Iron Accumulation and DNA Damage in a Pig Model of Intracerebral Hemorrhage

Yuxiang Gu, Ya Hua, Yangdong He, Lin Wang, Hua Hu, Richard F. Keep, and Guohua Xi

Abstract Cerebral iron overload causes brain injury after intracerebral hemorrhage (ICH) in rats and pigs. The current study examined whether an iron chelator, deferoxamine, can reduce ICH-induced DNA damage in pigs. Pigs received an injection of autologous blood into the right frontal lobe. Deferoxamine (50 mg/kg, i.m.) or vehicle was given 2 h after ICH and then every 12 h up to 7 days. Animals were killed at day 3 or day 7 after ICH to examine iron accumulation and DNA damage. We found that ICH resulted in the development of a reddish perihematomal zone, with iron accumulation and DNA damage within that zone. Deferoxamine treatment reduced the perihematomal reddish zone, and the number of Perls' ($p<0.01$) and TUNEL ($p<0.01$) positive cells. In conclusion, iron accumulates in the perihematomal zone and causes DNA damage. Systemic deferoxamine treatment reduces ICH-induced iron overload and DNA damage in pigs.

Keywords Cerebral hemorrhage · Deferoxamine · Iron · Perls' reaction · TUNEL

Introduction

Iron accumulation occurs in the brain after intracerebral hemorrhage (ICH) and results in brain injury [1–3]. Iron-induced brain injury including DNA damage may result from oxidative stress [4, 5]. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) is a DNA injury marker that is often used to detect double-strand DNA damage [4].

Deferoxamine (DFX), an iron chelator, is a FDA-approved drug for the treatment of acute iron intoxication and chronic iron overload due to transfusion-dependent anemia. DFX reduces brain edema, neuronal death and neurological deficits following ICH in rats [6–8]. DFX also reduces hemorrhagic transformation in a rat model of cerebral ischemia [9].

The current study examined whether systemic DFX treatment reduces brain iron accumulation and DNA damage in a pig model of ICH.

Materials and Methods

Animal Preparation, Intracerebral Infusion and DFX Treatment

Animal use protocols were approved by the University of Michigan Committee on the Use and Care of Animals. Male pigs (8–10 kg, Michigan State University) were sedated with ketamine (25 mg/kg, i.m.) and anesthetized with isoflurane. After a surgical plane of anesthesia was reached, animals were orotracheally intubated. The right femoral artery was catheterized for monitoring of blood pressure, blood gases and glucose concentrations. Body temperature was maintained at 37.5 ± 0.5°C.

A cranial burr hole (1.5 mm) was drilled 11 mm to the right of the sagittal and 11 mm anterior to the coronal suture. A 20-gauge sterile plastic catheter was then placed stereotaxically

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into the center of the right frontal cerebral white matter and cemented in place. Silicone elastomer tubing connected to the arterial catheter was filled with 5 mL of autologous arterial blood. An infusion pump was connected, and 1.0 mL of whole blood was injected over 15 min. After a 5 min break, another 1.5 mL of whole blood was injected over 15 min [10].

Brain Histology

Pigs were treated with DFX (50 mg/kg; i.m., given at 2 h after ICH and then every 12 h for up to 7 days) or vehicle. Pigs were reanesthetized on day 3 or day 7, and the brains perfused with 10% formalin. Paraffin-embedded brain was cut coronally into 10-μm-thick sections.

Enhanced Perls' Staining

Enhanced Perls' staining was performed to detect iron accumulation [11]. Paraffin sections were deparaffinized in xylol and alcohols of descending concentration, rinsed in distilled water, and incubated in Perls' Prussian blue staining solution (1:1, 5% potassium ferrocyanide/5% hydrochloric acid) for 45 min, followed by washing with distilled water. The sections were then incubated in 0.5% diamine benzidine tetrahydrochloride with nickel for 45 min.

Immunohistochemistry

Ferritin was examined by immunohistochemistry. The primary antibody was polyclonal rabbit anti-human ferritin IgG (DACO, 1:400 dilution). Normal rabbit IgG was used as a negative control.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL)

TUNEL staining was performed using a ApopTag Peroxidase Kit (Intergen). First, 3% hydrogen peroxide in 0.1MPBS was applied to sections for 5 min to quench endogenous peroxidases. After washing with PBS and equilibrating with the solution supplied, the specimens were incubated with TdT enzyme at 37°C for 1 h. The reaction was stopped by washing with buffer for 10 min. Anti-digoxingenin peroxidase conjugate was then applied to the slide for 30 min at room temperature. 3,3′ diaminobenzidine (DAB) was used for visualization. Omission of the terminal deoxynucleotidyl transferase was used as the negative control.

Photography for Cell Counting

Light microphotographs (×40, 4 fields from each distance) were taken at 200, 500 and 1,000 μm from the edge of the hematoma.

Statistical Analysis

Data from different animal groups and brain sites were expressed as mean ± SD and analyzed by Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

ICH resulted in the development of a reddish zone around the clot in pigs at day 3 and 7. Systemic DFX treatment reduced this reddish zone (Fig. 1). There were many Perls'-

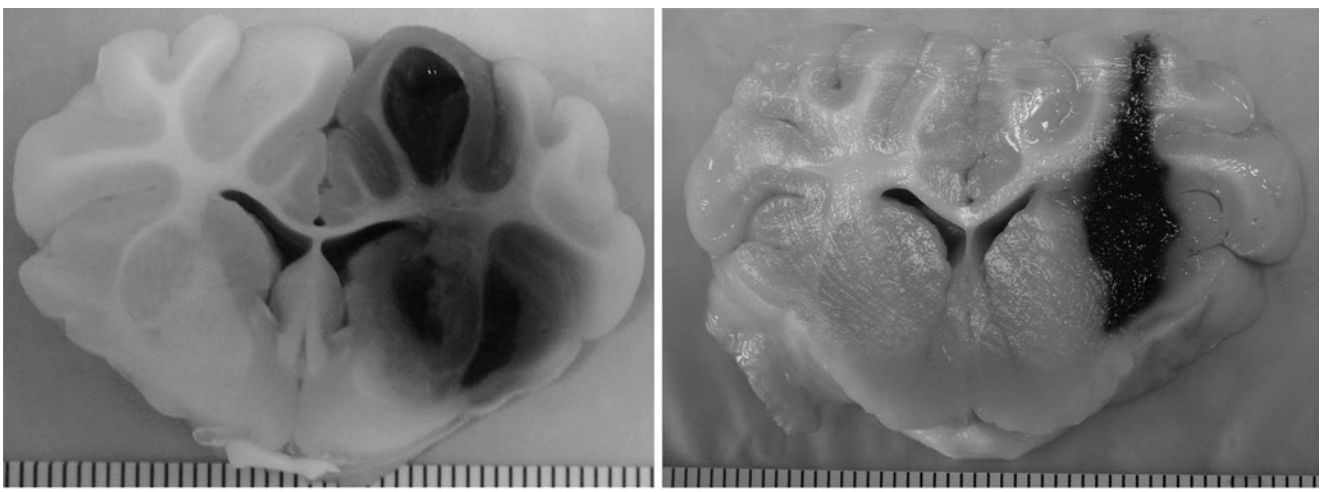


Fig. 1 Deferoxamine reduces the area of the reddish zone around the clot at 3 days after ICH

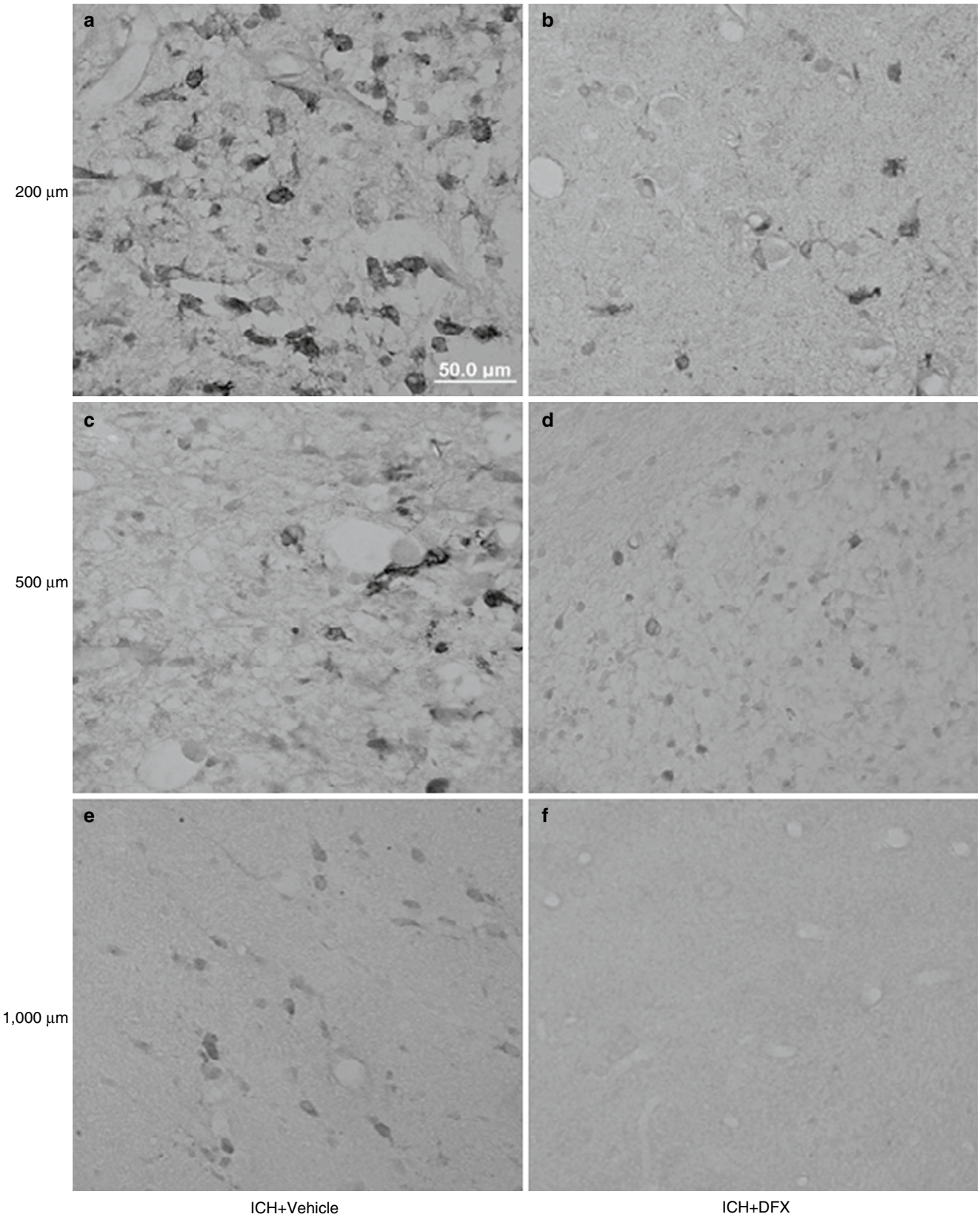


Fig. 2 Ferritin-positive cells in the perihematoma zone at different distances away from the edge of hematoma at day 7 after ICH. (**a**, **b**) 200 µm, (**c**, **d**) 500 µm and (**e**, **f**) 1,000 µm from the hematoma edge. The rats were treated by vehicle (**a**, **c**, **e**) or deferoxamine (**b**, **d**, **f**). Scale bar = 50 µm. (**g**) Quantification of ferritin-positive cells. Values are means ± SD, #$p < 0.01$ vs. ICH + Vehicle

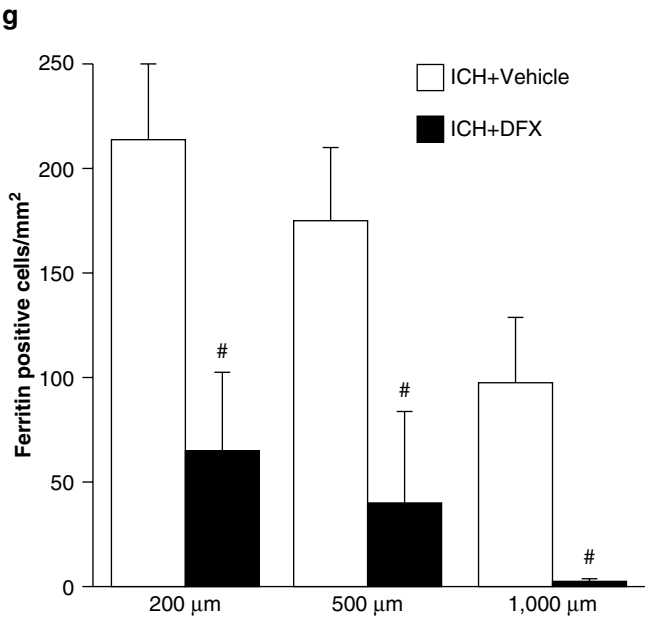


Fig. 2 (continued)

positive cells in the reddish zone, and DFX also reduced the number of these cells ($p<0.01$).

Ferritin was upregulated in the perihematomal area. Ferritin-positive cells around the hematoma were glia-like (Fig. 2). Most ferritin-positive cells were detected immediately next to the clot. There were fewer ferritin-positive cells in DFX-treated compared to vehicle-treated pigs (Fig. 2).

TUNEL staining was used to detect DNA damage in the brain. TUNEL-positive cells were found in the vicinity of the clot (Fig. 3). DFX treatment reduced the number of TUNEL-positive cells around the hematoma ($p<0.01$, Fig. 3).

Discussion

This study found that systemic DFX treatment reduces perihematomal iron accumulation and DNA damage in pigs. Our previous studies found that DFX is neuroprotective and can reduce brain edema and atrophy after ICH in young and aged rats [6–8]. DFX has protective effects in ICH models in two species (rats and pigs), as well as in young and aged animals, suggesting that DFX may also work in humans.

DNA damage was found in the perihematomal zone in pigs. Two pathways that can result in DNA damage are endonuclease-mediated DNA fragmentation and oxidative injury [12]. It is well known that reactive oxygen species can attack DNA directly, forming oxidative base damage and strand breaks [13], and that DNA damage by reactive oxygen species can be greatly amplified in the presence of free iron [14]. Our previous study showed that oxidative stress is a major cause of DNA damage in a rat model of ICH [4]. The current results suggest that iron released from the clot can cause DNA damage in pigs.

Iron overload following ICH is toxic to the brain. The duration over which clot lysis and iron release occurs is likely to be dependent on hematoma size. Our former data in rats [6–8] and present results in pigs show that DFX is effective in reducing brain injury in different ICH models with different sizes of clots.

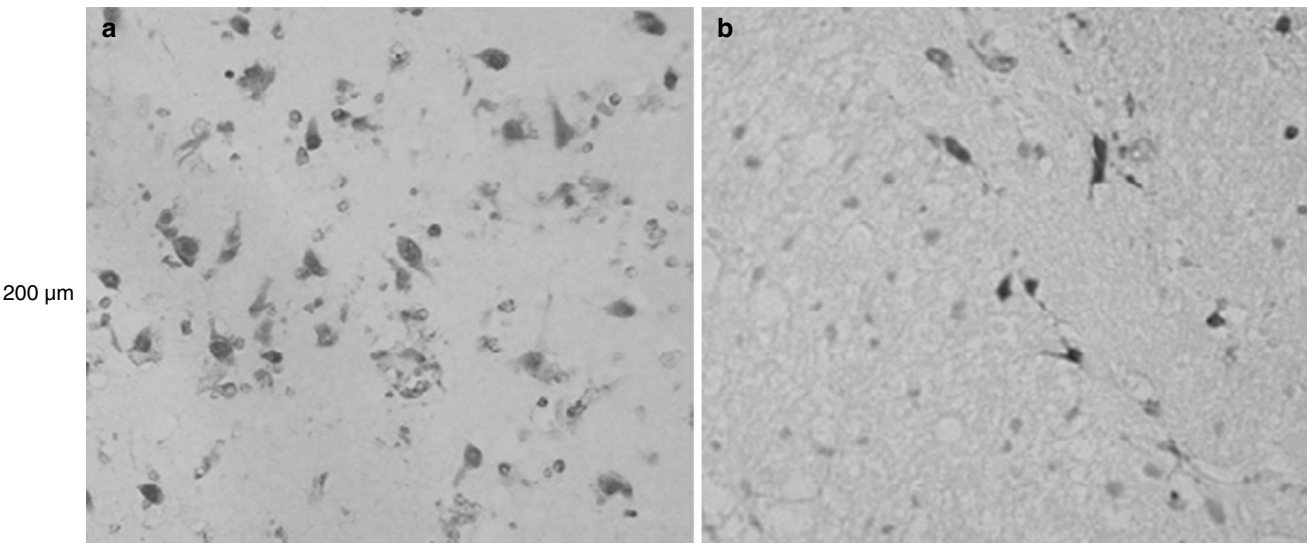
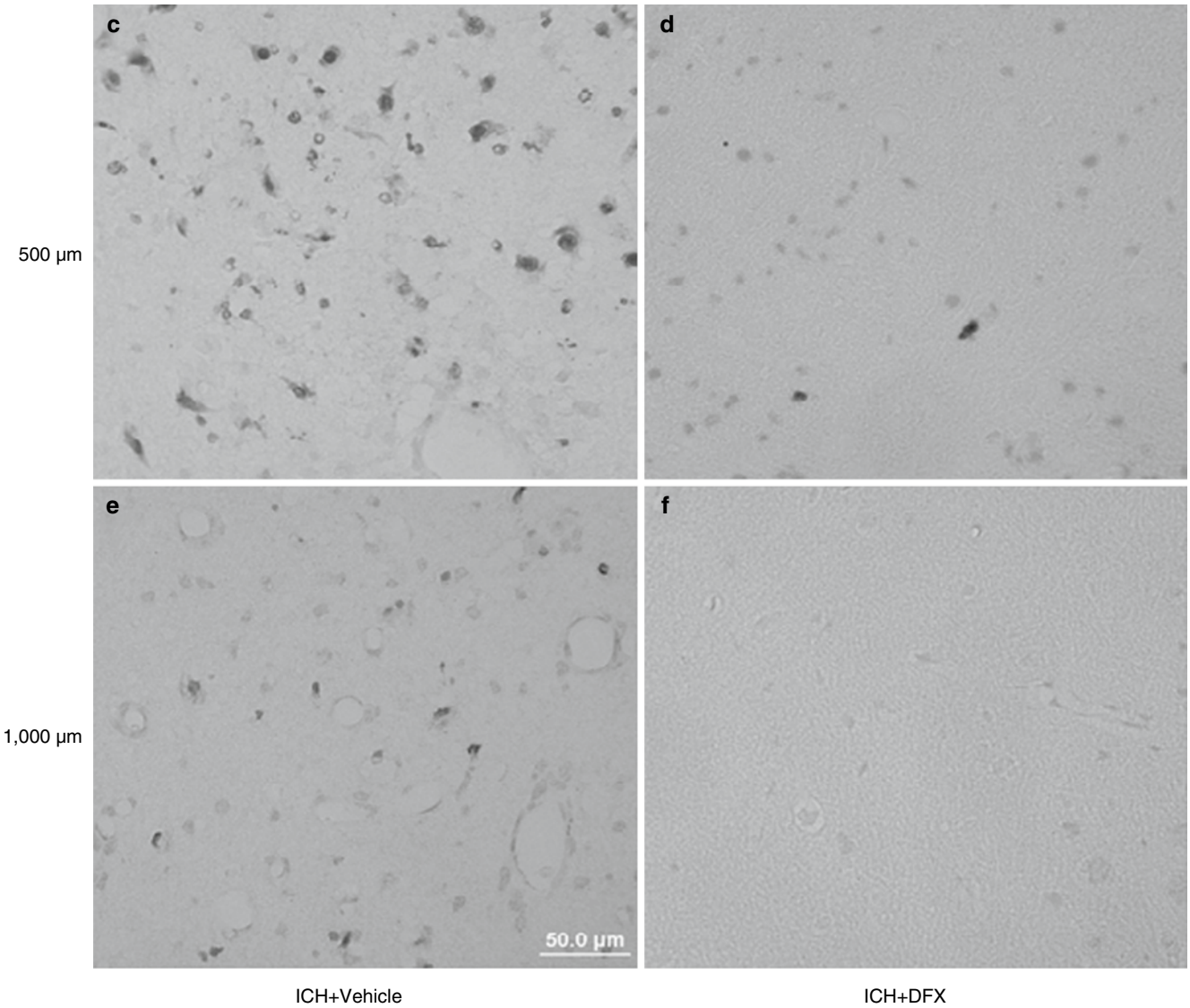


Fig. 3 TUNEL staining in the perihematoma zone at different distances away from the edge of the hematoma 3 days after ICH. (**a**, **b**) 200 µm, (**c**, **d**) 500 µm and (**e**, **f**) 1,000 µm from the edge. Rats were treated with vehicle (**a**, **c**, **e**) or deferoxamine (**b**, **d**, **f**). Scale bar = 50 µm. (**g**) Quantification of TUNEL-positive cells. Values are means ± SD, *$p<0.05$, #$p<0.01$ vs. ICH + Vehicle



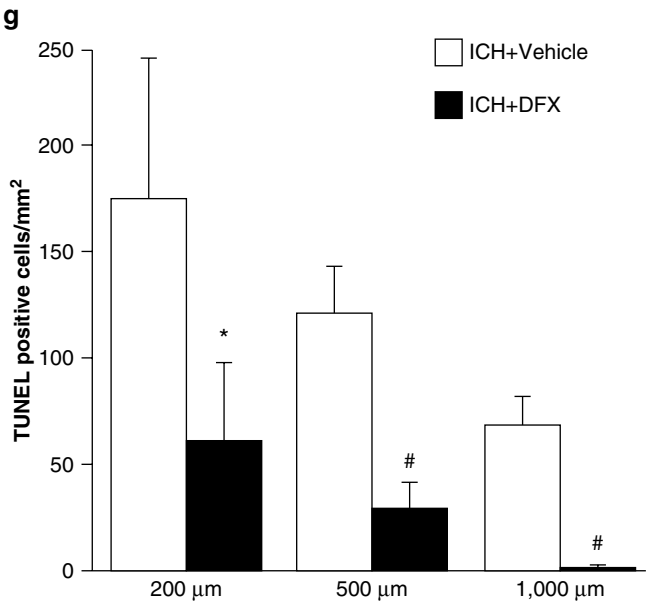


Fig. 3 (continued)

In conclusion, iron accumulation occurs in the pig brain, and systemic DFX treatment reduces ICH-induced DNA damage.

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Conflict of interest statement We declare that we have no conflict of interest.

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