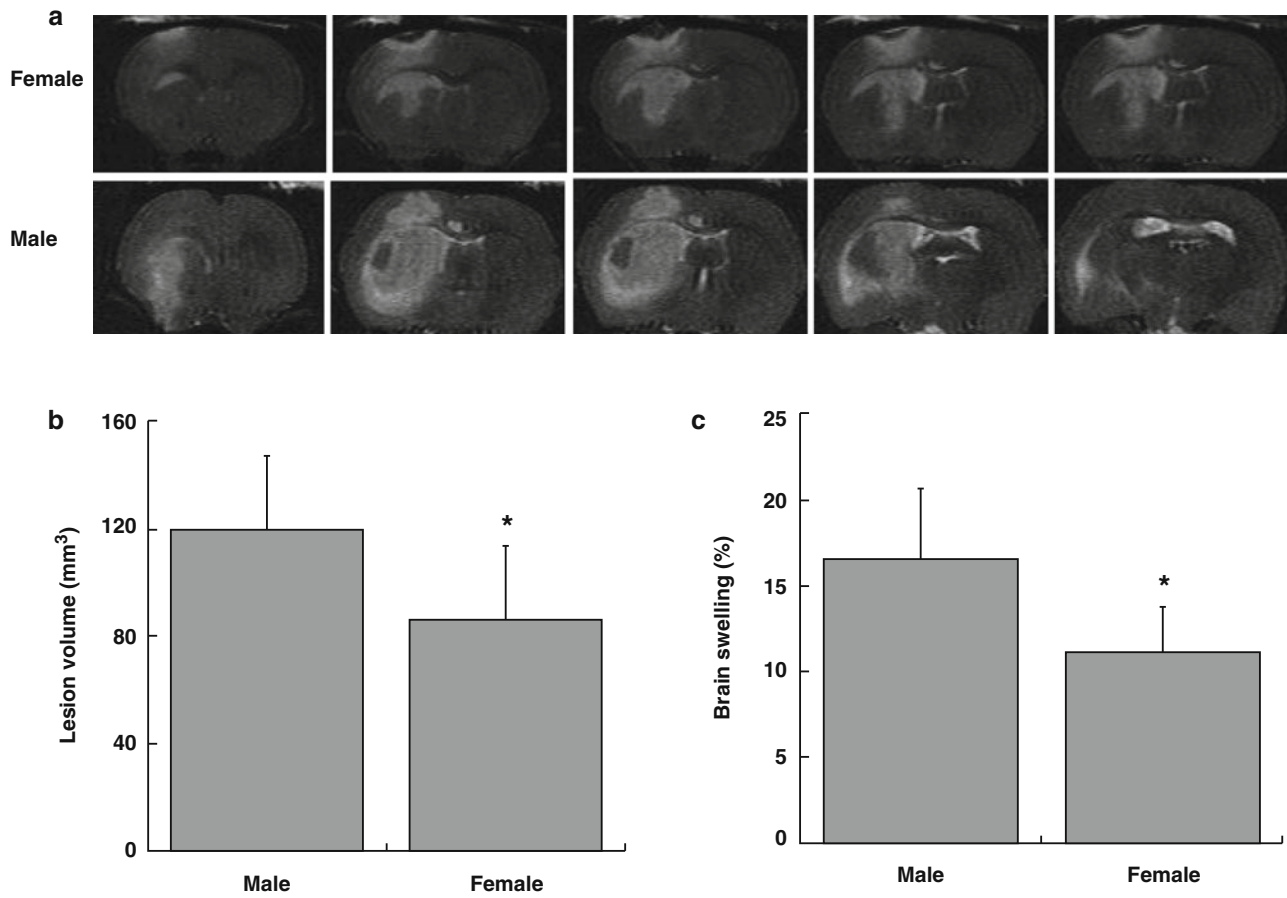
## Results

The LCR rats used in this study were from generation 29–33 of the breeding program. All physiological parameters were monitored during intracerebral infusions. No rats died postoperatively.

T2 lesion volumes at 24 h after iron infusion were larger in males ( $120 \pm 28 \text{ mm}^3$ ) than in females ( $87 \pm 27 \text{ mm}^3$ ,  $p < 0.05$ ); Fig. 1a, b). There was also more severe brain swelling in males ( $16.6 \pm 4.1 \%$ ) compared with females ( $11.1 \pm 2.6 \%$ ,  $p < 0.05$ ; Fig. 1a, c).

Albumin is normally excluded from the brain by the blood-brain barrier (BBB), and entry of albumin is a marker of BBB disruption. Intracaudate FeCl<sub>2</sub> injection caused marked BBB disruption in the ipsilateral hemisphere 24 h after injection (Fig. 2) with much higher albumin protein levels in the ipsilateral basal ganglia than in the contralateral ( $p < 0.01$ , Fig. 2). The albumin-positive area was larger in the ipsilateral hemisphere in males than females (Fig. 2) and albumin protein levels (Western blot) were significantly higher in males ( $7,717 \pm 1,502$  vs  $5,287 \pm 1,342$  pixels in females,  $p < 0.05$ ; Fig. 2).

HO-1 is a marker for brain stress that is expressed at very low levels in normal brain. The number of cells immunoreactive for HO-1 at 24 h after FeCl<sub>2</sub> injection was greater in male than female LCR rats (Fig. 3). Similarly, as assessed by Western blot, HO-1 protein levels in the ipsilateral basal ganglia were higher in males than females (HO-1/β-actin:  $1.31 \pm 0.44$  vs  $1.03 \pm 0.05$ ,  $p < 0.05$ ; Fig. 3).



**Fig. 1** (a) Representative T2 MRI scans of the brains of female and male LCR rats 24 h after an intracaudate injection of FeCl<sub>2</sub>. (b) Lesion volumes calculated from such MRI scans. (c) Brain swelling calculated

from such scans. Male LCR rats had significantly greater lesion volumes and brain swelling than females after FeCl<sub>2</sub> injection. Values are mean ± SD,  $n=6-7$ , \* $p<0.05$

## Discussion

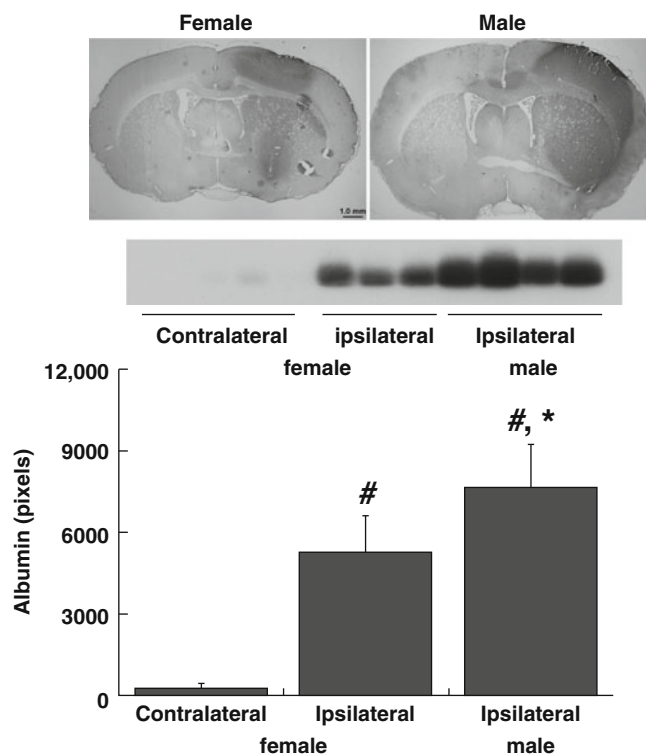
In the present study, we found that male rats had comparatively more severe hemispheric swelling, BBB disruption, and larger T2 lesions after intracerebral iron injection into the caudate. In addition, iron induced much stronger expression of HO-1 in males than females.

Brain iron overload has an important role in ICH-induced brain injury. The current results suggest that differences in iron-mediated damage contribute to gender differences in ICH-induced brain injury. It is still unknown why iron-induced brain swelling is less in female LCRs. For iron-induced brain injury, most research has focused on oxidative injury. The reduction in the iron-induced upregulation of brain HO-1 expression (a cellular stress marker) in

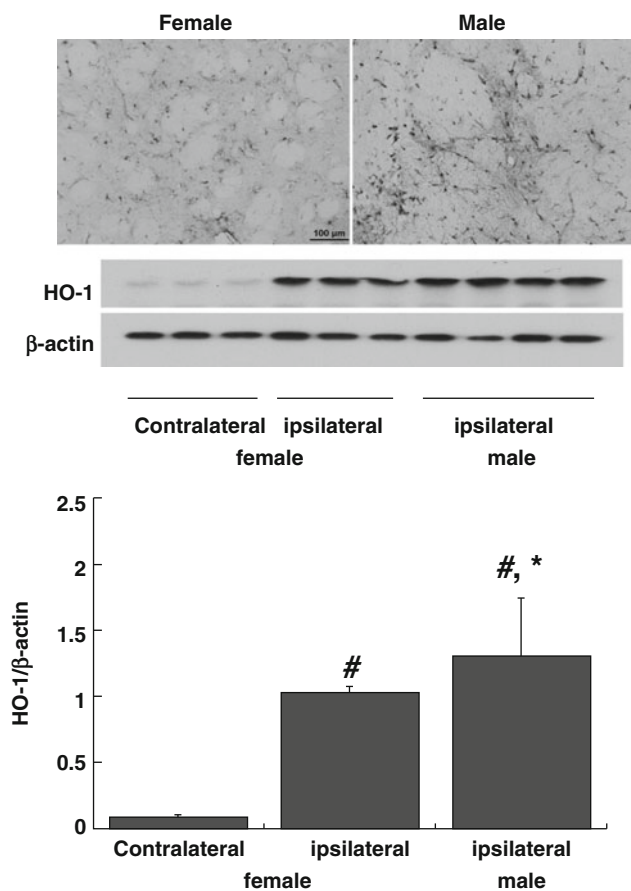
females may reflect reduced oxidative stress. This needs investigation.

Iron-induced brain edema could be vasogenic and cytotoxic. Our results showed that BBB leakage was more severe in males than females, suggesting less vasogenic brain edema in females. We have previously shown that females have less severe ICH-induced brain injury than males through an estrogen receptor-dependent mechanism [9]. More experiments are needed to determine whether estrogen and its receptors have a role in ameliorating iron-induced BBB disruption and vasogenic brain edema.

In conclusion, iron caused less brain damage, BBB leakage, and brain swelling in female LCRs compared with males. Gender differences in iron-induced injury may contribute to differences between females and males in ICH-induced brain injury.



**Fig. 2** Albumin immunohistochemistry and Western blot demonstrating protein extravasation into brain 24 h after intracaudate injection of  $\text{FeCl}_2$  in female and male LCR rats. Albumin extravasation is a marker of BBB disruption. *Bar graph* quantifies the Western blot data.  $\text{FeCl}_2$  injection caused unilateral BBB disruption that was significantly greater in male rats. Values are mean  $\pm$  SD,  $n=3-4$ ,  $*p<0.05$  male vs female;  $\#p<0.01$  ipsi- vs contralateral



**Fig. 3** Heme oxygenase 1 (*HO-1*) immunohistochemistry (ipsilateral caudate) and Western blot (basal ganglia) 24 h after intracaudate injection of  $\text{FeCl}_2$  in LCR and HCR rats. The *bar graph* quantifies the Western blot normalizing the data to  $\beta$ -actin.  $\text{FeCl}_2$  injection caused unilateral HO-1 upregulation and that upregulation was significantly greater in male rats. Values are mean  $\pm$  SD,  $n=3-4$ ,  $*p<0.05$  male vs female;  $\#p<0.01$  ipsi- vs contralateral

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