A New Hippocampal Model for Examining Intracerebral Hemorrhage-Related Neuronal Death

Effects of Deferoxamine on Hemoglobin-Induced Neuronal Death

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Background and Purpose—There is an urgent need to develop a model in which to study the mechanism of intracerebral hemorrhage-induced neuronal death in vivo.

Methods—This study was divided into 2 parts: (1) Rats received either an infusion of hemoglobin, ferrous iron, or saline into the right hippocampus; (2) Rats had an infusion of hemoglobin and then were treated with either deferoxamine or vehicle. Rats were killed for hippocampus size, DNA damage, and neuronal death measurements.

Results—Compared with saline, hemoglobin or iron injection caused hippocampal neuronal death. Systemic use of deferoxamine reduced hemoglobin-induced DNA damage, hippocampal neuronal death, and atrophy.

Conclusions—This article demonstrates a new model and indicates that iron has a key role in hemoglobin–induced neuronal death. (Stroke. 2007;38:2861-2863.)

Key Words: cerebral hemorrhage ■ hemoglobin ■ iron ■ neuronal death ■ deferoxamine

Recently delayed brain atrophy has been found in a rat model of intracerebral hemorrhage (ICH),1,2 but ICH-induced neuronal death has been difficult to quantify. It is, therefore, important to develop an ICH model for quantification of neuronal loss.

Both in vivo and in vitro experiments have demonstrated that hemoglobin and iron contribute to brain injury after ICH.3,4 Recent studies have also shown iron overload occurs in the brain after ICH and contributes to ICH-induced DNA damage and brain atrophy.2 However, whether or not hemoglobin causes neuronal death in vivo has not been well studied.

The present study develops a new model for examining the mechanisms of ICH-related neuronal death involving injection of hemoglobin or iron into the rat hippocampus. The effects of systemic use of deferoxamine (DFX) on hemoglobin-induced neuronal death were also investigated.

Materials and Methods

Animal Preparation and Intracerebral Infusion

The University of Michigan Committee on the Use and Care of Animals approved the animal protocols. Adult male Sprague-Dawley rats (275 to 350 g, Charles River Laboratories, Portage, Mich) were anesthetized with pentobarbital (45 mg/kg, i.p.). Physiological parameters were recorded immediately before intrahippocampal injections and were in the normal range. Core body temperature was maintained at 37.5°C. Saline, hemoglobin, or FeCl₂ was infused into the right hippocampus stereotactically (coordinates: 3.8 mm posterior, 3.2 mm ventral, and 3.5 mm lateral to the bregma).

Experiment Groups

There were two parts in this study: (1) Rats (n = 6, each group) received an intrahippocampal injection of 10 μL of either saline, bovine hemoglobin, or FeCl₂; (2) Rats (n = 6 each group) received an intracerebral infusion of 10 μL bovine hemoglobin (150 mg/mL) and were treated with either deferoxamine (100 mg/kg, i.p. given immediately after hemoglobin injection, then every 12 hours for up to 7 days) or vehicle. All rats were killed at days 1 and 7, and the brains used for histology and DNA damage measurement.

Histological Studies

Histological studies were performed as previously described.5 Hematoxylin and eosin was used for staining. The brain sections from 1 mm posterior to the blood injection site were scanned and the hippocampus was outlined and measured, and the number of neurons was quantitatively analyzed in the CA1 region by a blinded observer. All measurements were repeated 3 times, and the mean value was used.

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

TUNEL staining was performed as previously described.6 TUNEL-positive cells in the hippocampus were counted.

Statistical Analysis

All the data in this study are presented as mean ± SD. Data were analyzed by ANOVA test or Student t test. A level of P < 0.05 was considered statistically significant.

Results

TUNEL-positive cells were found in the ipsilateral hippocampus 1 day after injections of hemoglobin (171 ± 111...
cells per slice) or FeCl₂ (173±5 cells per slice), but not saline. Many TUNEL-positive cells could still be detected at day 7 (Figure 1). DFX reduced TUNEL-positive cells in the ipsilateral hippocampus at days 1 (43±19 versus 247±116 cells per slice in the vehicle-treated group; \( P < 0.05 \)) and 7 (Figure 1).

Hemoglobin but not saline injection induced significant loss of neurons (Figure 2). Intrahippocampal injection of ferrous iron also caused marked loss of CA1 neurons (Figure 2B). Systemic use of DFX reduced hemoglobin-induced neuronal death (Figure 2A and 2C).

As well as neuronal death in the hippocampus, hemoglobin and iron resulted in significant hippocampal tissue loss at day 7 (Figure 3A and 3B). DFX again reduced hemoglobin-induced hippocampal atrophy (Figure 3C and 3D).

**Discussion**

Rat ICH models, involving infusion of autologous blood into the caudate and cortex, have been used extensively to study mechanisms of brain injury after ICH. However, it has been difficult to quantify neuronal death as it appears to be diffuse with no defined infarct. Therefore, there is an urgent need to develop a model to measure neuronal death after ICH. Our present results suggest hippocampal injection model is such a model.

Both hemoglobin and iron caused hippocampal neuronal loss and DFX reduced hemoglobin-induced neuronal death, indicating a role for iron in the neuronal toxicity of hemoglobin. Our previous experiments have demonstrated that iron overload occurs in the brain after ICH and iron contributes to ICH-induced brain edema formation, brain atrophy, and

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**Figure 1.** A and B, TUNEL staining in the hippocampus 7 days after an injection of saline (A) or hemoglobin (B). Scale bar=50-μm. C, TUNEL-positive cell counting in the ipsilateral hippocampus 7 days after an intrahippocampal injection of 10 μL either saline, hemoglobin (Hb), or ferrous iron (Fe²⁺). Values are mean±SD, n=6, #P<0.01 vs Saline, *P<0.05 vs Hb. D, TUNEL-positive cell counting 7 days after an intrahippocampal injection of 10 μL hemoglobin with or without deferoxamine treatment. Values are mean±SD, n=6, *P<0.05 vs Hb+Vehicle.

**Figure 2.** A, Hippocampal CA1 neurons 7 days after an intrahippocampal injection of 10 μL either saline, hemoglobin plus vehicle treatment (Hb+Vehicle), or hemoglobin plus deferoxamine treatment (Hb+DFX). B, Hippocampal CA1 neurons 7 days after an intrahippocampal injection of 10 μL either saline, hemoglobin, or ferrous iron (Fe²⁺). C, Hippocampal CA1 neurons 7 days after an intrahippocampal injection of 10 μL hemoglobin with or without deferoxamine treatment. Values are mean±SD, n=6, #P<0.01 vs saline group, ##P<0.05 vs Hb+Vehicle.
prolonged neurological deficits.\textsuperscript{2,7} The present results show iron can induce neuronal death in vivo. Although this study did not examine whether DFX treatment only delays the death of dysfunctional neurons, our recent study on a caudate ICH model indicates that DFX reduces long-term brain tissue loss and neurological deficits.\textsuperscript{2}

Promising evidence has suggested that oxidative stress may play a key role in DNA damage after ICH. First, 2 oxidative DNA injury markers, 8-hydroxyl-2'-deoxyguanosine (8-OHdG) and apurinic/apyrimidinic sites, are markedly increased in the perihematomal zone.\textsuperscript{8} Second, hemoglobin activates caspases in neurons, but caspase inhibition does not reduce hemoglobin neurotoxicity.\textsuperscript{9} Third, antioxidants reduce hemoglobin-induced neuronal death and caspase-3 activation in primary neuronal cultures.\textsuperscript{9}

Our previous studies have demonstrated DFX reduces edema formation, neurological deficits, and brain atrophy after ICH.\textsuperscript{2,7} DFX also reduces brain 8-OHdG levels after ICH.\textsuperscript{7} In the present study we demonstrated that DFX reduces DNA damage (TUNEL-staining) and neuronal loss induced by hemoglobin in vivo. DFX-related neuronal protection may be attributable to its effect of iron chelation because DFX reduces free iron levels in the CSF and brain ferritin immunoreactivity after ICH.\textsuperscript{2,10}

In conclusion, this paper demonstrates a new model for studying ICH-related neuronal death in vivo. It indicates that iron has a key role in hemoglobin–induced neuronal death and deferoxamine may be a useful treatment for ICH patients.

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Disclosures
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References
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