Unique Properties Associated with the Brain Penetrant Iron Chelator HBED Reveal Remarkable Beneficial Effects after Brain Trauma

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Abstract

Iron is postulated to contribute to secondary injury after brain trauma through various pathways including oxidative stress and inflammation. Therefore, one goal is to limit iron toxicity by either directly limiting iron activity, or limiting the secondary cascade mediated by iron, therefore rescuing the brain from damage after trauma. The *N,N'*-Di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid monohydrochloride (HBED) is a unique iron chelator that has the ability to cross the intact blood–brain barrier; it has a higher affinity to iron, and it has a longer half-life than most commonly used chelators. A controlled-cortical impact model of traumatic brain injury (TBI) was induced in mice. Mice were subcutaneously injected with HBED immediately after TBI, then at 12 h after, followed by a twice-a-day regimen until an end-point of 3 days. Neurobehavioral tests were performed daily. Cortical injury volume, hemispheric enlargement, and hippocampal swelling were quantified. Perls' iron immunostaining along with markers of gliosis, oxidative stress, and aquaporin (AQP) 4 were also performed. Data revealed that HBED treatment significantly decreases motor deficits and improves recovery after TBI. It also reduces cortical injury volume by $36.6 \pm 6.8\%$ ($p < 0.001$), hippocampal swelling by $23.4 \pm 3.8\%$ ($p < 0.05$), and total hemispheric volume by $13.3 \pm 2.7\%$ ($p < 0.01$). These effects are related to a reduction in microgliosis and oxidative stress markers in the impacted corpus callosum area by $39.8 \pm 7.3\%$, and by $80.5 \pm 0.8\%$ ($p < 0.05$), respectively. AQP4 staining is also attenuated in the hippocampus of HBED-treated mice. Therefore, our results suggest that HBED should be considered as a therapeutic tool to facilitate the recovery process following brain trauma.

Keywords: blood; edema; microglia; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is one of the leading causes of death, notably in young males. Brain injury after trauma is categorized into two pathological mechanisms. Primary injury is the direct impact on brain tissue, whereas secondary injury involves the multiple biological interactions that follow. Such interactions might include an increase in oxidative stress, electrolyte imbalance, mitochondrial dysfunction, and inflammatory responses.

Abnormal accumulation of iron is a specific feature of various chronic neurological diseases such as Parkinson's disease and Alzheimer's disease; this accumulation tends to persist over the long term.¹ Although the majority of iron is found bound to iron-binding proteins, this balance shifts after trauma toward an increase in the chelatable iron pool. This shift is usually due to bleeding, cell death, and other mechanisms.

Iron chelators such as deferoxamine (DFO, clinically named Desferal®) have shown improvement in cognitive function after

TBI even though they are accessible to the brain only where the blood–brain barrier (BBB) is damaged.² The *N,N'*-Di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid monohydrochloride (HBED) is an iron chelator and hydroxyl radical inhibitor^{3,4} that has been proven to cross not only the intact BBB but also into brain cells and mitochondrial membranes.^{4–6} Further, it has higher affinity and selectivity toward iron compared with DFO.⁷ HBED binds ferrous iron and converts it into ferric iron and it is mostly excreted in the stool rather than in urine.⁷ Thus, it “deactivates” ferrous iron, which can become harmful to brain cells when in free state, and alleviates iron's harmful effect. These specific characteristics give HBED an advantage over the commonly used iron chelators. Consequently, HBED has been tested for its bioavailability and efficiency in rodents and primates.⁷ It has been demonstrated to be non-toxic in clinical settings other than skin irritation at the site of administration.⁸

It is expected for an iron chelator with such characteristics as HBED to show neuroprotective effects in terms of behavioral and

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anatomical outcomes following an acute trauma such as TBI. Moreover, because microglia activation is directly associated with neuroinflammation, it is likely that decreases in microgliosis would indicate a protective effect through microglial stabilization.⁹

Oxidative stress is one of the major pathways that augment injury after brain trauma. One of the proposed paramount properties of HBED is reducing oxidative stress by two different pathways; first by chelating iron, and second, by functioning as an H-donating agent.^{3,4} Moreover, oxidative stress could also result in trauma-induced edema. Edema, also frequent in intracranial bleeding, has considerable morbid consequences and can lead to death. One of the well-studied mechanisms associated with cell edema involves aquaporin (AQP) channels.¹⁰ Studies have demonstrated the deleterious role of AQP4 after TBI.^{11,12} Iron has been shown to augment oxidative stress and AQP4 channels expression in astrocytes.^{10,11} A potent iron chelator such as HBED is likely to attenuate oxidative stress, decrease AQP expression, and reduce brain edema after TBI.

In this current study, a penetrating traumatic impact was inflicted on C57BL/6 male adult mice. Neurological deficits and lesion volumes were assessed through and at 72 h, respectively. To test the possible beneficial effects of HBED, it was administered after the TBI insult and compared with control. Finally, the role of HBED treatment in TBI was assessed through behavioral testing, injury volumes, and various immunohistochemical staining.

Methods

Mice

All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health PHS Policy on the Humane Care and Use of Laboratory Animals. Mice were maintained in a temperature-controlled animal room on a 12-h light/dark cycle. Four cohorts of C57BL/6 male mice were used. This 3-day study used a total of 36 randomized mice (4.5 ± 0.5 months). Two cohorts ($n=12$ each) were used as controls and HBED treatment. Procedures in sham cohorts were performed with two groups equally assigned with and without HBED treatment ($n=6$ each). No mice died or were excluded from this study. All procedures, including HBED and vehicle delivery, functional outcomes, and anatomical and histological analyses were performed, assessed, and analyzed in a blinded manner.

Controlled-cortical impact model of TBI

The controlled cortical impact (CCI) model of TBI was induced by following the procedure we have described earlier with some modifications.¹³ Briefly, mice were anesthetized with 4% isoflurane and were maintained with 1.5–2% isoflurane during surgical procedures. Mice were placed in the stereotaxic apparatus and the skull was exposed by a 1-cm incision using a scalpel blade. The skin was displaced with a cotton tip, and a circular craniotomy approximately 4 mm in diameter was made in the right parietal bone. Mild-to-moderate impact on the right hemisphere was inflicted using the CCI model (PCI3000 PinPoint Precision Cortical Impactor, Hatteras Instruments, Cary, NC) with an impact tip diameter of 3 mm, a velocity of 3 m/sec, and a compression distance and time of 1.5 mm and 100 msec, respectively. A sterile cylindrical-shaped ring was placed around the craniotomy and sealed by a glass coverslip to preserve brain tissue and minimize external confounding factors. After closing the incision, the mice were placed into a temperature-controlled recovery chamber for at least 1 h before being moved to a clean home cage. During the entire surgical procedure mouse rectal temperature was monitored

and maintained at $37.0^\circ\text{C} \pm 0.5^\circ\text{C}$ using a controlled heating pad (Fine Science Tools, Vancouver, BC, Canada).

HBED dose and administration

The HBED iron chelator (Strem Chemicals, Inc., Newburyport, MA) was dissolved in sterile phosphate buffered saline,^{7,14} and then filtered through a 20- μm Whatman filter. HBED was administered in a 50-mg/kg subcutaneous dose. The dose used in this study is based on previous studies showing the effect of this dose in removing excess iron from the brain and other organs in naïve animals.^{6–8,15,16} Each mouse received a loading dose of 100 mg/kg immediately after the onset of TBI and then received a dose of 50 mg/kg/12 h for 72 h. Thus, each mouse received 150 mg/kg during the first 24 h and 100 mg/kg/24 h for the remaining days. HBED has significant bioavailability after such a subcutaneous protocol.⁷ More specifically, in previous studies, HBED levels in brain were detectable at 1 h, peaked at 3 h, and remained stable for at least 6 h, which gives it an advantage over other iron chelators such as DFO, which has a shorter half-life.⁶

Neurologic deficit scores

Functional outcomes after TBI were assessed by neurological deficit scores (NDS) and rotarod tests daily. These outcomes were assessed in the same order and at the same time of day, with 1 h of rest between the tests.^{17,18} As we maintained our mice in a reversed light/dark cycle facility, these behavioral protocols were performed during their active, awake period. The NDS behavioral test assesses motor function by examining body and front limb symmetry, circling and compulsory circling behavior, and climbing. Higher scores are given for more severe injuries and lower scores for mild to no injury. The scores were graded from 0 to 4 in increasing deficit order for each function assessed. Each mouse was assessed by two independent investigators and the scores for each function were combined. The average of both investigators' final score is reported.

Rotarod

The accelerating rotarod tests mice for their motor ability, coordination endurance, and balance (Rotamex-5, Columbus Instruments, Columbus, OH). Baseline was the final read after 3 consecutive days of training where each mouse had three sessions of training in the morning and another three sessions in the afternoon. Rotational speed began at 8 rpm and ended at 50 rpm, and the latency to fall was collected automatically by the machine software.^{17,19}

Histology and quantification

Histology and quantification were conducted as described.^{17,18} Briefly, 16 sections equally distributed throughout the trauma area were processed. Cresyl violet staining was used to assess anatomical tissue injury, hemispheric enlargement, hippocampal swelling, and bleeding amounts. Perls' iron staining was used to evaluate iron following a previously described protocol.^{20,21} Immunohistochemistry staining was performed using anti-Iba1 (1:1500, Wako Chemicals, Richmond, VA), anti-AQP4 (1:2000, Thermo Fisher Scientific, Waltham, MA), and anti-4-HNE (1:750, Abcam, Cambridge, MA). Secondary matched antibodies and 3,3'-diaminobenzidine protocols were provided by Vector Laboratories (Burlingame, CA). All brain sections were simultaneously stained. Slides were scanned using an Aperio ScanScope CS and analyzed with ImageScope software (Leica Biosystems, Wetzlar, Germany).

For quantification of brain pathology (cortical lesion, hippocampal swelling, hemisphere volumes, and Perls' iron), all sections were quantified. Injured brain areas were outlined. Using these areas, known distances between sections, and section thickness, a total brain injury volume was calculated. For Perls' staining and blood quantification, brain compartments were outlined. Ipsilateral

cortical and hippocampal microgliosis, 4-HNE, and AQP4 expression were analyzed by placing identically sized boxes of 1000×1000 pixels in these regions, whereas 500×500 pixel squares were used to assess 4-HNE and microgliosis in the impacted corpus callosum. Hemispheric microgliosis and AQP4 expression were analyzed by outlining of the ipsilateral hemisphere, including the lesion area, and these data are presented as the signal per area quantified. A positive pixel count algorithm was optimized for each stain such that the appropriate signal level was detected and the data were reported as positivity ratio or positive pixel count per given area.²²

Statistical analysis

To compare NDS and anatomical differences between the experimental and control groups, *t* test with equal variants was used. Generalized regression data analysis was used for progression of NDS performance over time. Mixed model regression data analysis and Tukey's post hoc comparison test between matched groups was used for rotarod assessment. All data were checked for normality and equal variances and are presented as mean $\pm$ standard error of mean; *p*-values <0.05 were considered significant.

Results

HBED improves behavioral outcomes

In support of our working hypothesis, HBED administration temporally influences functional outcomes (Fig. 1). At the 2-day time-point, HBED-treated mice displayed $57.5 \pm 6.6\%$ lower combined NDS (5.29 ± 1.35 vs. 2.25 ± 1.21 , $p < 0.001$). The most prominent differences were observed in front limb asymmetry (0.66 ± 0.57 vs. 0.12 ± 0.22 , $p < 0.001$) and circling behavior (1.08 ± 0.51 vs. 0.38 ± 0.11 , $p < 0.001$). At the 3-day time-point, HBED-treated mice displayed significantly lower combined NDS (4.16 ± 1.00 vs. 1.62 ± 1.30 , $p < 0.001$), front limb asymmetry (0.50 ± 0.36 vs. 0.04 ± 0.14 , $p < 0.05$), and circling (0.79 ± 0.39 vs. 0.04 ± 0.14 , $p < 0.001$). Using the rotarod, the assessed motor functions were not significantly different between the HBED and control groups (Fig. 1).

HBED reduces cortical injury, hippocampal swelling, and total hemisphere volume

HBED treatment resulted in an apparent reduction in TBI-induced brain damage (Fig. 2). On day 3 after TBI, HBED-treated mice had $36.6 \pm 6.8\%$ smaller cortical lesion volumes ($11.33 \pm 0.51 \text{ mm}^3$ vs. $7.37 \pm 0.85 \text{ mm}^3$, $p < 0.01$). We also observed that the hemispheric enlargement in HBED-treated mice was $13.3 \pm 2.7\%$ smaller ($98.39 \pm 1.99 \text{ mm}^3$ vs. $86.95 \pm 3.31 \text{ mm}^3$, $p < 0.001$). Similarly, HBED treatment also resulted in $23.4 \pm 3.8\%$ less hippocampal swelling ($13.36 \pm 0.88 \text{ mm}^3$ vs. $10.49 \pm 0.63 \text{ mm}^3$, $p < 0.001$). There were no significant differences in sham groups between untreated and treated mice for hemispheric volume ($87.34 \pm 5.57 \text{ mm}^3$ vs. $82.88 \pm 4.75 \text{ mm}^3$), and hippocampal volume ($7.26 \pm 0.80 \text{ mm}^3$ vs. $5.99 \pm 0.85 \text{ mm}^3$).

HBED's effect on AQP4 immunohistochemical staining

To gain knowledge on potential mechanisms for HBED in attenuating hippocampal brain swelling, immunohistochemistry staining for AQP4 was performed and revealed a significant reduction in AQP4 expression in the hippocampus of treated animals by $81.8 \pm 5.6\%$ ($32.16 \times 10^5 \pm 10.18 \times 10^5 \text{ AU}$ vs. $5.83 \times 10^5 \pm 1.80 \times 10^5 \text{ AU}$, $p < 0.05$). AQP4 staining was not significantly different between the control and HBED groups in cortical injury areas ($71.23 \times 10^5 \pm 2.95 \times 10^5 \text{ AU}$ vs. $24.54 \times 10^5 \pm 12.03 \times 10^5 \text{ AU}$) or total hemisphere ($1.71 \pm 0.22 \text{ AU}$ vs. $1.37 \pm 0.23 \text{ AU}$; Fig. 3).

FIG. 1. HBED treatment improves functional outcomes after traumatic brain injury. At 24, 48, and 72 h following traumatic brain injury, investigators blinded to groups performed two separate neurobehavioral tests: neurological deficit scoring (NDS) and rotarod. (A) HBED-treated mice displayed a significantly improved rate of recovery in focal neurological deficits. HBED mice had significantly better neurological function as identified by lower NDS scores at the 48- and 72-h end-points (left). On regression testing of NDS scores, a significant improvement over time was detected (right). (B) Motor functions that were assessed by rotarod test were not significantly different between HBED and control groups. All comparisons include $n = 12$; ** $p < 0.01$. HBED, *N,N'*-Di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid monohydrochloride.

HBED's effect on Iba1 immunohistochemical staining (a microgliosis indicator)

To monitor the potential effect of HBED on microgliosis, immunohistochemistry staining for Iba1 was performed and revealed a significant reduction in Iba1 staining in the traumatized portion of the corpus callosum by $39.8 \pm 7.3\%$ ($3.08 \times 10^5 \pm 0.35 \times 10^5 \text{ AU}$ vs. $1.94 \times 10^5 \pm 0.26 \times 10^5 \text{ AU}$, $p < 0.05$). Iba1 staining was not significantly different between the HBED and control groups in cortical injury areas ($48.01 \times 10^5 \pm 6.90 \times 10^5 \text{ AU}$ vs. $40.75 \times 10^5 \pm 5.54 \times 10^5 \text{ AU}$), or in the hippocampus ($15.96 \times 10^5 \pm 2.61 \times 10^5 \text{ AU}$ vs. $11.88 \times 10^5 \pm 2.01 \times 10^5 \text{ AU}$). Our data revealed significant reduction of Iba1 staining in total hemisphere by $42.4 \pm 7.9\%$ ($8.98 \pm 1.13 \text{ AU}$ vs. $5.16 \pm 0.70 \text{ AU}$, $p < 0.001$; Fig. 4). There were no significant differences in sham groups between untreated and treated mice for cortex ($20.10 \times 10^5 \pm 4.66 \times 10^5 \text{ AU}$ vs. $17.05 \times 10^5 \pm 5.83 \times 10^5 \text{ AU}$), corpus callosum ($0.08 \times 10^5 \pm 0.03 \times 10^5 \text{ AU}$ vs. $0.01 \times 10^5 \pm 0.04 \times 10^5 \text{ AU}$), and hippocampus ($2.51 \times 10^5 \pm 0.88 \times 10^5 \text{ AU}$ vs. $1.05 \times 10^5 \pm 0.90 \times 10^5 \text{ AU}$).

HBED's effect on extravasated blood

Because TBI also results in intracranial hemorrhages (ICHs), we investigated the role of HBED in bleeding outcomes after TBI. The results at 3 days were that HBED-treated mice showed no

FIG. 2. HBED treatment improves outcomes after traumatic brain injury. Both HBED and control mice were subjected to controlled cortical injury (CCI). HBED mice received treatment immediately after trauma and then every 12 h for a straight 72 h. Control mice received CCI in the same manner as HBED mice. Thereafter, brains were harvested by perfusion and fixation. Cryosections were stained with cresyl violet for lesion volume quantification. (A) Representative macrographs of cresyl violet-stained sections obtained from HBED ($n=12$), control ($n=12$). Scale bar=2 mm. (B) HBED treatment resulted in a significant decrease in the total ipsilateral hemispheric volume as compared with the control ($n=12$). (C) HBED treatment resulted in a significant decrease in the ipsilateral cortical injury volume compared with control ($n=12$). (D) HBED treatment resulted in a significant decrease in hippocampal swelling compared with the control ($n=12$); * $p<0.05$; ** $p<0.01$, *** $p<0.001$. Scale bar=2 mm. *N,N'*-Di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid monohydrochloride. Color image is available online at www.liebertpub.com/neu

significance in total hemispheric blood compared with the mice that received vehicle ($15.60 \times 10^{-3} \pm 0.20 \times 10^{-3}$ AU vs. $8.80 \times 10^{-3} \pm 2.04 \times 10^{-3}$ AU), and no significant difference was observed between the control and HBED groups in the cortical injury areas ($11.11 \times 10^{-2} \pm 2.08 \times 10^{-2}$ AU vs. $8.18 \times 10^{-2} \pm 1.21 \times 10^{-2}$ AU), or in the hippocampus area ($28.06 \times 10^{-4} \pm 97.06 \times 10^{-4}$ AU vs. $22.05 \times 10^{-4} \pm 8.05 \times 10^{-4}$ AU).

HBED's effect on Perls' staining

Considering that we did not detect significant differences in overall intracranial bleeding and considering that lysis of erythrocytes releases large amounts of hemoglobin molecules, we also investigated changes in iron accumulation by performing Perls' staining (Fig. 5). The staining showed no significant difference in the total hemispheric Perls' stain ($24.05 \times 10^{-4} \pm 4.07 \times 10^{-4}$ AU vs. $21.56 \times 10^{-4} \pm 2.45 \times 10^{-4}$ AU). Perls' staining was not significantly different between control and HBED groups in the cortical area ($30.12 \times 10^{-3} \pm 8.54 \times 10^{-3}$ AU vs. $35.20 \times 10^{-3} \pm 17.04 \times 10^{-3}$ AU) or the hippocampus ($29.05 \times 10^{-4} \pm 6.05 \times 10^{-4}$ AU vs. $42.00 \times 10^{-4} \pm 9.06 \times 10^{-4}$ AU; Fig. 5).

HBED's effect on 4-HNE immunohistochemical staining

Addressing the potential role of HBED in reducing oxidative stress, immunohistochemistry for 4-HNE revealed a significant reduction in 4-HNE expression in the corpus callosum of treated animals by $80.5 \pm 0.8\%$ ($32.16 \times 10^5 \pm 10.18 \times 10^5$ AU vs. $5.83 \times 10^5 \pm 1.80 \times 10^5$ AU, $p<0.05$). 4-HNE staining was not significantly different between the control and HBED groups in cortical injury areas ($37.83 \times 10^5 \pm 7.50 \times 10^5$ AU vs. $15.27 \times 10^5 \pm 2.82 \times 10^5$ AU) or the hippocampal region ($70.79 \times 10^4 \pm 18.25 \times 10^4$ AU vs. $28.52 \times 10^4 \pm 16.51 \times 10^4$ AU; Fig. 6). There were no significant differences in sham groups between untreated and treated mice for the cortex ($10.36 \times 10^5 \pm 0.67 \times 10^5$ AU vs. $11.20 \times 10^5 \pm 0.92 \times 10^5$ AU), corpus callosum ($0.04 \times 10^5 \pm 0.01 \times 10^5$ AU vs. $0.10 \times 10^5 \pm 0.44 \times 10^5$ AU), or hippocampus ($1.36 \times 10^4 \pm 1.32 \times 10^4$ AU vs. $4.62 \times 10^4 \pm 2.62 \times 10^4$ AU).

Discussion

This is the first study to assess the HBED iron chelator potency as a neuroprotective agent after open-head severe TBI. Our data

FIG. 3. HBED affects aquaporin-4 (AQP4) immunoreactivity. Brain sections were immunostained for AQP4 immunoreactivity to further understand changes in hippocampal brain swelling. (A) Representative of AQP4 protein expression at 1× showing immunoreactivity in HBED and control mice. Scale bar=2 mm (left). (B) Ipsilateral total hemispheric immunoreactivity for AQP compared between control and HBED groups. (C,E) The regions in gray boxes in panel A were magnified at 10× for right side on cortex and hippocampus. (D,F) Although there is no significance in cortical AQP4 immunoreactivity, quantification of the AQP4 signal shows significant reduction in AQP4 immunoreactivity in HBED-treated mice in the traumatized hippocampus compared with the control group. $N=5/\text{group}$; $*p<0.05$. N,N' -Di(2-hydroxybenzyl)ethylenediamine- N,N' -diacetic acid monohydrochloride. Color image is available online at www.liebertpub.com/neu

revealed that HBED alleviates the extent of the brain injury and limits deteriorated functional outcomes after TBI. HBED improves overall general motor functions, especially front limb symmetry and circling behavior after trauma. These significant behavioral findings are accompanied by significant reductions in hemispheric volume, injury volumes, and hippocampal swelling. In an attempt to probe the mechanisms that govern these protective effects, we assessed the microgliosis in different brain locations and found a significant reduction in Iba1 staining of the injured corpus callosum, whereas an overall reduction of microgliosis was also noticed in cortical and hippocampal areas, and all over the ipsilateral hemisphere. To investigate these changes in brain swelling, we assessed the expression of AQP4 at similar locations and found that

it is significantly lower in the hippocampus of the HBED-treated animals. To further assess the neuroprotective role of HBED, we looked into the 4-HNE immunoreactivity, a marker for oxidative stress, and found significant reduction in the immunoreactivity in corpus callosum suggesting attenuated oxidative stress. Together, these results demonstrate that HBED should be considered as a potential therapeutic agent in addition to the standard of care for these patients.

There are three main iron chelators approved by the FDA: DFO (desferrioxamine B; Desferal®), deferasirox (Exjade®), and deferiprone (Ferriprox®).^{14,23–25} Even though DFO is recognized for its possible use in treating experimental TBI, there are some notable issues associated with its use. DFO is not injected intravenously for

FIG. 4. HBED attenuates microgliosis. Brain sections were immunostained for Iba1 immunoreactivity to monitor microgliosis. **(A)** Representative macrographs at $1\times$ showing Iba1 immunoreactivity in HBED and control mice. Scale bar = 2 mm. **(C,E)** The regions in gray boxes in panel A were magnified at $10\times$ for the right side of the cortex and hippocampus. Although there is no significance in cortical and hippocampal microgliosis, a significant difference toward reduction in microgliosis was found in the total hemisphere. Scale bar = $200\ \mu\text{m}$. **(G)** The regions in gray boxes in panel A were magnified at $10\times$. The microglia in the corpus callosum exhibited amoeboid morphology, whereas those in the intact area show activated morphology. **(B)** Ipsilateral total hemispheric immunoreactivity for Iba1 compared between control and HBED groups shows significance. **(D,F)** Represents ipsilateral cortical and hippocampal immunoreactivity for Iba1 compared between control and HBED groups. **(H)** Quantification of the Iba1 immunoreactivity shows a significant reduction in Iba1 immunoreactivity in HBED-treated mice in the traumatized corpus callosum area compared with the control group. Scale bar = $100\ \mu\text{m}$. $N = 12/\text{group}$; $*p < 0.05$; $***p < 0.001$. *N,N'*-Di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid monohydrochloride. Color image is available online at www.liebertpub.com/neu

FIG. 5. HBED did not significantly affect the Perls' staining. As an estimation of iron, Perls' staining was performed. **(A)** Macrographs showing the sections obtained from HBED and control-treated mice. Scale bar = 2 mm. **(C,E)** The regions in gray boxes in panel A were magnified at 10 $\times$. Scale bar = 200 μ m. **(B,D,F)** Quantification of Perls' staining shows no significant difference in the iron content in HBED and control-treated groups. $N = 12/\text{group}$. N,N' -Di(2-hydroxybenzyl)ethylenediamine- N,N' -diacetic acid monohydrochloride. Color image is available online at www.liebertpub.com/neu

two main reasons: first, it is a small molecule and is eliminated rapidly through the kidney, with a very short plasma half-life in humans.^{23,26} The initial half-life of DFO after intravenous infusion is 0.28 h and the terminal half-life is 3.05 h. Second, the injection of an intravenous bolus of DFO can cause acute hypotension that is rapid and can be lethal.^{16,27,28} Therefore, effective therapy usually requires subcutaneous or intravenous administration by a portable infusion pump for 9 to 12 h daily, which can decrease the adherence of patients to this treatment.^{6,7} In addition, using HBED allows safe routes of administration compared with the intranasal route, which is intended to bypass the BBB and reduce systemic exposure as in DFO. Subcutaneous and intramuscular injections of DFO are often associated with local injection site reactions. Deferasirox and deferiprone, although orally active, have some associated toxicities. Deferasirox has been reported for possible renal and hepatic toxicity, and gastrointestinal hemorrhage.^{23,29} Deferiprone has also

been reported for possible neutropenia and agranulocytosis.^{14,30} On the other side, HBED has a longer half-life and a relatively safer toxicity index.^{6-8,24} Moreover, HBED has the advantage of being safe to administer systematically and acutely without being sequestered rapidly by kidneys and without side effects such as severe hypotension. Because HBED is a synthetic product, patients allergic to DFO would be unlikely to experience a similar response with HBED treatment.¹⁶ Subcutaneous administration of HBED to iron-loaded monkeys consistently induced iron clearance that was 2 to 3 times greater than that induced by an equimolar dose of DFO and no adverse effects of chelator administration were observed.⁷ HBED has the ability to penetrate the BBB and mitochondrial membrane, which provides an in-depth effect against iron toxicity.^{1,6}

Clinically, it is common for hemoglobin to be present at the area of contusion after TBI due to disruption of blood vessels. The lysis of a red blood cell releases between 250 and 290 million molecules of

FIG. 6. HBED decreases oxidative stress. Brain sections were immunostained for 4-HNE immunoreactivity to monitor oxidative stress. **(A)** Representative of macrographs at $1\times$ showing Iba1 immunoreactivity in HBED and control mice. Scale bar = 2 mm. **(B,D)** The regions in gray boxes in panel A were magnified at $10\times$ for the right side of the cortex and hippocampus. Although there is no significance in cortical and hippocampal oxidative stress, a trend toward reduction in oxidative stress was found in the total hemisphere. Scale bar = $200\mu\text{m}$. **(F)** the regions in gray boxes in panel A were magnified at $10\times$. **(C,E)** Represent ipsilateral cortical and hippocampal immunoreactivity for 4-HNE compared between control and HBED groups. **(G)** Quantification of the 4-HNE immunoreactivity shows a significant reduction in 4-HNE immunoreactivity in HBED-treated mice in traumatized corpus callosum area compared with the control group. Scale bar = $100\mu\text{m}$. $N=5/\text{group}$; $*p<0.05$. N,N' -Di(2-hydroxybenzyl)ethylenediamine- N,N' -diacetic acid monohydrochloride. Color image is available online at www.liebertpub.com/neu

hemoglobin and each molecule contains four heme molecules. In addition, non-heme iron tends to accumulate in the brain after a brain injury that is accompanied by damage of the BBB, which could result in the release of iron from transferrin to the plasma.³¹ Consequently, iron overload would play a major role in secondary brain injury following TBI. Therefore, the use of iron chelators to sequester iron presents a unique therapeutic approach to limit TBI-mediated brain injury.

The reduction in cortical injury volume aligns with the reduction of hippocampal swelling. This protective effect could be summarized in many underlying pathways and mechanisms. Because inflammation and oxidative stress play an important role in the

development of neurological deficits after TBI, we hypothesized that microglial activation would play a key role. Microglial activation is shown to be significantly increased in iron loaded cells.^{23,32} Further, administration of the iron chelator minocycline significantly attenuates the microglial response both in the hippocampus and the cortex as reflected by the reduced level of Iba1 expression in immature brains under stress.³³ Microglia contribute not only to inflammation processes but also to the control of oxidative stress. The potential sources of oxygen radicals following TBI include catecholamine oxidation, mitochondrial leakage, and oxidation of extravasated hemoglobin. This harmful process can compromise the BBB following TBI even more, leading to

increased free iron in the brain by breakdown of hemoglobin and/or ferritin. Hydroxyl radicals are a primary source of free radicals that can lead to various injuries to the central nervous system. One of the major pathways for their formation is the Haber-Weiss reaction. HBED, as an iron chelator, scavenges free iron available for the Fenton reaction, thus decreasing the generation of hydroxyl radicals.³⁴ Our data show an overall trend of reduction in microglial activation throughout all cerebral compartments in HBED-treated mice. Interestingly, the significant reduction of microgliosis was located specifically in the corpus callosum. This can provide an explanation for the observed improvement of functional outcomes in the HBED-treated mice.

This iron chelator is shown to be a potent antioxidant agent not only by chelating iron, but also by functioning as an H-donating antioxidant.^{3,4} Studies have shown that ferric iron chelated by HBED is not reducible by free oxygen radicals. Therefore, the ferric ion binding by HBED keeps the redox interaction inactive. One of the important markers for oxidative stress is 4-HNE and it has been reported to be highly augmented after trauma.³⁵ When testing the antioxidant property of HBED by performing 4-HNE immunoreactivity in our experimental conditions, we found that HBED significantly attenuated the 4-HNE immunoreactivity in the corpus callosum with an accompanied trend for reduction pattern in the ipsilateral cortex and hippocampus.

Vascular edema is commonly observed after injury and it is a result of BBB damage mediated by iron-generated free radicals.^{36–38} Moreover, AQP4 channels are also implicated in the formation of brain edema and the increase in intracerebral pressure.¹¹ Previous studies have shown the positive correlation between AQP4 expression and the extent of astrocyte activation.³⁹ Astrocytes are known to be activated in concussed areas of the brain and this activation is directly and indirectly affected by iron and iron-generated free radicals.⁴⁰ Because AQP4 is closely related to astrocyte reactivity, it has been shown that minimizing oxidative stress or using iron chelators such as DFO can decrease the expression of AQP4 and hence improve brain edema.^{3,24} Similarly, we also found that HBED resulted in significant decrease in the hippocampal swelling, which could be the consequence of reduced oxidative stress and reduced expression of AQP4.

It has been described that after TBI, the expression of iron-handling proteins such as ferritin, transferrin, and transient receptor potential canonical will significantly increase in response to high levels of iron.⁴¹ However, administration of iron chelators significantly inhibited the increase of these proteins, which can imply the role of iron chelators in the protection of cellular functions and integrity. Therefore, we believe that iron chelator administration can attenuate the stress of a high-iron environment in the brain that can impede the internal cellular balance.⁴¹ However, the mechanisms underlying this relationship require further study.

Although there are several studies that have investigated the ability of HBED to excrete iron, similar studies in the context of TBI are lacking. In our experimental conditions, we found that Perls' staining was not significantly different in HBED-treated and vehicle-treated TBI groups, although this drug has the ability to deactivate harmful iron and quench the iron overload that results from bleeding, cellular death, and imbalanced oxidative stress.⁴² Nevertheless, there might be several possibilities that hindered not having a significant difference in Perls' staining. For example, (1) although HBED iron chelator has a higher affinity for binding iron than deferoxamine and other commonly used iron chelators, it is reported that ferritin also has a high ability toward binding ferrous iron.^{7,43–45} Consequently, it is possible that at the time-point of our

study, iron was not freely available for HBED; rather, it was bound to ferritin. (2) Our time-point is focused on the third day after the TBI, whereas, iron contributes to many biochemical interactions at later time-points following brain trauma. Levels of iron are reported to increase in a non-significant manner after TBI by day 4 and significant differences in iron after trauma are reported by day 7.⁴⁶ However, this does not preclude the dangerous effects of iron during the acute phase (i.e., during the initial few days after trauma) where most of the acute cell injury takes place.^{47,48} (3) In accordance with our study, previous studies have shown that iron chelators have a minimal effect on total brain iron after ICH; however, they tend to regulate the free iron especially in ICH.^{33,49–51} (4) The nature of the CCI model of TBI and the vascular anatomy at the impact site do not allow for a consistent prediction of bleeding. This leads to some variations in bleeding among the animals; although, the overall injury remains consistent. Interestingly, we observed a higher standard deviation in the amount of blood (quantified by running an algorithm) in the brain of control and HBED groups. This could be another possibility of having higher standard deviation in Perls' staining, which could potentially overshadow any actual difference present between the groups.

Because HBED has passed Phase I in clinical trials and is shown to be safe to administer to humans,^{8,14,31} the door is open for direct Phase II clinical trials testing it in patients with TBI. As shown in this study, proven ability of HBED to reduce edema and to improve behavioral outcomes will allow for more clinical applications, not just for traumatic cranial injuries but also potentially for other acute brain insults.

A potential limitation of this work is that it tested the effect of one iron chelator instead of different isoforms of this drug or other iron chelators that would have allowed us to compare data. The main focus of this study is on a 3-day end-point. We chose a 3-day treatment regimen because this is the time frame where iron is reported to play a major detrimental role after TBI.^{47,48,52} Future studies of HBED's pharmacokinetics, dose response, and therapeutic window will provide additional insight in to HBED's mechanism of action. Further, performing more specialized behavioral tests would have allowed us to discover HBED's effect over cognitive functions.

In conclusion, the HBED iron chelator binds to ferrous iron and inhibits oxidative stress directly and indirectly through its iron chelating properties. Due to its ability to cross the BBB and mitochondrial membrane, as well as its higher affinity and selectivity for iron than other chelators,^{6,7,16} HBED can significantly reduce neurological deficits following TBI. Here, the data illustrate improved motor function along with reduced hippocampal swelling, cortical injury volumes, and hemispheric edema after 3 days of drug administration. Our results suggest that HBED can help facilitate the recovery process following brain trauma.

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