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Review

Natural Antioxidants May Prevent Posttraumatic Epilepsy: A Proposal Based on Experimental Animal Studies

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Head injury or hemorrhagic cortical infarction results in extravasation of blood and breakdown of red blood cells and hemoglobin. Iron liberated from hemoglobin, and hemoglobin itself, are associated with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS have been demonstrated to be involved in the mechanism of seizures induced by iron ions in the rat brain, an experimental animal model for posttraumatic epilepsy (PTE). ROS are responsible for the induction for peroxidation of neural lipids, *i.e.*, an injury of neuronal membranes, and also could induce disorders in the excitatory and inhibitory neurotransmitters. Antioxidants, such as a phosphate diester of vitamin E and C (EPC-K_1) and antiepileptic zonisamide, have been known to prevent the epileptogenic focus formation, or to attenuate seizure activities in the iron-injected rat brain. Natural antioxidants, such as α-tocopherol, and condensed tannins, including (−)-epigallocatechin and (−)-epigallocatechin-3-O-gallate, adenosine and its derivative, melatonin, uyaku (Lindera Strychnifolia), fermented papaya preparations, Gastrodia elata Bl., and Guilingji, have been demonstrated to scavenge ROS and/or RNS and to be prophylactic for the occurrence of epileptic discharge in the iron-injected rat brain.

Key words: posttraumatic epilepsy, iron-induced epileptic seizures, antioxidant, reactive oxygen species, reactive nitrogen species

Posttraumatic epilepsy (PTE) is characterized by epileptic seizures due to brain damage secondary to head injury. Clinically, PTE is classified into early epilepsy (or early seizures) and late epilepsy. Early epilepsy is defined as a convulsion occurring within 1 week of head trauma. Patients with early epilepsy have a high risk of late epilepsy. The occurrence of PTE varies greatly according to the severity of the injury. For example, according to the review by Pagni in 1990 [1], the overall incidence of PTE in different series of consecutive, unselected trivial and severe injuries is about 3-5%, and about 8-9% if the large number of open head injuries is included. And, the occurrence of PTE rises in combat injury to 12-24% in non-missile combat and up to 34-53 % in missile injury [1].

In Japan (population 120 million), the occurrence of posttraumatic epilepsy is presumed to be approximately 150,000 annually and has been shown to be about 10% of all hospitalized patients with head injury and about 1% of all out-patients with head injury [2]. This is a big problem both medically and socially. Offering a possible mechanism for the development of PTE, computerized

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tomography (CT) studies have demonstrated that the most powerful factor of early and late epilepsy is focal hemorrhagic brain damage [3]. Moreover, the possible epileptogenic role of hemosiderin has been evaluated by brain magnetic resonance imaging (MRI) [4]. The possible pathophysiological mechanisms of PTE have been studied using an animal model of PTE, originally developed by Willmore *et al.* [5-7], in which epileptic seizures in the rat brain are induced by iron injection.

Involvement of Free Radicals in the Seizure Mechanism

Free radicals, in addition to contributing to neuronal injury in cerebral ischemia and hemorrhage, may be involved in neuronal degeneration in schizophrenia, tardive dyskinesia, normal aging, and Parkinson's and Alzheimer's diseases [8]. Concerning epileptic seizures, excitatory amino acid receptor activation by glutamate or N-methyl-D-aspartic acid (NMDA) has been known to accompany generation of reactive oxygen species (ROS), *e.g.*, superoxide anion radical ($O_2\cdot^-$) hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$), and reactive nitrogen species (RNS), *e.g.*, nitric oxide (NO) and peroxynitrite anion ($ONOO^-$) [9-11]. In fact, free $\cdot OH$ is detectable after pentylenetetrazol-induced seizure and kindling [12]. ROS and RNS are related in their metabolic pathway in that $ONOO^-$, formed from NO and $O_2\cdot^-$, is a potent oxidant that may exert injurious effects in the brain.

RNS, especially NO, is produced in several epilepsy animal models. NO is thought to function in the brain as a neuromodulator of cerebral blood flow and to play a role in learning and memory. However, the presence of excess NO may cause neuronal cell injury. NO may be associated with convulsive seizures in that excess synthesis and release of NO occurs with the stimulation of NMDA receptor [13, 14]. Proconvulsant effects of NO have been found not only in NMDA-induced seizures [15] but also in experimental seizures induced by arginine [16], pentylenetetrazole (PTZ)-induced seizures [17-22], convulsions induced by hyperbaric oxygen [23, 24], and in El-mice [25] seizures.

Anticonvulsant effects of NO have been documented as well. Anticonvulsant effects have been reported in kainate-induced seizures [26-29] and PTZ limbic seizures [17], in seizures in immature rats [30], in seizures induced by penicilline [31], by α-guanidinoglutaric acid [32], bicuculline [33], lithium-pilocarpine [34], and amygdala kindling [35], and in NMDA-induced seizures [36, 37]. This evidence suggests that endogenous NO may behave either as a proconvulsant or an anticonvulsant in the brain. NO may have neuroprotective properties, as it is a potent antioxidant. NO can scavenge $O_2\cdot^-$, $\cdot OH$, lipid peroxyl radicals ($LOO\cdot$), and thiyl radicals such as $GS\cdot$ produced from reduced glutathione (GSH). Moreover, nitrosoglutathione (GSNO), produced from GS, is known to be an extremely potent antioxidant that could effectively protect neurons against oxidative damage [38].

The role of NO in the brain is diverse and complicated with contradictory evidence reported regarding its proconvulsant and anticonvulsant effects. These conflicting findings may be explained by different experimental conditions. Variables include experimental animal models used, methods for inducing convulsions, methods of administration of NO synthase (NOS) inhibitors, and the stage of seizures (pre-convulsive, convulsive, or post-convulsive stage). Excitatory amino acid-induced convulsions may reflect pathophysiological mechanisms of PTE with the increased release of excitatory amino acids. For example, excitatory amino acid glutamate released from a presynaptic terminal acts on NMDA and α-amino-3-hydroxymethylisoazole-4-propionic acid (AMPA) receptors. When postsynaptic membranes are sufficiently depolarized by Na^+ influx and blocking of NMDA channels by Mg^+ is reduced, Ca^{2+} influx is accelerated and activates NOS. NO production activates guanylcyclase, and the generated cyclic GMP is related to initiation and propagation of seizures [39]. On the other hand, excess NO generated by accelerated NOS activity inhibits glutamate binding to NMDA receptor in a negative feed back manner [40, 41], *i.e.*, it contributes to the termination of seizures.

An Experimental Model of PTE

Iron-induced seizures in rodents is a widely used experimental animal model for PTE. A single injection of several microliters of ferrous or ferric chloride into the rat or cat sensorimotor cortex by stereotaxic procedures results in chronic recurrent focal paroxysmal electroencephalographic discharges as well as behavioral convulsions [5-7]. Generally, epileptiform discharges are induced 15 min after ferric chloride injection into the rat sensorimotor cortex, with discharges detected for more than 6 months

after the injection in chronic experiments [42]. Histological findings show the depopulation of Golgi-impregnated neurons, astocytic gliosis, loss of dendritic spines, decreased dendritic branching, and dendritic varicosities that are similar to the pathological findings in human epileptogenic foci [7, 43, 44]. Hemoglobin also is known to induce epileptiform discharges as does the iron ion, suggesting that the epileptogenic effect of hemoglobin may depend on iron released from globin [45]. However, either ferric or ferrous ions are more commonly and preferentially used as an experimental model of PTE because of ease of handling and more stable effects than hemoglobin analogues. Our work has focused on the pathogenesis of PTE, especially on the relationship between the generation of free radicals and the development of PTE and on the rational use of antioxidants as treatment.

Generation of ROS and RNS by Injection of Iron Ions into Rat Brain: A Possible Mechanism for Epileptogenesis of Head Trauma

Head injury or hemorrhagic cortical infarction results in extravasation of blood with breakdown of red blood cells and hemoglobin. Iron liberated from hemoglobin and hemoglobin itself are known to generate ROS [46-48]. Transient formation of ROS is found after the injection of iron salt into the rat cerebral cortex [49, 50]. ROS, especially ·OH, are responsible for the induction of peroxidation of unsaturated fatty acids that are components of neuronal membranes. Such damage to neuronal membranes may result in depolarization. On the other hand, ROS accelerate production of neurotoxic guanidino compounds, endogenous substances known to be convulsants in the brain [51]. Such reactions may be followed by excitatory and inhibitory neurotransmitter changes, especially increased release of excitatory amino acids such as aspartic acid [52], and decreased release of inhibitory amino acid such as γ-aminobutyric acid [53]. Such transmitter changes may be related directly to epileptogenecity. Accelerated release of excitatory amino acids may trigger excitotoxicity at the NMDA receptor in acute seizures and may be followed by the formation of a chronic epileptogenic focus [51, 54]. Excessive activation of excitatory amino acid neurotransmitter receptors during seizures is known to generate NO and ROS, including $O_2{\cdot}^-$, H_2O_2, and ·OH [55-57], followed by accelerated production of neurotoxic guanidino compounds in the pattern of a vicious circle.

Effects of Antioxidant on PTE

Vitamin E (tocopherol), vitamin C, and glutathione are the most well known radical-scavenging antioxidants in animals. Carotenoids may also act as radical-scavenging antioxidants. Many natural phenolic antioxidants have been found in plants, including vegetables, teas, and Chinese and Japanese herbal medicines.

Pre-treatment with a free radical scavenger or antioxidant, such as α-tocopherol, prevents the development of iron-induced epileptiform activity in rats, decreases the formation of peroxides at the iron injection site, hastens the resolution of brain edema, and also prevents the development of cavitation and gliosis [58-60]. Zonisamide is known to be effective as an anticonvulsant in a wide variety of animal models of epilepsy [61, 62] and in humans with epilepsy [61, 63]. We observed that zonisamide scavenged ·OH and NO and suggested that the mechanism of the antiepileptic effect of zonisamide may involve the protection of neurons from free radical damage and stabilization of neuronal membranes [64, 65].

α-Tocopheryl-L-ascorbate-2-O-phosphate diester (phosphate-diester of vitamin E and C: EPC-K_1) is a novel hydroxyl radical scavenger [66]. We have demonstrated that EPC-K_1 dose-dependently inhibits the production of thiobarbituric acid reactive substances (TBARS) and protein carbonyl (P-Carb), both indices of biogenic macromolecular peroxidation induced by ferric ions *in vitro*, and that the occurrence of ferric ion-induced epileptic discharges is delayed and/or suppressed by prior and simultaneously used EPC-K_1 [67]. Thus, EPC-K_1 prevented the induction of early convulsions that is the major risk factor of PTE. In chronic experiments, supplement of EPC-K_1 into diet restored body weight, and TBARS content increased in the focal area induced by iron-injection into the cortex and significantly lowered percent induction of epileptic discharges in electrocorticograms until 6 months after iron injection [67].

On the other hand, there is considerable evidence of the prophylactic and/or inhibitory effects of natural antioxidants on PTE, as follows:

1) α-Tocopherol. α-Tocopherol prevents the development of iron-induced epileptic seizures [58-60]. Moreover, it has been reported to delay significantly the onset of electroenchephalographic seizures induced by

intracerebral ferrous chloride injection [68]. α-Tocopherol may be a rational and practical way to prevent PTE.

2) Condensed tannins. Condensed tannins are widely distributed in the plant kingdom and are present in high amounts in teas, red wine, and fruits such as the persimmon. Persimmon juice has been used in Japan as a traditional medicine for the treatment of hypertension and to prevent stroke. Persimmon tannins have been reported to prolong the life span of stroke-prone spontaneously hypertensive rats (SHRSP) [69]. We first estimated ROS-scavenging activities of condensed tannins by an electron spin resonance (ESR) spectrometer with a spin trapping technique in 1987. We found that the condensed tannins (−)-epigallocatechin and (−)-epigallocatechin-3-O-gallate, procyanidine B-2 3,3'-di-O-gallate, procyanidine B-5 3,3'-di-O-gallate, procyanidine C-1 3,3',3"-tri-O-gallate, and crude persimmon tannin scavenged $O_2\cdot^-$ and ·OH [70]. An electroencephalographic recording demonstrated that pre-treatment with epigallocatechin (50 mg/kg iv.) and (−)-epigallocatechin-3-O-gallate (200 mg/kg iv.) prevented or slowed the occurrence of epileptiform discharges induced by iron ion injection into the rat brain [71].

3) Adenosines. Adenosine is known to act as a neurotransmitter and neuromodulator in the peripheral and central nervous systems. Adenosine depresses neuronal activity by acting at specific extracellular receptors [72]. Systemic injection of adenosine prevents audiogenic-, kainate-, and picrotoxine-induced seizures [73, 74]. Carbamazepine, an anticonvulsant, has been reported to bind with high affinity to adenosine receptors in the brain [75]. Further, adenosine is thought to be released during seizures in metabolically active areas and to inhibit seizure activity with adenosine and its analogues acting to inhibit seizure propagation [76].

ROS-scavenging activities of adenosine and its analogue have been demonstrated by an ESR technique as with adenosine and 2-chloroadenosine scavenging ·OH generated by the Fenton reagent in a dose-dependent manner. Adenosine (5 mg/kg) or 2-chloroadenosine (1 mg/kg), injected intraperitoneally 30 min prior to the iron injection into rats, suppresses or delays the occurrence of epileptiform discharges induced by iron ions [77].

4) Melatonin. Melatonin is a pineal hormone that regulates circadian rhythm. Many pharmacological actions of melatonin in oxygen radical pathophysiology have been elucidated by Reiter and his colleagues [78]. Melatonin has been found to protect cells, tissues, and organs against oxidative damage induced by a variety of free radical-generating agents and processes. Melatonin as an antioxidant is effective in protecting nuclear DNA, membrane lipids, and possible cytosolic proteins from oxidative damage. Melatonin exhibits potent antioxidant activities by scavenging ·OH and other free radicals [79], by stimulating glutathione peroxidase activity [80], and by inhibiting nitric oxide synthase [81]. NO-scavenging activity of melatonin was demonstrated by us [82]. Melatonin inhibits iron-induced epileptic discharges in rats by suppressing peroxidation [83].

5) Uyaku. Uyaku (Tendai Uyaku) is the dried root of Lindera strychnifolia F.Villaris (Sieb. et Zucc.). It is a traditional Asian medicine used in China as an astringent, carminative, stomachic, or tonic, for asthma, cholera, congestion, dyspepsia, dysmenorrhea, fluxes, gonorrhea, hernia, malaria, menorrhagia, stomach ache, stroke, and urinary difficulties [84]. Uyaku has been used as a folk drug for good health and for the treatment of stomach and renal diseases, neuralgia, and rheumatism in some districts of Japan, including Shingu, Wakayama prefecture, Japan. Uyaku extract, both from roots and leaves, shows strong superoxide dismutase (SOD)-like activity [85], and our recent study demonstrated that uyaku inhibited lipid peroxidation and carbonyl protein formation in the rat brain tissue induced by iron ions. This *in vitro* evidence suggests a possible preferable effect of uyaku on iron-induced epileptic activity [86].

6) Fermented Papaya. Bio-normalizer is a white, sweet, granular, natural health food commercially sold in Japan and the Philippines. It made by yeast fermentation of Carica papaya Linn, Pennisetum pupureum Schum. (Napier grass), Swartz (vegetable), and glucose as the main carbon source. Bio-normalizer is a potent ·OH scavenger, and significantly inhibits thiobarbituric acid reactive substances formation in iron-induced seizure focus of rats [87]. Moreover, Bio-normalizer decreases the release of monoamine metabolites in iron-induced epileptogenic focus in the rat, while iron-induced lipid peroxidation relates to the turnover rate of monoamines and seizures [88].

PS-501 is also a sweet granular papaya preparation made by yeast fermentation that has been sold as a natural health food in Japan. Recently, significant improvement in the impairment of short- and long-term memory induced by scopolamine in mice was demonstrated by PS-501 oral administration [89]. PS-501 scavenged

·OH and inhibited lipid peroxidation, oxidative DNA damage, and rat brain tissue injury induced by iron ions [90, 91], suggesting an inhibitory effect on iron-induced seizures.

7) Gastrodia elata Bl. (GE) and its components. GE is a traditional herbal medicine widely used to treat convulsive disorders and dizziness in China. GE significantly inhibits the increase in lipid peroxide levels and increases SOD activity in the rat brain with ferric chloride-induced epilepsy [92]. Recently, 5 active components from GE were clearly identified by electrophoresis by Zhao *et al.*; they are gastrodin, 4-hydroxybenzyl alcohol, vanillyl alcohol, 4-hydroxybenzaldehyde, and vanillin [93]. The antioxidant actions of 4-hydroxybenzyl alcohol and vanillin have been demonstrated at the cellular and molecular level in the brain by us [94]. Hsieh *et al.* [95] demonstrated anticonvulsive and free radical-scavenging activities of vanillyl alcohol in ferric chloride-induced epileptic seizures in rats, suggesting that the anticonvulsant effect of GE may be attributable, at least in part, to its vanillyl alcohol component.

8) Guilingji. Guilingji is a prescripted mixture of 18 different traditional Chinese medicinal herbs and animal components that includes a powder mixture of the following: Ginsen radix, Cornu cervi pontatrichum, Fructus lycii, Caryophlli flos, Radix achyranthis bidentatae, Herba cynomorii, Fructus psoraleae, Semen cuscutae, Cortex eucommise, Herba cistanchis, Glycyrrhizae radix, Fructus amomi, Hippocampus kelloggi, Rhyzoma rehmonoiae, SquCama maniti, Fossilia sppiriferis, Halitum, and sparrow brain [96]. Guilingji has been used in China as an antiaging agent for 400 years. Pharmacological studies have shown that the oral intake of Guilingji increases the level of ascorbic acid in the adrenal cortex and protects from exhaustion induced by administration of hydrocortisone [97]. In addition, Guilingji has been reported to prolong the mean life span and increase the survival rate of mice [98]. We have found that Guilingji possesses a significant scavenging effect on free radicals *in vitro* [99]. Pretreatment with Guilingji of rats with ferric chloride-induced seizures decreases levels of TBARS and increases SOD activity in the brain [100]. Decreasing TBARS elevation and increasing SOD attenuation in the brain with iron-induced seizures are suggested to be important characteristics of antiepileptogenic agents.

Concluding Remarks

Head injury or hemorrhagic cortical infarction results in the extravasation of blood and breakdown of red blood cells and hemoglobin. Iron liberated from hemoglobin and hemoglobin itself are associated with the generation of

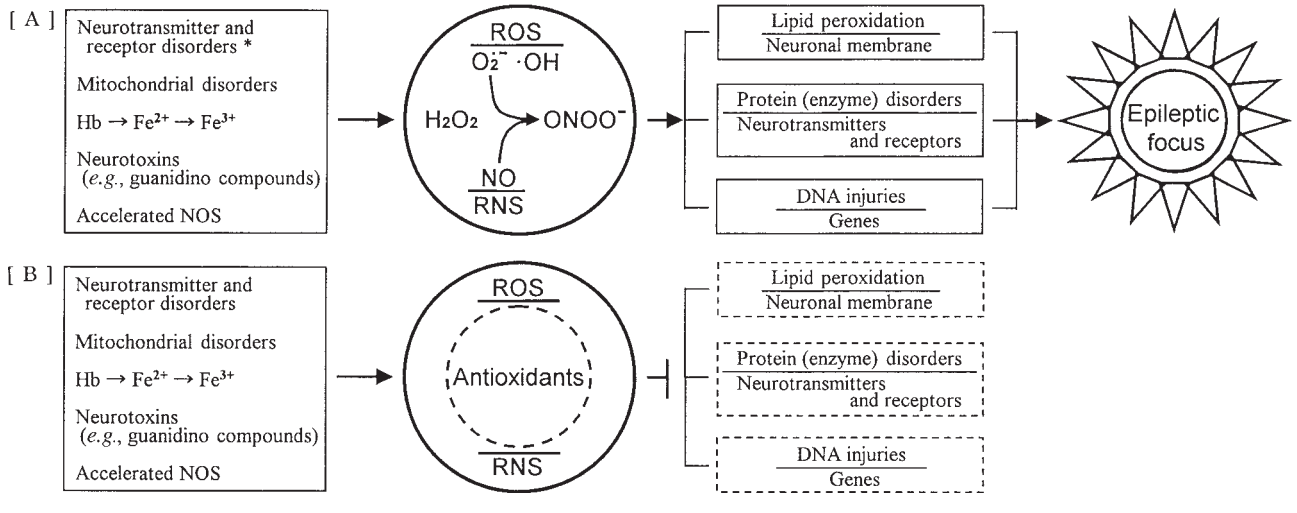

Fig. 1 Possible Anticonvulsant Effect of Antioxidant

A, Involvement of ROS and RNS in seizure mechanism: ROS and RNS, induced by neurotransmitter and receptor disorders, iron ions and/or neurotoxins, result in neuronal disorders, which lead to epileptic focus formation. *Neurotransmitter and receptor disorders may be in a vicious cycle, coupling with ROS and RNS. **B**, Anticonvulsant effect of antioxidants: Antioxidants inhibit ROS- and RNS-induced neuronal damage, and prevents epileptic focus formation.

ROS. ROS may also be produced by accelerated metabolism, *e.g.*, accelerated mitochondrial respiration, neurotransmitter and receptor disorders, and neurotoxins, such as guanidino compounds. RNS may also be involved in the seizure mechanism. ROS and RNS induce disorders in neuronal membranes and in the neurotransmitter systems that finally result in formation of epileptic focus formation in the brain. The possible involvement of ROS and RNS in epileptogenecity or in the seizure mechanism is summarized in Fig. 1. Synthetic antioxidants, such as the antiepileptic drug zonisamide and EPC-K_1, are known to prevent effectively the occurrence of posttraumatic epilepsy and/or to alleviate seizure activity. In the same way, natural antioxidants such as those described above may be useful alternative medications or supplements for preventing the occurrence of posttraumatic epilepsy and/or for attenuating epileptic seizure activities.

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