Murine Red Blood Cell Fragility Is Not Affected by Either Vitamin E Depletion or Supplementation (44017)

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Abstract. **Male ICR mice were pair-fed semipurified diets containing 0, 55 (control), and 500 IU/kg of vitamin E. Plasma and hepatic concentrations of vitamin E were determined and found to parallel the vitamin E levels in the diet. Even though plasma vitamin E levels were virtually zero in mice fed the depleted vitamin E diet for up to 304 days, there was no statistical difference in the red blood cell fragility between these animals and controls, as determined by a hypoosmotic fragility test. The diet with enriched vitamin E concentrations also did not affect the fragility of the red blood cell (RBC). Even after 300 days of zero dietary vitamin E, mice appeared healthy, demonstrating neither neurologic dysfunction nor failure to thrive. The data indicates that mice, unlike several other species, are more resistant to vitamin E depletion and may have other mechanisms to compensate for loss of this important antioxidant.**

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The present report is an initial attempt to assess the effect of varying dietary amounts of vitamin E on red blood cell (RBC) hemolysis in mice. Vitamin E is a well known inhibitor (1) of oxidative breakdown of cell membranes. The RBC hemolysis test has been reported in other species to be an extremely sensitive indicator of functionally important alterations of vitamin E levels in tissue (2, 3). Vitamin E, as an important dietary antioxidant (4), is involved in multiple protective roles that may include protection against oxidation of specific cell mediators (5, 6).

In this study, dietary vitamin E in the form α-tocopherol acetate (α-TOH) was used as the manipulated dietary variable. Biological membranes contain oxidizable polyunsaturated fatty acids which make the membrane susceptible to oxidative damage from highly reactive, nonselective free radical species. α-TOH, the most active form of vitamin E, may prevent this oxidative damage through the process of chain breaking inhibition (1). This occurs when an α-TOH molecule, a replenishable source, is oxidized instead of a membrane component. The antioxidant is capable of containing and terminating the free radical oxidation peroxyl radicals, while the oxidation of a fatty acid in the membrane may elicit the propagation of oxidation to other components of the membrane (7). The phytyl tail of α-TOH is important for the retention of the molecule in the membrane (8, 9). Although the mechanism remains to be elucidated, it has been shown that α-TOH exchanges between rat plasma and RBC membranes with a $t_{1/2}$ of 2.2 hr, thus providing evidence that an increase in serum α-TOH may increase RBC membrane α-TOH (9). These characteristics of α-TOH make it a strong candidate for protection of the RBC membrane.

A modification of the RBC hemolysis test (3) was

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used. In this procedure, RBC are exposed to an increasingly hypotonic sodium chloride (NaCl) solution which causes the cell volume to increase. When a RBC reaches a certain critical volume due to a hypotonic change in the NaCl environment, the hemoglobin diffuses from the intracellular to the extracellular space to relieve the imposed stress on the cellular osmotic equilibrium. The volume of the cell at which the diffusion takes place is believed to be inversely proportional to the fragility of the cell (10). Fragility is reportedly decreased as a function of increasing vitamin E levels (2, 11–14). Our modified hemolysis procedure allows the plasma to be removed before determination of RBC fragility for subsequent analysis of blood solutes. Vitamin E levels were also measured in liver.

Materials and Methods

Experimental Diets and Animals. Six-week-old male mice (ICR) strain Harlan Sprague-Dawley (Indianapolis, IN) were housed one per cage. The experimental protocols were approved by the Institutional Animal Care and Use Committee. The animal room had a controlled temperature of 25°C with a 12-hr light cycle. Mice were pair-fed and allowed water *ad libitum*. All special diets were certified by Bioserve (French Town, NJ) for vitamin E levels, screened for toxins, and an analysis for protein, carbohydrate, fiber, and fat were given. The animals were weighed on a Harvard balance, four times per month, before feeding.

Diets. Animals were pair-fed a semipurified diet containing various amounts of α-tocopherol acetate. The composition of the diets were as follows: 17.98% protein (all from casein), 5.07% fat (tocopherol stripped corn oil), 5.13% fiber, 3.29% ash, 10.00% moisture, and 61.53% carbohydrate. Caloric profile (kcal/g): protein 0.767, fat 0.448, carbohydrate 2.436, and ethanol 0.000. The fatty acid distribution was (g/kg semipurified diet): saturated, 6.95; monounsaturated, 13.3; and polyunsaturated, 29.75. Specifically the fatty acid composition was (g/kg semipurified diet): palmitic, 5.75; stearic, 1.1; oleic, 13.3; linoleic, 29.35; linolenic, 0.4; and arachidonic acid, 0.1. Trace elements included: selenium, 141 μg/kg; zinc, 41 mg/kg; and magnesium, 500 mg/kg. Other components (g/kg diet): thiamine · HCl, 0.6; riboflavin, 0.6; pyridoxine, 0.7; niacin, 3.0; calcium panthothenate, 1.6; folic acid, 0.2; biotin, 0.02; B_{12}, 0.001; vitamin K, 0.06; vitamin A, 500,000 IU; and vitamin D_3, 10,000 IU.

The only difference in the diets was the amount of α-tocopherol in each diet, as follows: vitamin E–free diet, 0 IU/kg; control vitamin E diet, 55 IU/kg; and vitamin E–enhanced diet, 500 IU/kg.

Red Blood Cell Hemolysis. Mice were anesthetized with urethane and blood was collected from the incised carotid artery into a heparinized syringe. The blood sample was centrifuged at 2000 rpm for 5 min and the plasma was removed using a Pasteur pipette and stored for subsequent analysis. The removed volume was replaced with an approximately equal amount of isotonic 0.9%, pH 7.4, buffered NaCl solution. To determine the hemolysis (3), the mixed RBC suspension was added to tubes of increasing concentration of buffered NaCl solution from 0.1% to 0.8%. The tubes were incubated at 30°C for 0.5 hr in a shaker-waterbath. After incubation, the samples were centrifuged at 2000 rpm for 5 min and the relative concentration of the hemoglobin was determined in the supernatant fluid by the absorbance at 540 nm. To calculate the percentage hemolysis, the absorbance (A) for the 0.8% buffered NaCl solution (which represents the 0% hemolysis and any previous hemolysis) was subtracted from the A of the other buffered NaCl solutions. The equation for calculation of percentage hemolysis is

$$\left(\frac{A_{n\%} - A_{0\%}}{A_{100\%} - A_{0\%}}\right) \times 100.$$

The $A_{n\%}$ is the A for various concentrations of NaCl. The 100% hemolysis value was the experimentally determined absorbance ($A_{100\%}$) at 0.1% NaCl minus the 0.8% NaCl absorbance. The 0% hemolysis was experimentally determined to be the absorbance ($A_{0\%}$) of the tube containing 0.8% NaCl. The percentage hemolysis with changing NaCl concentration was plotted using Cricket Graph version 1.3.2 from Cricket Software (Malvern, PA) on a Mac LC II. The 50% hemolysis value was interpolated from the graph. A sample is shown in Figure 1. Data was compared using t tests. $P < 0.05$ were considered significant.

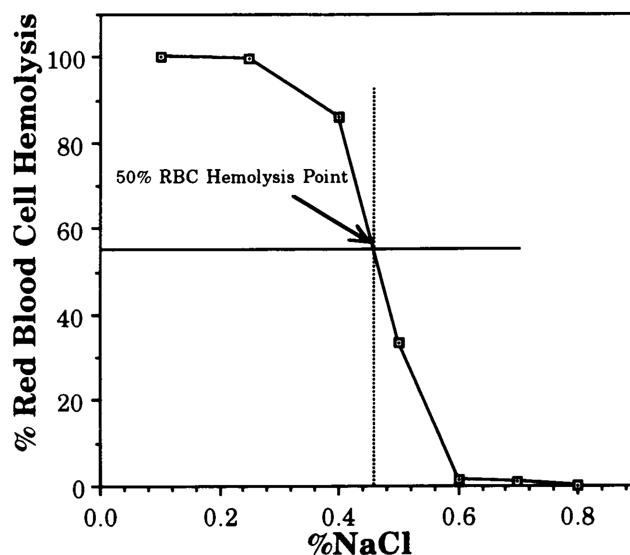

Figure 1. A plot of percentage RBC hemolysis versus percentage NaCl is illustrated for a single animal. The interpolated 50% hemolysis point is shown where the two lines intersect.

HPLC Analysis of α-Tocopherol. Plasma was

placed in 2-ml cryostat tubes and stored in a −70°C freezer until assay. Plasma and tissue vitamin E were quantified using an HPLC technique previously described (15, 16).

Results

Vitamin E Concentrations. Table I shows plasma and liver vitamin E concentrations for mice on all diets. Mice on the vitamin E–deficient diet (0.0 IU/kg semipurified diet) for 64–304 days had plasma devoid of vitamin E (0.0 μg/dl). Hepatic values (7.0 ± 3.7 [5] μg/dl) were substantially depleted when compared with the control (55 IU diet). Mice fed the deficient diet seemed healthy. Their coats appeared normal and they showed no evidence of motor impairment. The body weights were not significantly different from that of pair-fed 55 IU controls (data not shown). There were no changes in the plasma or liver levels of vitamin E during the course of the observations shown in Table I. In other words, steady-state vitamin E level had already been reached by Day 64.

Mice on the 500 IU vitamin E diet had plasma vitamin E values (666 ± 115 [16] μg/dl) four times greater than those of mice on the 55 IU diet and hepatic vitamin E values (323 ± 63 [10] μg/dl) five times those of mice on the 55 IU diet during the 112- to 128-day period that the mice were on the diet.

Hematocrits and NaCl Concentration at 50% and 6% RBC Hemolysis. Table II shows the NaCl concentration for 50% hemolysis and 6% (initial hemolysis point) hemolysis of the RBC for the four diets. These values were not significantly different, indicating that the fragility of the RBC for the mice on each diet was not affected by the amount of vitamin E in their diet, even when the duration of treatment was extended to 10 months. This was confirmed by comparing hemolysis curves from individual mice euthanized at different times within any treatment group. The hemolysis curves were remarkably similar no matter what the duration of the treatment. The hematocrit values were not significantly different for animals on their respective diets, which helps confirm the hypothesis that vitamin E supplementation or depletion in mice had no effect on the stability of mouse RBC.

Table I. Plasma and Liver Vitamin E Concentrations for Experimental Diets

Vitamin E in diet[a]	Plasma vitamin E concentration[b]	Liver vitamin E concentration[c]	Duration (days)
0	0.0 ± 0.0 (16)	7.0 ± 3.7 (5)	64–304
55	170 ± 21 (26)	60 ± 5.5 (12)	64–328
500	666 ± 115 (16)	323 ± 63 (10)	112–128

[a] Values are reported in IU Vitamin E/kg semipurified diet/day.
[b] Plasma values are reported in μg/dl (mean ± SEM [*n*]).
[c] Tissue values are reported in μg/g of wet tissue (mean ± SEM [*n*]).

Discussion

Plasma and liver levels of vitamin E obtained from control mice in these experiments are in agreement with those reported by others in mice (17–19). Dietary supplementation of vitamin E increased levels of plasma and liver vitamin E concentration. The zero vitamin E diet led to significantly lower concentrations of vitamin E in liver and zero values for vitamin E in plasma. However, no change in the RBC fragility was found after either significant increases or depletion of both plasma and liver vitamin E levels. This observation was unexpected in view of the data reported in other species, such as humans, rats, and rabbits (2, 11–14, 20). Our results agree with a study that measured the osmotic fragility of RBC in dogs, where no difference in hemolysis was found between those on a vitamin E–deficient diet and those on a standard vitamin E diet (10). The results in the mice are consistent with the apparent absence of illness or failure to thrive in the mice deprived of vitamin E. The unaffected growth of the deficient mice confirms a similar observation by Davies *et al.* (21). This contrasts greatly with the devastating effects of vitamin E deficiency reported in rats (22–24), where RBC hemolysis is greatly enhanced by vitamin E depletion (11).

Perhaps a species peculiarity exists which allowed mice to survive without apparent symptoms with the diet conditions reported here. Higher dietary levels of PUFA might, however, alter the requirement for vitamin E. RBC hemolysis seems to be unaffected in spite of markedly diminished vitamin E level. The basis for this resistance of mice to vitamin E deficiency is not clear. One possibility concerns the ability of mice to synthesize ascorbic acid, a water-soluble antioxidant, that may provide vitamin E–deficient animals protection from lipid peroxidation. Negre-Salvayre *et al.* have shown that ascorbic acid was effective in reducing lipid peroxidation and had a synergistic effect with α-TOH in human RBC (25). Maellaro *et al.* (26) have shown in vitamin E–deficient animals an ascorbic acid decrease at the onset of lipid peroxidation. However, no data that we are aware of conclusively show ascorbic acid to be able to compensate totally for a loss of α-TOH in any system, including mice.

Even after many months with no measurable vitamin E (below 2 μg/dl) in plasma, there was still residual vitamin E detectable in the liver. It might be thought that tissue and plasma levels would be in equilibrium with measurable levels in both. Since this was not the case for liver, it is possible that other cells, including RBC or their cell membranes, may have contained enough, albeit drastically reduced, vitamin E to confer protection from oxidation or resistance to osmotic stress.

The present studies were performed to establish

Table II. Hematocrit and NaCl Concentration at 50% RBC Hemolysis Values for Experimental Diets

Vitamin E in diet[a]	Hematocrit	Percentage NaCl at 50% RBC hemolysis	Percentage NaCl at 0.6% RBC hemolysis	Duration (days)
0.0	46 ± 0.5 (19)[b]	0.463 ± 0.007 (21)	1.1 ± 0.4 (8)	64–304
55	48 ± 0.1 (32)	0.477 ± 0.005 (45)	1.1 ± 0.2 (35)	64–328
500	47.8 ± 0.75 (17)	0.478 ± 0.003 (19)	0.6 ± 0.3 (18)	112–128

[a] Reported in IU vitamin E/kg semipurified diet/day.
[b] Values are reported mean ± SEM (N).

baseline data for an investigation of endothelium-dependent functions in mice with altered vitamin E levels. In view of the inability of dietary vitamin E manipulation and altered vitamin E levels to affect the RBC fragility in mice, it would be of particular interest should dietary manipulation of vitamin E nevertheless influence phenomena that reflect actions of oxidation-sensitive mediators in this species.

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