

The difference in seizure incidences between young and adult rats related to lipid peroxidation after intracortical injection of ferric chloride

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ABSTRACT

Introduction: Clinical studies have shown that the incidence of early post-traumatic seizures is higher in children than in adults. It has been proposed that iron-induced lipid peroxidation plays an important role in the development of epileptogenic foci. This study examined some of the hypothesised reasons for the difference in the incidence of early post-traumatic seizures between children and adults.

Methods: 12 young rats and 12 adult rats were randomised into four groups. Groups 1 and 2 were control groups, comprising six young rats and six adult rats, respectively, and they were administered an intracortical injection of saline. Groups 3 and 4 were injury groups, comprising six young rats and six adult rats, respectively, and they were administered an intracortical injection of FeCl₃. All the rats were observed for six hours post-injection for the occurrence of seizures, and were then killed. The injected hemispheres were extirpated and tested for the malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity as indices of oxidative damage.

Results: Seizures were observed only in Group 3. Increased MDA levels and decreased SOD activity were observed in Group 3 (ANOVA, p-value is less than 0.001). Increased MDA levels and decreased SOD activity were significantly higher in rats with seizures (Group 3) than in those without seizures (independent t-test, p-value is less than 0.001).

Conclusion: Different levels of lipid peroxidation induced by an intracortical ferric chloride injection may account for the different incidence rates of seizures between young and adult rats.

Keywords: early post-traumatic seizures,

epileptogenesis, lipid peroxidation

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INTRODUCTION

Clinical studies have consistently found that the incidence of early post-traumatic seizures in children is higher than in adults, although the incidence varies widely. Annegers et al reported a cumulative incidence of 30.5% in children and 10.3% in adults with severe head injury,⁽¹⁾ whereas Desai et al reported an incidence of 8.7% in children and 3.1% in adults⁽²⁾ and De Santis et al reported a difference of 5% and 2.7% in children and adults, respectively.⁽³⁾

Head injury, with extravasation of red blood cells followed by haemolysis and deposition of iron compounds within the brain tissue, is associated with the development of post-traumatic seizures.^(4,5) The same thing happens when iron salt or heme is injected intracortically into the rat brain, inducing seizures and epileptic discharges.^(6,7) It has been proposed that oxygen free radicals that are generated by an iron-mediated reaction in the brain can peroxidise lipid compounds in the neuron membrane to produce epileptogenic foci.^(6,8,9) The role of lipid peroxidation in epileptogenesis is supported by other studies, which have shown that exogenous antioxidants are also effective in suppressing iron-induced seizures.^(7,10-14)

The levels of activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), are low in the immature brain.⁽¹⁵⁻¹⁸⁾ The low level of activity of these antioxidants makes the immature brain more susceptible to oxidative stress, which is shown as higher lipid peroxidation.⁽¹⁹⁻²³⁾ The amount of oxidative stress can be assessed by measuring the products of peroxidation and the activities of endogenous antioxidant enzymes.⁽²⁴⁾ Malondialdehyde (MDA) is a secondary product of lipid peroxidation that has been used to evaluate oxidative stress, while the antioxidant enzymes that have been used to evaluate oxidative stress include SOD and GPx.^(7,14,24-27) Under

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conditions of oxidative stress, there is an increase in the MDA level and a decrease in the level of SOD and GPx activities.^(26,28)

This study tested the hypothesis that the higher levels of lipid peroxidation in the immature brain and the role of lipid peroxidation in the development of epileptogenic foci, accounts for the difference in seizure incidence rates between children and adults.

METHODS

12 young Wistar rats (14 days old) and 12 adult Wistar rats (90 days old) were used in this experimental study.⁽¹⁹⁾ The animals were placed in individual cages under controlled conditions (a 12-hour day and night cycle in 26°C room temperature) with free access to food and water. All the rats were anaesthetised with an intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg).⁽¹⁴⁾ Epileptic foci were induced by injecting FeCl₃ into the left sensory-motor cortex using the procedure described by Willmore et al.⁽⁶⁾ The rats were placed in a stereotaxic device, and burr holes of 2 mm in diameter were made in the cranial bone at a point 1 mm posterior and 2 mm lateral to the bregma. A needle (25 G) of a micro-syringe was inserted through the hole with the tip inserted into the cortex, about 1.6 mm deep in the exposed dura mater.⁽⁷⁾ The injury groups were injected with 5 µL of a freshly prepared aqueous solution of 50 mM FeCl₃ (Sigma Chemical Co, St Louis, MO, USA). The control groups were injected with the same volume and pH of saline.

The rats were randomised and divided into four groups. Groups 1 and 2, the control groups, comprising six young rats and six adult rats, respectively, were injected with saline. Groups 3 and 4, the injury groups, also comprising six young rats and six adult rats, respectively, were injected with FeCl₃. All the rats were observed for six hours post-injection for seizure events, after which they were killed and decapitated. Their left hemispheres were extirpated and tested for the MDA levels and SOD activities.

150 mg of left hemisphere brain tissue was homogenised with a phosphate buffer and 2 ml of ethylenediaminetetraacetic acid (EDTA).⁽²⁹⁾ 200 µL of the homogenised specimen was taken and mixed with 500 µL of aqua bidest, 200 µL of hydrochloric acid, 250 µL of 40% trichloroacetic acid and 250 µL of 1.34% sodium thiobarbituric acid. The mixture was incubated in boiling water for 25 minutes and left to cool at room temperature. It was then centrifuged at 3000 rpm for ten minutes and filtered through Whatman paper. The filtered fluid was added with aqua bidest to 3 ml and was ready

to be read on 532 nm.⁽³⁰⁾ The MDA level was expressed as µg/100 mg wet tissue.

200 mg of the sample was homogenised with phosphate buffer and 2 ml of protease inhibitor, and centrifuged at 4000 rpm at a temperature of 4°C for 15 minutes.⁽³⁰⁾ 200 µL of the supernatant was taken and mixed with 100 µL of EDTA, 100 µL of nitroblue tetrazolium (without chloride), 100 µL of xanthine, 100 µL of xanthine oxidase and phosphate buffer. The solution was incubated at a temperature of 37°C for 30 minutes. Subsequently, this mixture was centrifuged at 3000 rpm for ten minutes and filtered using Whatman paper. The filtered solution was added with a phosphate buffer to 3 ml, and was ready to be read on 580 nm. The SOD activity level was expressed as U/100 mg wet tissue.

All data was expressed as the mean ± standard deviation. One-way ANOVA and the least significant difference post-hoc test were used for multiple comparisons. Comparisons between two groups were analysed using the independent *t*-test. A *p*-value of < 0.05 was considered to be significant.

RESULTS

Seizures were observed only among the young rats that were administered a FeCl₃ intracortical injection (Group 3). Table I shows the MDA and SOD activity levels in the rat brains in each experimental group. The MDA level was significantly higher in Group 3 than in the other groups (ANOVA, *p* < 0.001). The SOD activity level was significantly lower in Group 3 than in the other groups (ANOVA, *p* < 0.001). Table II shows that the MDA level in rats that had a seizure was significantly higher than in those without a seizure (independent *t*-test, *p* < 0.001). The SOD activity level in rats that had a seizure was significantly lower than in rats without a seizure (independent *t*-test, *p* < 0.001).

DISCUSSION

It has been proposed that intracortical iron injection causes seizures and epileptiform discharges on electroencephalogram (EEG).^(6,7,14) The histological appearance of the epileptic model is similar to that observed in human post-traumatic epileptogenic foci.⁽⁸⁾ Haemoglobin is known to cause seizures that are similar to iron-induced seizures, which may be due to the iron released from the haemoglobin.⁽⁵⁾ In addition, D'Alessandro et al reported that the main factor that causes seizures is brain damage caused by focal haemorrhage, which can be shown on computed tomography (CT).⁽³¹⁾ An iron-induced seizure might

Table I. MDA levels and SOD activity levels.

Group	MDA ($\mu\text{g}/100$ mg wet tissue)	SOD (U/100 mg wet tissue)
Young rats/control (Group 1, n = 6)	0.141 \pm 0.020	9.763 \pm 1.017
Adult rats/control (Group 2, n = 6)	0.141 \pm 0.008	9.375 \pm 0.745
Young rats/injury (Group 3, n = 6)	0.232 \pm 0.026*	2.958 \pm 0.769*
Adult rats/injury (Group 4, n = 6)	0.113 \pm 0.031	10.190 \pm 0.377

Values are expressed as mean \pm standard deviation.

MDA: malondialdehyde; SOD: superoxide dismutase

* P < 0.001 compared with all groups.

Table II. MDA levels and SOD activity levels of seizure rats and non-seizure rats.

	Seizure rats	Non-seizure rats	P-value
MDA ($\mu\text{g}/100$ mg wet tissue)	0.232 \pm 0.026	0.132 \pm 0.024	< 0.001
SOD (U/100 mg wet tissue)	2.958 \pm 0.769	9.776 \pm 0.792	< 0.001

Values are expressed as mean \pm standard deviation.

MDA: malondialdehyde; SOD: superoxide dismutase

therefore be used as a model for post-traumatic epilepsy.^(6,7,12-14)

Iron facilitates the production of free radicals either via a Fenton or Haber-Weiss reaction.^(32,33) Free iron catalyses the conversion of radical superoxide to highly reactive hydroxyl radicals, compounds that can attack all molecules in living cells.⁽³²⁻³⁵⁾ Free iron is also required to initiate and propagate lipid peroxidation.⁽³⁶⁾

Studies using seizure models have been conducted in adult rats at a dosage of 100 mM iron salt.^(6,7,11,13,14) Willmore et al reported that an injection of 25 and 50 mM iron salt did not cause seizures. Convulsive seizures or generalised epileptiform discharges occurred with 100 mM iron salt, and the product of lipid peroxidation in rats that had seizures was higher than in rats without seizures.⁽¹¹⁾ A dosage of 50 mM was used in this study for all the groups of rats, with the assumption that in the clinical situation, the same degree of head injury increases the chances of early post-traumatic seizures among children. The dosage of 50 mM in this study was found to increase lipid peroxidation and induce seizures in the group of young rats that were administered an intracortical iron injection (Group 3).

Willmore and Rubin reported a significant increase in the lipid peroxidation products after an iron salt injection, within 15 minutes to four hours.⁽⁸⁾ Singh and Pathak observed an increase in lipid peroxidation at Day 23 after an iron salt injection, suggesting that lipid peroxidation is a long and continuous process, and not a transient event.⁽³⁷⁾ Based on the relatively long-lasting lipid peroxidation, seizure events were observed for six hours in this study, with the expectation that this would reduce observation bias.

Oxidative stress, followed by lipid peroxidation, can be assessed by measuring the lipid peroxidation products and antioxidant activities.⁽²⁴⁾ MDA is formed from the breakdown of polyunsaturated fatty acids and is the main secondary product of lipid peroxidation. MDA provides a simple way to determine the level of lipid peroxidation.^(7,11,14) SOD, an antioxidant enzyme that converts superoxide radicals to hydrogen peroxide, can also be used to measure the level of oxidative stress.⁽²⁴⁾ Under conditions of oxidative stress, lipid peroxidation products, such as MDA, are increased, and the levels of the antioxidants SOD and GPx are decreased.^(26,28)

The human body conserves antioxidants in order to neutralise or limit the oxidative damage caused by free radicals. The brain is particularly vulnerable to oxidative stress due to its high rate of oxidative metabolism and its relatively low antioxidant capacity.⁽¹⁸⁾ The results of many studies that have been conducted to observe the development of antioxidant enzymes in the developing brain are varied. Aspberg and Tottmar reported that the development of antioxidant enzyme activities vary. They found that the activity of MnSOD increased with age, but the CuZnSOD activity did not show the same pattern. While the GPx activity level was low during the first two weeks after birth, it increased to the adult level thereafter.⁽¹⁶⁾ Catalase tended to decrease to the adult level after reaching a peak on Day 10. This was possibly due to the low catalase content in the brain; this made it difficult to detect.⁽¹⁵⁾ Mavelli et al demonstrated an increase in both the CuZnSOD and MnSOD levels with age, but found a more significant increase in the ZnSOD levels.⁽¹⁵⁾ They also found that the increase in the GPx level was similar to the results reported by Aspberg

and Tottmar.^(15,16) Hussain et al clearly demonstrated that although the development of antioxidant enzymes varies in different regions of the developing brain, the overall level of enzyme activities tends to increase with age.⁽¹⁷⁾

Although the results of the above studies are varied, many other studies that have been conducted to observe the vulnerability of the immature brain to oxidative damage have clearly demonstrated that the immature brain is more vulnerable to oxidative stress than a mature one. This has been considered to be due either to the low antioxidant activity levels or to an inadequate compensatory mechanism of increasing antioxidants during oxidative stress. Koudelová and Mourek reported that the MDA levels in the immature brain were higher than in the mature brain when oxidative stress was encountered.⁽¹⁹⁾ Buonocore et al reported that the low activity level of the natural antioxidant system was responsible for the highest oxidative damage in the newborn.⁽²¹⁾ Bayir et al showed that there was a significant increase in the lipid peroxidation products and a decrease in the antioxidant reserves in children with a severe head injury.⁽²⁶⁾ Fullerton et al demonstrated that the more severe oxidative damage in the immature brain was due to an inadequate compensatory mechanism of increasing GPx and catalase levels during hypoxic-ischaemia.⁽³⁸⁾ Fan et al also reported that the immature brain did not have an adequate compensatory mechanism of increasing GPx levels in response to oxidative stress.⁽²⁷⁾ Ozdemir et al reported that the increase in the MDA level in immature rats after injury was not followed by an increase in the levels of SOD and GPx activities.⁽²⁸⁾ The results of our study also showed that the immature rat brain is more susceptible to oxidative stress induced by an intracortical iron salt injection. This was shown by the high levels of MDA and the low levels of SOD activities.

The neuronal membrane damage caused by lipid peroxidation decreases the activity levels of Na⁺/K⁺-ATPase.⁽³⁹⁻⁴¹⁾ Na⁺/K⁺-ATPase maintains the neuronal ionic gradient, and a lowering of its activity levels will decrease the convulsive threshold.⁽⁴⁰⁾ Free radicals and other lipid peroxidation products can damage the sulfhydryl group of excitatory amino acid transporters (EAAT) and downgrade its production.^(42,43) This condition reduces the ability of the EAAT to uptake glutamate. Glutamate is an important excitatory neurotransmitter in the brain. Lipid peroxidation decreases the release of γ -aminobutyric acid, which is a well-known inhibitory neurotransmitter.⁽⁴⁴⁾ Thus, lipid peroxidation causes a disturbance in the extracellular neurotransmitter

regulation. All the above conditions increase the synaptic transmission of excitatory glutaminergic and neurotoxic glutamates, which are closely correlated to the seizure pathophysiology.⁽⁴⁵⁾

In 1978, Willmore et al reported on the role of lipid peroxidation in iron-induced epileptogenesis.^(6,46) The role of lipid peroxidation in the development of seizures has also been observed in other studies. The examination of the epileptic foci in patients with intractable seizures has revealed higher levels of MDA and lower levels of SOD activities compared to the brain tissue surrounding an epileptic focus.⁽⁴⁷⁾ Seizures induced by L-cysteine and electroconvulsion further prove the role of lipid peroxidation in epileptogenesis.^(48,49) The observation that antioxidants can suppress seizures that are caused by an intracortical iron salt injection also supports the role of lipid peroxidation in epileptogenesis.^(7,10-14) Likewise, our results support the role of lipid peroxidation in seizures. It was also observed that the level of peroxidation in rats that had seizures was higher than in those without.

Based on the results of this study, it is concluded that the different levels of lipid peroxidation induced by an intracortical iron salt injection could account for the difference in the incidence rates of seizures in young and adult rats. This is the first study that shows that different levels of lipid peroxidation are associated with differences in seizure incidence rates in immature and mature brains. However, these results cannot be extrapolated to humans, and therefore, further studies should be conducted in the clinical setting to observe if the differences in lipid peroxidation between children and adults result in the differences in seizure incidence rates in children and adults after traumatic brain injury. Research in this clinical setting could utilise cerebrospinal fluid to measure the oxidative damage in the brain after traumatic brain injury.⁽²⁶⁾ Our results may explain the failure of conventional drugs in the treatment of early post-traumatic seizures in children.^(22,50) Further work should examine the effectiveness of exogenous antioxidants to suppress iron salt-induced seizures in the immature rat brain. Finding effective treatments for early post-traumatic seizures could lead to improved clinical outcomes after traumatic brain injury in children.⁽⁵¹⁾

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