Title: Effectiveness of deferoxamine on ferric chloride-induced epilepsy in rats

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Abbreviations

DFO= deferoxamine; EEG= electroencephalography; MRI= magnetic resonance imaging; SOD= superoxide dismutase; ICH= intracerebral hemorrhage.

Abstract

Iron overload has been regarded as a common cause for refractory epilepsies in patients after hemorrhagic strokes. This study is to examine the potential epilepsy control effect of deferoxamine (DFO), an iron chelator, on a ferric chloride-induced epilepsy rat model. Twenty four rats were divided into 4 groups: group I is blank control group, group II is sham group with intracortical injection of saline, group III is epilepsy group with intracortical injection of iron and saline treatment, group IV is treatment group with intracortical injection of iron and DFO treatment. For the DFO intervention group, a daily dose of 100 mg/kg DFO via peritoneal injection was applied for 14 days. Outcomes were evaluated by behavioral study, electroencephalography (EEG), magnetic resonance imaging (MRI) scan and tissue analysis. Epilepsies according to behavioral observations and EEG analysis were significantly suppressed after intervention of DFO. Reduction of iron content in the brain cortex was proved by diminished low signal area on T2-MRI images ($p=0.006$) and tissue analysis ($p<0.001$), simultaneously the superoxide dismutase (SOD) activity increased ($p < 0.001$). Western blot analysis demonstrated the decreasing of local transferrin after DFO treatment. DFO is efficient at Fe clearance, thus helpful in epilepsy control. This finding implies potential therapeutic value of DFO in patients with refractory epilepsy after hemorrhagic stroke.

Key words: iron; epilepsy; deferoxamine; electroencephalography; MRI; rats.

1. INTRODUCTION

Epilepsy is a common sequela after cerebral bleeding from brain trauma, tumors, or hemorrhagic strokes. This disorder is estimated to occur in 4% to 16% of patients after hemorrhagic stroke (Conrad et al., 2013; Giroud et al., 1994; Passero et al., 2002), and its incidence varies from 4.4% to 13.6% after brain trauma (according to the severity of the injury) at 3 years after discharge (Ferguson et al., 2010). Epilepsy presents in 25% of all symptomatic patients with cavernous malformations (CM) (Josephson et al., 2011). If a focal epileptogenic region persists, anti-epileptic drugs (AEDs) may be less effective.

The essential epileptogenic factor for these hemorrhagic diseases is believed to be the deposition of iron, which is liberated from hemoglobin. The over loaded iron variably exists as ferrous or ferric ion, mediates the generation of hydroxyl radicals (Yokoi et al., 1995), and affects the oxidative stress level of cortical neurons (Willmore and Rubin, 1981; Willmore et al., 1986). Therefore, iron clearance may be favorable for epilepsy control.

Deferoxamine (DFO), an iron chelator, has been proved to be very effective in ameliorating hemorrhagic brain injury by attenuating iron deposition, brain edema, and neuron death (Gu et al., 2009; Lee et al., 2011; Okauchi et al., 2010). In this study, we examined the iron scavenging role and anti-epileptic effect of DFO in a rat model of iron-induced focal epileptic seizures.

2. RESULTS

2.1 Effect of DFO on epilepsy control according to behavioral observations

The rats in Groups I and II showed no evidence of seizures. The accumulated Racine scores of the rats in Groups III and IV were 11.0 ± 2.7 and 12.3 ± 1.5 , respectively, on the first day after surgery. On days 7, 14, 21 and 28, the scores in Group III were 20.3 ± 3.2 , 21.33 ± 4.16 , 18.7 ± 1.53 and 15.3 ± 2.5 , respectively. The corresponding scores in Group IV were 15.3 ± 1.5 , 17.33 ± 3.79 , 9.0 ± 1.7 and 8.7 ± 1.5 respectively ($p=0.072$, 0.286 , 0.002 and 0.017 compared to Group III, $n=6$, Figure 1A). For stage 3-5 behavioral seizures in Group III and IV, it is 2.3 vs. 0.8 on the 14th day, 1.3 vs. 0.2 on the 21st day averagely. After one month, no stage 3-5 behavioral seizure was recorded on in Group IV (Figure 1B).

2.2 Effect of DFO on epilepsy control according to EEG analysis

EEG was recorded continuously for 28 days in Groups III and IV after FeCl_3 injection. Representative EEG was presented in Figure 2A-C and Figure 3A-B. The cumulative durations of high frequency high amplitude discharges (HAFDs) between the two groups were not significantly different until day 8, when the epilepsy activity in Group IV was significantly suppressed. After day 16, the mean cumulative duration of HAFDs waves was only about 5 minutes per day in Group IV, which was less than that in Group III ($n=3$, Figure 2D). Besides, amplitude in Group IV became lower in the last seven days (Figure 2B). For the number of HAFDs, it is less in Group IV in 11 days after one week DFO treatment (marked by #, $n=3$, Figure 2D). For the spike count, there's no significant difference between Group III and IV (Figure 3C).

However, spike amplitude became lower in the last seven days in Group IV as well (Figure 3B).

2.3 Effect of DFO on brain Fe content according to MRI

On the first day after FeCl₃ injection, a low signal area was observed in the cortex on the MRI T2 sequence in Groups III and IV (green dotted line area, Figure 4A). In group III, the low signal area on day 28 was similar with that on day 1 (2.80 ± 1.58 vs 3.84 ± 1.69 mm², $p=0.479$, $n=3$, Figure 4B). While in Group IV, the low signal area was smaller on day 28 (0.99 ± 0.63 vs 3.11 ± 0.26 mm², $p=0.006$, $n=3$, Figure 4A). MRI scan for all the rats in group III and IV was available in Supplementary material.

2.4 Effect of DFO on brain ferritin, transferrin, Fe content and SOD activity after intracortical injection of FeCl₃

Compared with the normal and sham control groups, ferritin, transferrin levels were significantly increased on day 28 in Group III. In comparison, the transferrin levels was less (0.19 ± 0.02 vs 0.42 ± 0.09 , $p=0.01$, $n=6$) in Group IV (Figure 4B). However, ferritin level was not significantly suppressed by DFO on the 28th day (0.09 ± 0.01 vs 0.11 ± 0.02 , $p=0.26$, $n=6$, Figure 4B). Fe content was significantly increased and SOD activity was significantly decreased on day 28 in Group III. In comparison with Group III on day 28, the whole-brain Fe content was 80% less (7.34 ± 1.89 vs 35.94 ± 2.74 , $p<0.001$, $n=6$, Figure 4C) and the SOD activity in the cerebral cortex was increased 5-fold (14.48 ± 2.59 vs 2.63 ± 0.45 , $p<0.001$, $n=6$) in Group IV (Figure 4C).

3. DISCUSSION

Approximately 30% of patients with epilepsy are drug-resistant (Cockerell et al.,

1997), and many of these patients have causative intracranial lesions, such as tumors, vascular abnormalities, or malacia after trauma or infarction. For cavernous malformations, epilepsy is often the initial symptom; however, hemosiderin deposition around the lesion, which can be identified by MRI on T2 or SWI sequences, indicates previous insidious hemorrhages. Resection of the lesion could prevent rehemorrhage; however, 15.1-17.9% of patients still exhibit seizure persistence after operation (Kwon et al., 2013; Stavrou et al., 2008; Yeon et al., 2009). Seizure control can be improved by performing extensive resection of the hemosiderin fringe rather than lesionectomy (59% vs. 24%, respectively, in a 3-year follow-up study; 32% vs. 8%, respectively, in a 5-year follow-up study) (Baumann et al., 2006; Wang et al., 2013), indicating that being 'iron free' is important for eliminating seizures in epilepsy (Kim et al., 2011). Intracranial hemorrhage has also been reported to be closely related to seizures in patients with arteriovenous malformations (AVMs). The risk of first-ever seizures in the first 5 years after diagnosis was higher for adults with AVM presenting with intracerebral hemorrhage (ICH) or focal neurologic deficits (23%) in comparison with adults who were incidentally discovered AVM (8%) (Josephson et al., 2011). For newly diagnosed malignant brain tumors, cortical hemosiderin was also found to be associated with epilepsy based on SWI findings (Roelcke et al., 2013). These results suggest that iron may participate in epileptogenesis in these diseases.

Iron-induced epileptic seizures in animal models closely resemble seizures in human post-traumatic epilepsy because of their common primary histopathological features

(Mori et al., 1990; Payan et al., 1970; Willmore et al., 1978). As a catalytic agent, iron is believed to induce a local oxidizing reaction in brain tissue. In the presence of iron, one electron is added to oxygen to produce the superoxide radical. Subsequently, another electron is added to superoxide, creating hydrogen peroxide (Liochev and Fridovich, 1994). Hydrogen peroxide can generate a free radical (a hydroxyl radical), which is responsible for the initiation of lipid peroxidation in neuronal membranes and the accelerated production of guanidine compounds in the brain, leading to epileptogenesis (Mori et al., 1990).

In previous studies, many chemicals used for intervention were shown to have anti-epileptogenesis effects on a rat model of iron-induced seizure, including acorus calamus, curcumin, and dehydroepiandrosterone (Hazra et al., 2007; Jyoti et al., 2009; Mishra et al., 2013). The common mechanism of these chemicals involves anti-oxidation and free radical scavenging. As an iron chelator, DFO has the ability to suppress the generation of free radicals caused by iron, which may inhibit the epileptogenesis more effectively.

In this study, we demonstrated the effect of DFO on suppressing epilepsy in an iron-induced seizure model. Behaviorally, epileptic seizures in Group IV became less severe according to the Racine scale after 3 weeks of DFO treatment. Continuous EEG recording revealed a significant decrease in the duration of HFADs after 1 week of intervention, as well as the episodes of attacks. On MRI, the local area of ferric chloride accumulation was decreased by intervention with DFO in Group IV based on the T2 sequence. Furthermore, tissue analysis also showed a decrease in the

intracerebral iron ion concentration and increased SOD levels. Western blot analysis demonstrated the decreasing of local transferrin, indicating the low brain iron overload from another perspective (Zhang and Pardridge, 2001; Zhao et al., 2016). However, since the GAPDH level can be increased by oxidative stress, the tissue level in Group III might be overestimated during western blot. Therefore in our study, we might underestimate the ratio of ferritin and transferrin/GAPDH (Ito et al., 1996). Although DFO infusions were only performed for two weeks, recurrent severe epilepsy was not observed by EEG recordings, indicating that DFO can control iron-induced epileptogenesis over the long term.

DFO has long been used for the treatment of acute iron intoxication and chronic iron overload, and has been shown to be very safe in clinical practice. In animal models, DFO has also been reported to improve neurological recovery after ICH. Okauchi et al. (Okauchi et al., 2010) found that acute DFO treatment after ICH at a dose of 50 mg/kg for more than 7 days could attenuate ICH-induced ventricle enlargement, caudate atrophy, and neurological deficits. Lee et al. (Lee et al., 2011) showed that early treatment with DFO significantly reduced neuronal cell death in a rat model of intracerebral hemorrhage. Gu et al. (Gu et al., 2009) also found that DFO could reduce iron deposition and brain injury in a piglet model of ICH. A multicenter, phase-I clinical study of ICH patients showed that consecutive daily administration of DFO at a dose of 62 mg/kg per day was not associated with an excess of serious adverse events or mortality (Selim et al., 2011). Accordingly, further clinical study should be conducted to assess the effect of DFO on suppressing epilepsy in patients

with epileptic seizures caused by intracranial iron deposition.

This study demonstrated the potential role of DFO in epileptic control by removing epileptogenic iron in a rat model. Further studies should investigate the mechanism by which DFO affects intra/extracellular iron and the relationship between remnant iron after therapy and long-term epileptic control. Thus far, we have not determined to which extent iron could be cleared over time. Additionally, many detailed mechanisms remain to be discovered.

Despite the limitations of this study, our findings collectively imply the potential therapeutic value of DFO in this iron induced seizure rat model, either in the cortex iron overload or in the epilepsy attack. Further clinical investigation is deserved to determine its effect on patients with CCMs or other brain hemorrhagic diseases.

4. EXPERIMENTAL PROCEDURE

4.1 Animals and Grouping

Thirty-two male Sprague-Dawley (SD) rats weighing 200-250 g were used in the experiments. All rats were raised and maintained in a specific pathogen-free (SPF)-II environment. All operative procedures were performed under strict aseptic conditions.

Before experimentation, the rats were allowed to acclimate to laboratory conditions.

All animal experiments were in accordance with the ARRIVE guidelines. All experiments were approved by the Animal Care and Use Committee of Huashan Hospital, Fudan University.

The rats were divided into four groups: Group I – the normal control, without injection or treatment (n=6); Group II – the sham control, with intracortical injection

of saline only (n=6); Group III – the ferric chloride (FeCl₃)-induced epilepsy group, with intracortical injection of freshly prepared FeCl₃ solution (100 mM, 5 µL) and subsequent intraperitoneal injection with saline (10 mL/kg/day) for treatment lasting 14 days (n=6); Group IV – the DFO treatment group, which was subjected to FeCl₃-induced epilepsy (as in Group III, n=6) and subsequently intraperitoneal injection with DFO (100 mg/kg, concentration: 10 mg/mL/day for 14 days). No unexpected death existed during the experiment. Actually, on the first day after modeling, only well-modeled rats with correct injection position and no reflux confirmed by magnetic resonance imaging (MRI) were used for further experiment. At first of the experiment, a total of 20 rats were received intracortical FeCl₃ injection. By MRI confirmation on the first day, 5 rats were injected too deep to the ventricle or basal ganglia, 3 rats were with reflux, only 12 rats were finally included in the following experiments, randomly assigned to Group III and IV. Six rats in both groups were then used for behavioral study, and three of them were under electroencephalograph (EEG) recording. All the 6 rats in both groups were used for MRI study and tissue analysis.

4.2 FeCl₃-induced Epileptic Model

After a midline scalp incision, the peri-cranial muscles and fascia were retracted laterally. Next, a bone hole (1 mm diameter) was drilled 1 mm posterior and 2 mm lateral to the bregma (the bone hole locates at the locus of the sensorimotor cortex following the coordinates of the stereotaxic atlas). Through this hole, 5 µL of freshly prepared aqueous solution containing 100 mM FeCl₃ was injected over a period of 5

min into the sensorimotor cortex at a depth of 0.5 mm using a microinjection syringe. The needle was held in place for an additional 30-60 seconds to prevent reflux. For rats that would undergo EEG recording, a bipolar electrode was implanted, one is on the surface of the sensorimotor cortex under the hole used for FeCl₃ injection; another reference electrode was simultaneously implanted on the contralateral frontal dura surface. Subsequently, the bone hole was covered with bone wax, and the wound was closed with stitches. After modeling, rats were randomly grouped into Group III or IV. EEG was recorded from days 1 to 28 after intracortical injection of FeCl₃. In the sham control animals (Group II), 5 µL of saline was injected into the sensorimotor cortex.

4.3 Behavioral Study

Behavioral analysis was based on the Racine scale(Racine, 1972), which includes “mouth and facial movements” (intensity stage 1); “head nodding” (stage 2); “forelimb clonus” (stage 3); seizures characterized by rearing (stage 4); and seizures characterized by rearing and falling (stage 5). The movements of the rats were recorded using a digital camera for 4 hours a day at the same time on days 1, 7, 14, 21 and 28 after surgery. The behavioral seizures were further scored from the “standalone videos” by two other personnel who were blind to the experiments.

Behavioral seizures were estimated on modified Racine scale at 5 minutes epochs, during which the highest seizure stage was considered. The severity of behavioral seizures was calculated by cumulative Racine score during the four hours. Besides, numbers of stage 3-5 attack was also considered for comparison. Six rats in each group were used for behavioral analysis, which were matched by synchronous EEG

1 recording. Two independent investigators analyzed the behaviors who were blinded to
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3 the animal group assignment.
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6 **4.4 Electroencephalography (EEG) Study**

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9 Wireless EEG-collecting implants (DSI, USA) were used to record EEG of rats 24
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11 hours per day for 28 days (sampling rate 200Hz). The EEG electrodes were implanted
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13 in the epidural space according to the manual, and in fact after the MRI scan. Each
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15 EEG file was analyzed manually by scanning through the EEG recording on the
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17 computer screen. Epilepsy severity was quantified by high frequency high amplitude
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19 discharge (HFAD), which was defined as a high-frequency (>10 Hz), high-amplitude
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21 (>2×baseline) discharge that lasted at least 5 seconds. The number and duration of
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23 HFAD were calculated for each rat in Groups III and IV. Epileptic spike was also
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25 analyzed in those two groups, which was defined as a spike discharge with duration
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27 <100ms or spike-and-wave duration <400ms. Number of spikes during epilepsy was
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29 calculated by Clampfit 9.0 program. Three rats in each group were used for analysis.
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31 The investigators were blinded to the animal group assignment.
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42 **4.5 Magnetic Resonance Imaging (MRI) Examination**

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45 MRI scan (Bruker Biospec 70/20 USR, German) was performed on days 1 and 28
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47 after FeCl₃ injection using T2 sequence in Groups III and IV. For each rats, the low
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49 density area was calculated on the central slice of iron deposition area. All the six rats
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51 in Group III and IV were underwent MRI scan. The investigators were blinded to the
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53 animal group assignment.
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58 **4.6 Western blot analysis**

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1 The cortex tissue of the injection area was collected for further analysis. All of the
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3 nuclear and cytoplasmic proteins (100µg) of the brain tissue were size-fractionated
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5 using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to
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9 Immobilon-P membranes (Millipore). The blotted membranes were incubated with
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11 primary antibodies against Ferritin, Transferrin and GAPDH (Abcam, HK Ltd)
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13 followed by incubation with an HRP-conjugated secondary antibody (Jackson). The
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15 immunoreactivity was detected using an enhanced chemiluminescence reaction
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19 system (Amersham Pharmacia Biotech).
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22 **4.7 Estimation of Tissue Iron Content and Superoxide Dismutase (SOD)**

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25 At day 28, all rats were sacrificed by overdose of anesthetics. The whole brain was
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28 rapidly removed to measure the wet weight. Then, the brain cortex was carefully
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30 separated under a microscope and homogenized in ice-cold phosphate-buffered saline
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32 (1/9, wt/vol). The supernatant was collected to detect SOD activity using
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35 commercially available kits. To measure iron content, the whole brain was dried in an
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38 oven at 60 °C to constant weight and subsequently mineralized in HNO₃. After
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41 reduction with thioglycolic acid, the Fe concentration was spectrophotometrically
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44 determined by measuring the absorbance at $\lambda = 535\text{nm}$ in the presence of
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47 bathophenanthroline.
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50 **4.8 Statistical Analysis**

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53 Data of behavioral, EEG, MRI studies, western blot, SOD activity and iron content
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56 are all analyzed by two independent researchers who are blinded to each group. Data
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59 of behavioral analysis, MRI scan and tissue analysis are presented as the means $\pm$ SD.
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1 Data were analyzed using independent-samples t-tests and one-way analysis of
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3 variance (ANOVA). P-value <0.05 was considered as statistically significant. All
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5 statistical analyses were performed using Graphpad Prism 5.0 for Windows.
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26 **6. DISCLOSURES**

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31 **7. FIGURE CAPTIONS**

32 **Figure 1. Racine scale for behavioral seizures.**

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34 (A) Cumulative Racine scores in Groups III and IV on the day 1, 7, 14, 21 and 28
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36 (mean±SD, *p<0.05, **p<0.01 compared to Group III, n=6); (B) Numbers of stage
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38 3-5 attacks in Groups III and IV on day 1, 7, 14, 21 and 28, n=6.
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45 **Figure 2. EEG recordings and HAFDs analysis in Group III and IV.**

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47 (A) EEG baseline; (B) HAFDs on day 28 in Group III; (C) HAFDs on day 28 in
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49 Group IV; (D) HAFDs duration and numbers during 28 days in Group III and IV,
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51 mean±SD, n=3. # marked the days that numbers of HAFDs in Group IV is less than
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53 group III.
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59 **Figure 3. EEG recordings and spike analysis in Group III and IV.**

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(A) Spikes, spike and waves on day 28 in Group III; (B) Spikes, spike and waves on day 28 in Group IV; (C) Spike numbers during 28 days in Group III and IV, n=3.

Figure 4. Brain MR scan and tissue analysis.

(A) Average low density area on Day 1 and Day 28 in Group III and IV (mean±SD, **p<0.01 compared to day 1, n=6); green dotted line area: low density area. (B) Western blot analysis for ferritin and transferrin (mean±SD, *p<0.05 compared to Group III, n=6). (C) Left: Fe content in the whole brain in four groups on Day 28. Right: SOD activities in the cortex in four groups on Day 28 (mean±SD, **p<0.01 compared to Group III, n=6).

Supplementary material. Brain MR scan for all the rats in Group III and IV.

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Figure 2

Figure 3

Figure 4