Dietary Vitamin E Levels Affect Outcome of Permanent Focal Cerebral Ischemia in Rats

H.B. van der Worp, MD; P.R. Bär, PhD; L.J. Kappelle, MD, PhD; D.J. de Wildt, PharmD, PhD

Background and Purpose—A supraphysiological amount of vitamin E in the standard diet of laboratory animals may provide partial protection against cerebral ischemic damage in stroke models. The aim of the present study was to test the effect of dietary vitamin E on infarct volume in rats subjected to permanent focal cerebral ischemia.

Methods—Male Wistar rats were raised on a vitamin E–deficient diet (n=10) or a control diet containing 62.7 mg vitamin E/kg (n=11) for 13 to 16 weeks, from the age of 3 weeks. The left middle cerebral artery (MCA) was permanently occluded by means of an intraluminal silicone-coated 3–0 suture. Blood flow in the left MCA territory was measured before and after occlusion with laser Doppler flowmetry. The area of infarction was measured in hematoxylin-eosin–stained brain sections by means of an image analysis system. The investigator was not aware of the vitamin E status of the rats.

Results—Blood flow in the left MCA territory in the second half hour after occlusion was 43±17% and 42±17% (mean±SD) of the baseline value in control and vitamin E–deficient rats, respectively. The mean infarct volume, measured after 48 hours of survival, was 61±19 mm³ in control rats and 137±76 mm³ in vitamin E–deficient rats (P=0.037).

Conclusions—After permanent focal cerebral ischemia, the infarct is larger in vitamin E–deficient rats than in rats raised on a diet with the usual, supraphysiological amount of vitamin E. This may have consequences for cerebral ischemia studies with experimental animals. (Stroke. 1998;29:1002-1006.)

Key Words: cerebral ischemia ■ lipid peroxidation ■ vitamin E ■ diet

Free radical formation and subsequent lipid peroxidation have been implicated as important factors in the pathogenesis of ischemic brain damage.1,2 In animal models, several antioxidants have been shown to be neuroprotective when given before or shortly after induction of focal cerebral ischemia.3–5 The naturally occurring antioxidant vitamin E scavenges lipid peroxyl radicals and thereby inhibits lipid peroxidation.6 In rats, the extent of postischemic cerebral lipid peroxidation has been reported to depend on the amount of vitamin E in the diet.7 To improve health and fertility and to prevent oxidation of food, animal diets are usually supplemented with supraphysiological doses of vitamin E. This may influence outcome in experimental studies of focal cerebral ischemia.

The aim of the present study was to test the effect of dietary vitamin E on cerebral infarct volume in rats subjected to permanent intraluminal middle cerebral artery (MCA) occlusion.

Materials and Methods

The experiments were performed according to a protocol approved by the institutional animal care committee. Male Wistar rats, weaned at 3 weeks of age, were raised on a vitamin E–deficient diet (n=10) or control diet containing 62.7 mg dl-α-tocopheryl acetate/kg (n=11) for 13 to 16 weeks. These special diets, containing 5.2% fat, were prepared at our request by Hope Farms (Woerden, the Netherlands) and differed only in content of dl-α-tocopheryl acetate. The composition of the diets was guaranteed by the manufacturer.

Vitamin E concentrations in food samples from this manufacturer are regularly determined by means of high-performance liquid chromatography, according to a method described by the Analytical Methods Committee of the Royal Society of Chemistry.8 Body weights (mean±SD) of the rats at onset of the diet were 82±11 and 78±9 g, respectively. The investigator was blinded to the diet allocation of the animals during all procedures and measurements.

The rats were anesthetized with 3% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen, intubated, and mechanically ventilated with a rodent ventilator (Rodent Ventilator 683, Harvard). Anesthesia was maintained during the operative procedures and the first 60 minutes after MCA occlusion with 1.5% isoflurane in the same gas mixture. The left femoral artery was cannulated to record arterial blood pressure every 30 seconds (Viggo-Spectramed DT-XX disposable transducer, Viggo-Spectramed BV). Arterial blood gases were measured before and 30 minutes after MCA occlusion (model 288 Blood Gas System, Ciba-Corning Diagnostics Corp). When necessary, respiratory adjustments were made to maintain normal blood gas values. Rectal temperature was measured continuously and maintained between 36.5°C and 37.5°C throughout the experiment.
by means of a feedback-regulated heating blanket (homeothermic blanket control unit, Harvard).

MCA occlusion was achieved by a minor modification of the intraluminal filament technique originally described by Koizumi et al. Briefly, the left common, external, and internal carotid arteries were identified through a ventral cervical midline incision. The superior thyroid, maxillary, lingual, and occipital branches of the external carotid artery were coagulated and cut. The pterygopalatine artery was ligated with a 5–0 silk suture. A 3–0 polypropylene monofilament suture, its tip slightly enlarged by treatment with boiling xylene and coated with silicone, was introduced into the lumen of the stump of the external carotid artery and gently advanced into the internal carotid artery (ICA) until a slight resistance was felt, occluding the origin of the MCA.

To verify MCA occlusion, the regional cerebral blood flow (CBF) in the cortex supplied by the left MCA was measured every 30 seconds by laser Doppler flowmetry (LDF) (Periflux PF3, Perimed) 2 minutes before (baseline) and 60 minutes after occlusion. A small craniectomy was made with a high-speed mini-drill to expose the left parietal cortex; the dura was left intact. The LDF probe (PF 302, Perimed) was stereotaxically placed on the exposed dura 3 mm posterior and 5 mm lateral to the bregma; large blood vessels were avoided. Regional CBF during occlusion was expressed as a percentage of baseline.

Capillary plasma glucose was measured before the operative procedures by means of a blood glucose sensor electrode (Companion 2, MediSense). α-Tocopherol in serum was analyzed by high-performance liquid chromatography, followed by spectrophotometry, according to a minor modification of the methods of Lee et al. and Nierenberg and Nann.

Forty-eight hours after induction of ischemia, the rats were killed by an intraperitoneal injection of 150 mg pentobarbital and transversally perfused with normal saline followed by a 4% phosphate-buffered formaldehyde solution. Brains were carefully removed and stored in the formaldehyde solution for a minimum of 7 days. After dehydration in a phosphate-buffered 25% sucrose solution, coronal cryopreserved sections (25 μm) were cut and stained with hematoxylin and eosin for histopathological evaluation. “Infarct” was defined as the area of pallor caused by loss of affinity for hematoxylin affecting all cell types except infiltrated inflammatory cells. The areas of the infarct, left hemisphere, and total brain were measured in each 20th section by means of a digital image analysis system (TIM, DIFA) and multiplied by the distance between sections to obtain the respective volumes.

All data are expressed as mean ± SD. Statistical evaluation was performed using Student’s t test and Levene’s test for equality of variances. Differences were considered to be statistically significant at P < 0.05.

Results

One rat died of an unknown cause 10 weeks after onset of the vitamin E–deficient diet. Two control rats and two rats fed the vitamin E–deficient diet died within 24 hours after MCA occlusion. Because we could not determine infarct volume accurately in these rats, they were excluded from further analysis, as was one control rat in which the suture had perforated the intracranial ICA. The only (control) rat from a different stock had an infarct volume of 252 mm³ and was excluded after an outlier test had indicated it as an extreme value (P < 0.005).

The serum level of α-tocopherol in control animals was 30.9 ± 6.6 μmol/L, which is in the range reported for rats fed vitamin E–supplemented chow in studies of cerebral ischemia and brain trauma. The α-tocopherol concentration in serum of vitamin E–deficient rats was 1.5 ± 1.1 μmol/L, which was significantly lower than that seen in control animals (P < 0.001), but somewhat higher than that in rats maintained on vitamin E–deficient diets for 16 to 18 weeks (0.4 ± 0.3 μmol/L) or 8 to 10 weeks (0.6 ± 0.5 μmol/L) in previous studies. There was no difference in the duration of the diet between the two groups, and the diet had no effect on body weight or plasma glucose levels (Table 1). Physiological parameters are shown in Table 2. There were no differences between groups for rectal temperature, blood gas data, heart rate, or mean arterial blood pressure. Regional CBF in the first 60 minutes after induction of ischemia was not different between the two groups (Table 3).

In both groups, most neurons, glia, and myelin in ischemic tissue had lost their affinities for hematoxylin, and most neurons showed eosinophilia. Occasional eosinophilic neurons with pyknotic nuclei (red neurons) surrounded by normal tissue were found just outside the pale area, and few neurons within the pale area showed no abnormalities. The pale infarcts could be differentiated readily from the surrounding normal tissue. Except for volume, there were no differences at the cellular level between the infarcts in control rats and those in vitamin E–deficient rats. The infarct volume was 2.2 times larger in vitamin E–deficient rats than in control rats (137 ± 76 versus 61 ± 19 mm³, respectively; P = 0.037). Infarct volume as a percentage of the left hemisphere was 23 ± 12% in vitamin E–deficient rats and 10 ± 3% in control animals (P = 0.038).

Discussion

In the present study, the infarct volume after permanent intraluminal MCA occlusion was 2.2 times larger in rats deficient in the antioxidant vitamin E than in rats raised on a standard diet containing 62.7 mg vitamin E per kilogram, whereas blood flow in the MCA territory was equally reduced in both groups. This supports the assumption that free radical damage is not the primary cause of infarcts after permanent intraluminal MCA occlusion. The infarct volume was 2.2 times larger in vitamin E–deficient rats than in control rats (137 ± 76 versus 61 ± 19 mm³, respectively; P = 0.037). Infarct volume as a percentage of the left hemisphere was 23 ± 12% in vitamin E–deficient rats and 10 ± 3% in control animals (P = 0.038).

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<tr>
<th>TABLE 1. Age, Body Weight, and Serum Glucose Concentration of Rats Raised on Control and Vitamin E–Deficient Diets</th>
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<td><strong>Control Rats</strong></td>
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<td>Age, wk</td>
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<td>Body weight, g</td>
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<td>Plasma glucose, mmol/L</td>
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<td><strong>Values are mean ± SD. n = 7 in both groups.</strong></td>
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<th>TABLE 2. Physiological Variables of Control and Vitamin E–Deficient Rats Before and in the First 60 Minutes After Middle Cerebral Artery Occlusion</th>
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<td><strong>Baseline</strong></td>
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<td>pH</td>
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<td>MABP, mm Hg</td>
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<td>Temp °C</td>
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MABP indicates mean arterial blood pressure; temp, rectal temperature. Values are mean ± SD. n = 7 in both groups. Differences are not significant.
formation and subsequent lipid peroxidation are pivotal steps in the pathogenesis of focal ischemic brain damage and points to an important role of this vitamin as an inhibitor of oxidative damage in this condition.

Vitamin E includes eight naturally occurring compounds in two classes, designated as tocopherols and tocotrienols, with different biological activities. For consistency, in this article the amounts of all dietary compounds with vitamin E activity are expressed as the equivalent of 1 mg of the synthetic form, a-tocopherol acetate, which is equal to 1 IU vitamin E. The lipid-soluble vitamin E inhibits the peroxidation of polyunsaturated fatty acids in cellular membranes or plasma lipoproteins. Dietary deficiency of this vitamin therefore enhances the susceptibility of biomembranes to oxidative damage. The vitamin E requirement for the most frequently used strains of rats is 27 mg/kg diet, when lipids compose less than 10% of the diet. However, to improve fertility and health, supplements of vitamin E to animal food have increased over the last few years. Unfortunately, the composition of the animal diet and its antioxidant content are often neglected by investigators.

Levels of vitamin E in animal food usually are not mentioned in articles on experimental cerebral ischemia, even when a-tocopherol concentrations in the brain are measured. In studies that focused on various cerebral effects of differing levels of dietary vitamin E, the vitamin E content of the control food usually varied between 20 and 100 mg/kg, with exception of up to 170 mg/kg, reflecting the variety in vitamin E concentrations in the “standard” rodent diet.

Our results demonstrate that the amount of vitamin E in the standard rodent diet provides substantial protection against focal cerebral ischemic damage. This is in line with earlier observations that lipid peroxidation after decapitation ischemia followed by reoxygenation was less in vitamin E–supplemented rats compared with control animals raised on a diet containing 20 mg/kg. Oral or intravenous administration of this vitamin in the acute phase of neurological injury has no therapeutic efficacy, because the vitamin is taken up only very slowly by cerebral tissues.

The availability of oxygen is a prerequisite for the peroxidation of lipids. However, in contrast to the absence of blood flow in some models of global cerebral ischemia, blood flow reduction in focal ischemia is not absolute but ranges from severe in the ischemic core to moderate in the penumbra. In the present study, cortical blood flow in the MCA territory during the second half hour of ischemia was reduced to about 42% of the preischemic baseline. Because free radicals are formed even at very low oxygen tensions, reperfusion is not a requirement for their formation. Hydroxyl radical formation during both focal ischemia and subsequent reperfusion was suggested by an increase of salicylate hydroxylation. This is supported by the present study: in the absence of recirculation the infarct volume was larger in vitamin E–deficient rats, suggesting increased oxidative degradation of biomembranes.

In this study, serum a-tocopherol levels in vitamin E–deficient rats were significantly decreased to 5% of the control value. During vitamin E deficiency, a-tocopherol concentrations in the brain, spinal cord, and nerves decline more slowly than in other tissues; after 16 weeks of deficiency, concentrations in the brain are still 18% of control values. The present study, in which rats were maintained on a vitamin E–deficient diet for a comparable period, demonstrates that such a concentration of vitamin E does not provide sufficient antioxidative protection. Whether shorter periods of deficiency and thus less decreased brain levels of vitamin E also result in enhanced lipid peroxidation and increased infarct volume remains to be answered in further studies. This is important, because the rats used in most experiments are much younger than those used in the present study.

In contrast to serum levels, brain a-tocopherol levels were not measured. In previous studies of permanent focal cerebral ischemia in the rat, levels of 31 nmol/g and 40 nmol/g were found. In one of these studies, a-tocopherol in ischemic tissue decreased to 63% of the control value, but others found no evidence of consumption of this antioxidant during permanent focal cerebral ischemia.

Because infarct volume was only measured 48 hours after onset of ischemia, the present study does not provide evidence that the effect of vitamin E is maintained for a longer period of time. However, except for volume, no pathological differences in ischemic tissue were found between the two groups. Therefore, it is very unlikely that the difference in infarct volume found at 48 hours would have disappeared at a later point in time, indicating a faster rate of infarct development in vitamin E–deficient rats as the cause of the observed difference.

Recent prospective clinical studies have shown an inverse association between dietary intake of vitamin E and the occurrence of cardiovascular disease. The effect of dietary vitamin E on outcome in acute ischemic stroke is less extensively studied than its preventive properties. In a small study, no difference in outcome after ischemic stroke was found between patients with serum concentrations of vitamin E above or below the mean value. A larger study applying a more subtle subdivision of serum concentrations of this vitamin would be required to test the effect of dietary intake of vitamin E on stroke outcome more accurately. However, such a study will always be hampered by the fact that its serum concentrations do not precisely reflect those in the brain.

In conclusion, our results support the postulate that free radical formation and subsequent lipid peroxidation are important factors in the pathogenesis of ischemic brain injury.
caused by permanent MCA occlusion. The amount of vitamin E in the standard rodent diet provides partial protection against this oxidative damage. The effects of increasing supplements of vitamin E to animal food should be tested in further studies. Researchers should be aware of a possible confounding effect of the dietary amount of vitamin E on outcome in animal studies of cerebral ischemia and should note the concentration of this vitamin in the food used.

Acknowledgments

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References


Editorial Comment

The authors convincingly demonstrate that in their model of cerebral infarction, a diet enriched in vitamin E results in a reduction in the size of the infarction. The reduction was not merely statistically significant but significant in a practical sense, since the infarct volume was reduced by 50%. It is unfortunate that the studies were not carried out beyond a 48-hour period of survival. Recent studies of cerebral ischemia indicate that significant loss of neurons may occur after 48 hours. It is not yet totally clear whether those “delayed” increments in infarct size are due only to apoptosis or to other forms of selective neuronal necrosis, or whether in addition there is a delayed increment in necrosis of all the tissue elements. In the present study, infarction is defined in the latter sense. In any case, it would be of interest to see to what extent protection by vitamin E is manifest 1 or 2 weeks after the ischemic event.
The authors speak of the vitamin E levels in the protecting diet as “supraphysiological.” They base this opinion on the amount of vitamin E heretofore thought sufficient to provide the needs of rodents. However, as is also the case in humans, there has never been a set of objective criteria upon which to base the assessment of a “normal” requirement for vitamin E. It may be that evidence of frank deficiency is too crude a means for determining a normal daily requirement. In another study, this one of mice, higher amounts of vitamin E were required to protect the endothelium of pial arterioles from damage that resulted in loss of ability to produce endothelium-derived relaxing factor following the application of acetylcholine. In studies of humans, a daily dose of 400 U has sometimes been found beneficial in the prevention of cardiovascular disease while lesser amounts have failed to show a benefit. The 400-U dose is far in excess of current “daily requirements.”

The proposed mechanism of vitamin E action is the scavenging of oxygen-centered free radicals. It would be interesting to know whether the authors can achieve an equal amount of protection with some other radical scavenger. Regardless of mechanism, the effects of vitamin E have obvious implications for humans. Since 400 U/d seems to be without any adverse effects, it may be advisable to incorporate this supplement into the daily diet.

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Reference
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