The Role of Dietary Antioxidant Insufficiency on the Permeability of the Blood-Brain Barrier

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Abstract

Our previous studies implicated vitamin E deficiency as a risk factor for equine motor neuron disease, a possible model of human amyotrophic lateral sclerosis, and showed direct effects of this deficiency on brain vascular endothelium. To gain better understanding of the pathogenesis of equine motor neuron disease, we determined the effects of dietary antioxidant insufficiency and the resultant brain tissue oxidative stress on blood-brain barrier permeability. Rats (n = 40) were maintained on a diet deficient of vitamin E for 36 to 43 weeks; 40 controls were fed a normal diet. Permeability of the blood-brain barrier in the cerebral cortex was investigated using rhodamine B, and lipid peroxidation was measured as a marker for oxidative stress. Animals on the vitamin E–deficient diet showed less weight gain and had higher brain lipid peroxidation compared with the controls. These results suggest that a deficiency in vitamin E increases brain tissue oxidative stress and impairs the integrity of the blood-brain barrier. These observations may have relevance to the pathogenesis of amyotrophic lateral sclerosis and other neurologic diseases.

Key Words: Amyotrophic lateral sclerosis, Blood-brain barrier, Equine motor neuron disease, Oxidative stress, Vitamin E deficiency.

INTRODUCTION

Equine motor neuron disease (EMND) is an acquired, sporadic disorder of the horse in which both the distribution and nature of pathologic alterations closely resemble those of human motor neuron disease (1–4). As in the human disorder, the cause of this disease is not known. In the horse, muscle-fiber typing studies indicate that the antigrophy muscles are preferentially affected, and that Type I fiber atrophy predominates (5). α-Motor neurons innervate these fibers in rodents (3). Therefore, it is likely that motor neurons on horses and humans have higher levels of oxidative metabolism and greater numbers of mitochondria that generate more reactive oxidative species, and that the fibers are particularly susceptible to oxidative damage.

Our epidemiologic observational studies have demonstrated pronounced deficiency of the chain-breaking antioxidant vitamin E in all EMND horses during the progressive phase of the disease; this is likely primarily due to inadequate dietary intake (6). Moreover, lipopigment accumulation in central nervous system (CNS) vessels and concurrent pigmentary retinopathy provide histologic evidence of membrane lipid peroxidation due to vitamin E deficiency in EMND (7).

The composition of the cerebrospinal fluid and interstitial fluid is controlled in large part by the blood-brain barrier (BBB) and in much smaller part by the blood-cerebrospinal fluid barrier, which together provide the interface between brain function and nutritional input (8). The processes that determine the movement of molecules across the BBB are regulated in the brain capillary wall by diffusion, pinocytosis, carrier-mediated transport, and transcellular transport (9). Diffusion across the lipid cell membranes of the BBB only occurs when the solute is lipid-soluble or when the membrane contains specialized channels. As a result, diffusion is the principle mechanism for BBB exchange of respiratory gases and highly lipid-soluble compounds. Carrier-mediated transport in brain endothelial cells enables molecules with low lipid solubility (e.g., glucose, monocarboxylic acids, neutral l-amino acids, and vitamins) to cross the BBB (9). The important role of vitamin E in biologic membranes as an antioxidant and stabilizing agent is well established (10), but practically no data exist on possible effects of low dietary levels of vitamin E on BBB integrity and function.

Based on the aforementioned data, we hypothesized that dietary insufficiency of vitamin E predisposes animals to oxidative stress and hence impair the function of the BBB. In support of this hypothesis, we found that horses fed a diet high in pro-oxidants and low in vitamin E had an increased risk of EMND (11), but the mechanism by which oxidative stress increases the risk of EMND is not understood. Vitamin E is known to function as an antioxidant that neutralizes highly reactive oxygen species, and by doing so reduces cell membrane lipid peroxidation. Therefore, we hypothesized that vitamin E is a critical factor in preserving BBB integrity.
Protects polyunsaturated fatty acids within phospholipid membranes and in plasma lipoproteins against oxidative damage. Oxidation of polyunsaturated fatty acids leads to the production of lipid hydroperoxides (12).

In the present study, we determined the effects of low dietary levels of vitamin E on the development of cerebral oxidative stress and the integrity of the BBB in rats. The results provide further support for the hypothesized role of vitamin E deficiency in functional impairment of the BBB and the development of CNS diseases.

**MATERIALS AND METHODS**

**Experimental Design**

To evaluate the impact of feeding a diet deficient in vitamin E on the permeability of the BBB, rats were randomly assigned to either diet deficient of vitamin E (treatment) or normal diet (control) groups. The animals were followed for intervals from 36 to 43 weeks, and the levels of antioxidants (vitamin E), lipid peroxides, and the permeability of the BBB were evaluated.

**Animals**

Thirty-day-old Sprague-Dawley male rats were acquired from Charles River Breeding Laboratory (Wilmington, MA). Before enrollment, 10 rats were randomly selected, and baseline data were determined. The baseline data included weight, blood hemolysis, plasma vitamin E levels, CNS tissue vitamin E concentrations, and permeability of the BBB to rhodamine B as described in the succeeding paragraphs.

**Sample Size**

We assumed that 5% of the rats would have increased permeability of the BBB irrespective of the diet. Among the animals that receive a diet deficient in vitamin E, 50% might have increased permeability of the BBB. The probability of rejecting the null hypothesis of no difference between the 2 groups in regard to the permeability of BBB was set at 0.05. The statistical power of concluding that there was no difference between the 2 groups in the permeability of BBB was assumed to be 95%. Using these assumptions and the sample size formula suggested by Fleiss (13), we determined that at least 31 rats would have to be included in each group. To allow for attrition because of natural death or other reasons, we included 40 rats per group. Animals were randomly assigned to either the treatment or control groups.

**Treatment and Maintenance**

The treatment group received a diet that was deficient in vitamin E provided by BIO-SERV (Frenchtown, NJ). The diet consisted of sucrose (50%), vitamin-free casein (20%), cornstarch (15%), cellulose (5%), tocopherol stripped corn oil (5%), AIN-76 mineral mixture (including 0.02 g/kg sodium selenite; 3.5%), AIN-76 vitamin mixture (without vitamin E; 1%), L-methionine (0.3%), and choline bitartrate (0.2%). The control group received the same diet with supplementation of 100 IU/kg vitamin E (all-rac-α-tocopherol acetate). The 2 diets were stored at 4°C until use and protected from light for the duration of the experiment. All the rats received food and water ad libitum.

Rats were marked individually and housed in pairs in plastic cages at the animal facilities at the College of Veterinary Medicine, Cornell University. Body weights were recorded weekly. Animal care and handling conformed to the regulations, policies, and principles required by the Institutional Animal Care and Use Committee at Cornell University.

**Determination of Vitamin E Levels**

Samples of plasma were analyzed for α-tocopherol using high-performance liquid-liquid partition chromatography. Under conditions of subdued light, the samples were exhaustively extracted into heptanes, dried in a stream of N2, and redissolved in ethanol-dioxane (1:1; v/v) to which acetonitrile (1.5 volumes) was added. The high-performance liquid-liquid partition chromatography analysis involved a reverse-phase system with a mobile phase of acetonitrile-tetrahydrofuran-methanol–1% ammonium acetate (68:22:7.3; v/v) and a C-18 stationary phase. α-Tocopherol was detected by molecular fluorescence (excitation at 291 nm, emission at 330 nm [6.33 min]) using a Perkin-Elmer 650-10S spectrofluorometer (Perkin-Elmer, Norwalk, CT). The entire system is computer controlled (Waters Maxima) and includes automated multiple-sample injection (Waters WISP), pump control (Waters 510 high-performance liquid-liquid partition chromatography), and data acquisition (Waters Associates, Inc., Milford, MA). Analytic quality control is assured by the use of α-toc as an internal standard. These determinations were performed at the Diagnostic Laboratory, College of Veterinary Medicine, Cornell University.

**Determinations of Lipid Hydroperoxides**

As an index of lipid peroxidation, we measured the plasma levels of lipid hydroperoxides. The test was performed using a commercial kit (lipid hydroperoxide assay kit; Cayman Chemical, Ann Arbor, MI), which is based on extraction of lipid hydroperoxides into chloroform, and the extract is used directly in the assay. The test is based on the redox reactions with ferrous iron, and the test results are read using a 96-well microplate reader. The test principle is based on assaying the oxidation of ferrous ions to ferric ions by hydroperoxides under acidic conditions. The resulting ferric ions are detected using thiocyanate ion as chromogen. The test was performed according to the manufacturer recommendations using the 96-well plate reader. The concentration of the hydroxide in the original sample was calculated from the standard curve using the formulas suggested by the manufacturer.

**Evaluation of BBB Permeability**

The disruption of the permeability of the BBB was assessed using a modification of the rhodamine extravasation approach (14). At termination of the study, 36 to 43 weeks postenrollment, all animals were anesthetized with isoflurane following the Institutional Animal Care and Use Committee protocol at Cornell. Five milliliters of blood from the heart was collected cautiously, the serum was separated for vitamin E determination, 1 ml of plasma was processed...
immediately for lipid hydroperoxidase determination, and the remainder was stored at −80°C for further analyses.

Rhodamine B (Fisher Scientific, Atlanta, GA; 0.5% solution in saline) was injected via the intracardiac route at an amount of 2.5 μL/g body weight while the animal was still anesthetized. Thirty minutes later, 3 ml of blood was collected from the heart in a heparinized tube for brain/plasma ratio determination. While the animal was still anesthetized, the thoracic cavity was subsequently opened, and the right atrium was identified and punctured with a stab incision. Saline was then perfused through the left ventricle until the perfusion fluid visualized at the right atrium was colorless (60 ml of saline on average). The animal was humanely killed via this exsanguination process, and the whole intact brain was immediately harvested. The brain was weighed, divided on the median plane, and each half was separately weighed. One half was immediately anchored in optimum cutting temperature compound in a freezing boat and immersed in chilled isopentane (in liquid N₂) for 30 to 60 seconds. The boats were subsequently sealed with foil and stored at −70°C for digital photography. The other half was processed for fluorometric studies.

**Fluorometric Studies**

The rhodamine concentration in the brain harvested from the vitamin E-deficient and control groups was determined using a microplate fluorometer (Safire, Tecan, France). The half of the brain assigned to this study was diced on aluminum foil and transferred into a 2-ml microcentrifuge tube. An equal volume of phosphate-buffered saline was added. Microbeads were added at the rate of 2% (v/v), and the tissues were homogenized using a bead beater for 2 minutes. After homogenization and centrifugation, the extracted dye in the supernatant was harvested into

![Figure 1](http://jnen.oxfordjournals.org/)

**Figure 1.** Mean weights of vitamin E-deficient (Defi) and control (Cont) rats over time. Forty rats per group were obtained at 1 month of age and randomly placed into the diet groups.

![Figure 2](http://jnen.oxfordjournals.org/)

**Figure 2.** Higher mean plasma lipid hydroperoxide concentrations in vitamin E-deficient compared with control rats (n = 40 per group, p < 0.05).
a 2-ml microcentrifuge tube and stored at −20°C until fluorescence determination. At the time of determination, 1:4 μl (in phosphate-buffered saline) of the thawed rhodamine was transferred into special plates, and its fluorescence was determined (excitation, 540 nm; emission, 650 nm) with a Tecan Safire luminescence spectrometer. The tissue content of rhodamine was computed from a standard curve derived from known amounts of the dye and expressed per gram of tissue.

**Digital Fluorescence Microscopy**

At the time of processing, frozen blocks were sectioned in a cryostat at 8 μm, and the sections were placed onto glass slides. The sections were then mounted on Vectashield (Vector Laboratories, Burlingame, CA) to slow the loss of fluorescence. Some sections were treated with Velashield mounting medium that contained 4',6-diamidine-2'-phenylindole dihydrochloride to visualize cell nuclei. The sections were then visualized using a fluorescence microscope (Olympus AX70 reflected fluorescence microscope with a mercury burner; Olympus America Inc., Lake Success, NY). The slides were examined at 10× with the triple cube (U-MW1Y) to visualize rhodamine B and 4',6-diamidine-2'-phenylindole dihydrochloride simultaneously. At least 10 fields were examined from each slide from the cerebrum.

**Statistical Analysis**

The significance of differences between the groups with respect to vitamin E levels, weight, and plasma levels of lipid hydroperoxides was determined using the regression analysis technique in SAS statistical software (15). The significance of the difference in the permeability of the BBB...
with regard to rhodamine concentration in the 2 groups was evaluated using the Wilcoxon rank sum test. The level of significance was considered at $p < 0.05$.

**RESULTS**

**Impact on Body Weight**

The weights of the animals were significantly affected by the type of diet despite apparently similar dietary intake. Figure 1 shows the changes in weight over time between the 2 groups. Animals on the vitamin E–supplemented diet continued to grow, whereas the growth rate in the deficient group stabilized. The rate of weight gain in the first 10 weeks for both groups was faster than the rate of gain after 10 weeks of age, but weight gain continued after this age at significantly lower rates. Regression analysis demonstrated that the average difference in the rate of weight gain between rats maintained on the vitamin E–deficient and control diets was significant ($p < 0.005$).

**Vitamin E Concentrations**

There were significant differences in the plasma vitamin E levels in animals that were kept on the vitamin E–deficient diet compared with controls. Animals that were kept on the deficient diet had, on average, plasma vitamin E levels of $0.23 \pm 0.56 \mu g/ml$. Thirty-seven percent of the animals on the deficient diet had no detectable plasma levels of vitamin E, and only 3 animals had values greater than 1 $\mu g/ml$. In contrast, the plasma vitamin E values for control animals ranged from 5.94 to 21.62 $\mu g/ml$ (mean, $12.0 \pm 3.37 \mu g/ml$).

**Lipid Peroxidation**

Animals kept on the vitamin E–deficient diet had a significantly higher concentration of plasma lipid hydroperoxides in comparison to animals kept on the normal diet ($31.95 \pm 11.15$ vs $21.90 \pm 7.9 \mu M$; $p < 0.05$; Fig. 2). These results are interpreted as evidence of increased lipid peroxidation in the vitamin E–deficient group and marked oxidative stress.

**Rhodamine B Fluorometry**

Figure 3 shows representative images of brains from animals in the vitamin E–deficient and control groups. An image from a control rat not injected with rhodamine B is shown in Figure 3A. Fluorescence was confined to the lumens of blood vessels in tissues from control animals injected with rhodamine (Fig. 3B). There was no escape of rhodamine B into the perivascular space or in the brain parenchyma. In contrast, Figures 3C and D demonstrate strong rhodamine B fluorescence in the lumens and walls of blood vessels and in the surrounding parenchyma. Animals in the deficient diet group had significantly higher concentrations of rhodamine B in the tissues compared with the controls ($12.1$ vs $3.7 \mu g/g$; $p < 0.05$; Fig. 4).

**DISCUSSION**

Previous studies have shown that horses afflicted with EMND had significantly lower levels of plasma and tissue vitamin E compared with controls (6, 11, 16, 17) and increased accumulation of endothelial lipopigments in the small vessels of the spinal cord (7). Moreover, EMND was produced in 50% of animals on a vitamin E–deficient diet that was supplemented with high levels of pro-oxidants (11). These data have led to the development of the hypothesis that oxidative stress affected the permeability of the BBB in vitamin E–deficient animals.

The BBB is composed of brain capillaries with continuous epithelial-like tight junctions that adhere capillary endothelial cells together and has approximately a 5,000 times greater surface area than that of the blood–cerebrospinal fluid barrier, which is limited to the area occupied by the choroid plexus (9, 18, 19). The important role of vitamin E in biologic...
membranes as an antioxidant and stabilizing agent is well established (10), and the present results support the hypothesis that dietary deficiency in vitamin E compromises the integrity and the function of the BBB.

The mechanism by which the integrity of the BBB is compromised by vitamin E deficiency is not fully understood. It has been suggested that free radicals produced during ischemia play a role in the disruption of the BBB (20), and several studies associate the breakdown of the BBB with oxidative stress (21–26). We demonstrate here that rats maintained on a vitamin E–deficient diet had significantly greater plasma concentrations of lipid hydroperoxides than animals on the normal diet. The combination of low levels of plasma vitamin E and the increased concentrations of lipid hydroperoxides are therefore strong arguments for oxidative stress in the vitamin E–deficient rats.

The relationships between oxidative stress and the BBB are also not understood. One potential direct effect of oxidative stress may be via the superoxide radical, which could cause endothelial cell injury, increased vascular permeability, and edema (27–32). In support of this hypothesis, treatment with superoxide dismutase suppresses oxidative stress and hence reduces neuronal death (27, 33). We did not measure superoxide dismutase activity in the present study, but this etiologic scenario remains plausible. Another possible mechanism for the involvement of superoxide radical is through the interaction with nitric oxide whereby the resultant highly reactive hydroxyl and peroxynitrite radicals alter the endothelial permeability (34, 35). Elevated levels of both superoxide and nitric acid might affect capillary endothelial cell tight junction integrity (36, 37), and other indirect mechanisms for BBB injury by oxidative stress may be involved. The integrity of the BBB has been reported to be affected in several infectious and pathologic conditions, including meningitis and human immunodeficiency virus infections (38–40), stroke (41, 42), and multiple sclerosis (9, 43, 44).

Because of its contribution to the integrity of cell membranes, there has been a longstanding interest in the potential protective role for vitamin E in neurologic disorders such as epilepsy and amyotrophic lateral sclerosis. In experimental epilepsy models, therapy with vitamin E improved BBB function (45, 46). Because all of the rats in the present study were male, it is of interest that its effects on the BBB in experimental epilepsy were found to be more efficacious in male than in female rats (47). Dietary supplementation of vitamin E in transgenic mouse models of familial amyotrophic lateral sclerosis delayed the clinical onset and slowed disease progression, but does not prolong survival (48, 49). The results of studies of the potential therapeutic effect of vitamin E in human amyotrophic lateral sclerosis and other neurodegenerative disorders have been mixed (50–54).

In agreement with other studies in rats (55, 56), we found that animals on a vitamin E–deficient diet had low plasma vitamin E levels and significantly lower weight gain and body weights. We also found that vitamin E–deficient rats had significantly greater leakage of rhodamine B from the vessels than the controls. This implies that the deficiency altered the integrity of the BBB because the lack of vitamin E was the only experimental factor and hence allowed the exogenous, low molecular weight substance (479.02 g/mol) to pass into the normally protected brain parenchyma. Based on these observations, we infer that the increased incidence of EMND among horses may similarly be related to the deficiency in vitamin E, although it is not likely the major factor that causes neurodegeneration. Rather, the vitamin E deficiency may compromise the BBB, thereby allowing neurotoxic substances to enter the CNS and injure neurons. Further research on vitamin E–deficient animals under natural circumstances may help pinpoint some of the deleterious neurotoxins.

REFERENCES

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