Vitamin E deficiency and erythrocyte deformability in the rat

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It has been suggested that erythrocyte deformability is decreased in vitamin E deficiency due to oxidative damage to the cell membrane. Male Wistar rats (66 to 88 g) were fed ad libitum an AIN76-based diet containing tocopherol-stripped corn oil without added vitamin E for 8 weeks (−E; n = 8). Control animals were fed ad libitum the same diet containing 50 IU/kg dl-α-tocopheryl acetate (+E; n = 7). Vitamin E deficiency was confirmed by depressed mean (± SEM) plasma α-tocopherol levels (μmol/L), as measured by high performance liquid chromatography [−E: 0.5 ± 0.1; +E: 20.3 ± 1.8] and elevated hydrogen peroxide-induced hemolysis (%) [−E: 92.6 ± 2.4; +E: 4.2 ± 1.6; P < 0.05 by Student's t *test]. The only alteration in a complete blood count was a depression in reticulocyte number (× 10^{12}/L) [−E: 0.19 ± 0.02; +E: 0.46 ± 0.03; P < 0.05]. Erythrocyte deformability was measured at standard shear stress under conditions of increasing osmolality in the ektacytometer. Elongation index (the ratio of length to width of the diffraction pattern of the deformed cells) was plotted against osmolality to generate an osmotic deformability profile. EI_{max} (the maximum elongation index) and O_{hyper} (the osmolality at which the elongation index is half of EI_{max} on the hypertonic arm of the curve) were significantly increased in samples from the −E group (P < 0.05 by Student's* t *test). In summary, erythrocyte deformability as measured by the ektacytometer was not decreased by a subclinical vitamin E deficiency in the rat. In fact, a small but significant increase in maximum deformability was observed in erythrocytes from vitamin E-deficient rats.* (J. Nutr. Biochem. 5:298–302, 1994.)

Keywords: erythrocyte deformability; vitamin E deficiency; rat; erythrocyte; ektacytometer; α-tocopherol

Introduction

Vitamin E is the major lipid-soluble antioxidant responsible for protecting membranes against lipid peroxidation.[1] In vitamin E deficiency, erythrocyte survival is reduced, and the cells show increased sensitivity to oxidant-induced hemolysis in vitro. The hematologic manifestations of vitamin E deficiency are of two types, a hemolytic anemia and impaired erythropoiesis; the predominance of each component varies with species.[2–4] While vitamin E-deficient swine and nonhuman primates show ineffective erythropoiesis, the hemolytic component predominates in the human and the rat but does not usually progress to anemia. However, hemolytic anemia can occur with vitamin E deficiency in both premature infants and rats challenged by a greater then normal oxidant stress.[2–4]

The oxidative damage to the erythrocyte membrane in vitamin E deficiency may alter the ability of the cell to deform. Deformability is critical for normal survival of the red cell, as its resting diameter exceeds that of the capillaries and endothelial slits,[5] and a change in this physiological property may contribute to the decrease in cell survival. While it is often stated that a decrease in erythrocyte deformability accompanies vitamin E deficiency,[3] the evidence in support of this idea is limited. Studies reporting erythrocyte filterability as a measure of cell deformability in vitamin E deficiency have yielded conflicting results, depending on the in vitro treatment of the cells before the measurement. A second limitation of these studies may be the methodology employed as erythrocyte filterability is less sensitive than some other techniques to altered deformability due to membrane damage.[6,7] Levander et al.[8] reported decreased filterability of red cells from vitamin E-deficient rats in some but not all experiments, and this find-

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ing was dependent on incubation of the cells in buffer for 6 hours prior to the measurement. The loss in filterability was exacerbated by lead poisoning. In contrast, erythrocyte filterability was not altered under similar incubation conditions in the vitamin E-deficient mouse nor did vitamin E status have a major effect on the depression in filterability associated with ozone treatment in this species.[9] Erythrocytes obtained from vitamin E-deficient premature infants were less filterable than those from control infants if incubated with hydrogen peroxide for 15 minutes but not if incubated with buffer.[10] Taken together, these studies have not shown convincingly that vitamin E deficiency causes sufficient oxidative damage to erythrocytes to render them less deformable in vivo. It is of interest to know whether depressed erythrocyte deformability contributes to the reported decrease in cell survival in vitamin E deficiency.

In this study, we report the use of the ektacytometer to investigate the influence of vitamin E deficiency on erythrocyte deformability in the rat. The measurement is sensitive to small changes in deformability[6] and has the capacity to differentiate the contribution of each of the cellular determinants of deformability (internal viscosity, cell geometry, and membrane deformability).[11] Ektacytometry has previously been used to detect depressed whole cell and membrane deformability in response to oxidative stress imposed on erythrocytes in vitro.[12]

Methods and materials

Experimental design

Male Wistar rats (66 to 88 g) were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). The animals were acclimatized overnight and randomly assigned to treatment groups the following day. Tap water was supplied ad libitum, and the animals were housed individually in stainless-steel cages. Eight animals were fed ad libitum for 8 weeks an AIN76-based diet[13,14] that contained 5% tocopherol-stripped corn oil, 20% vitamin free casein, and no added vitamin E (−E). Eight control animals were fed ad libitum the same diet containing 50 IU/kg dl-α-tocopheryl acetate (+E). One animal in the +E group was humanely killed during the study due to an injury. Daily feed intake was measured, and the rats were weighed weekly. At the end of the 8-week trial, vitamin E status was assessed using a combination of plasma α-tocopherol concentration and an in vitro hydrogen peroxide erythrocyte hemolysis test. A hematologic assessment and measurements of erythrocyte deformability were carried out as described below.

Sample collection and storage

Blood samples were obtained by a combination of cardiac puncture and decapitation under methoxyflurane (Metofane, Janssen Pharmaceutica, Mississauga, Ontario, Canada) anesthesia and placed on ice. Blood samples taken for a complete blood count were collected in EDTA. Blood samples for other analyses were collected in heparinized polystyrene tubes. Samples to be analyzed for α-tocopherol concentration were immediately covered with foil to avoid exposure to light. All analyses were performed on the day of blood sampling with the exception of plasma α-tocopherol concentration. The latter measurements were performed on plasma samples that had been stored for less than 3 weeks at −70° C.

Plasma α-tocopherol analysis

Plasma α-tocopherol concentration was determined by high performance liquid chromatography (HPLC) under ambient laboratory conditions using the modified procedure of Catignani and Bieri.[15] All samples and standards were protected from light (foil wrap) during preparation and stored in the refrigerator until analysis. Stock standards were stored at −20° C. Plasma samples were deproteinized with a solution of ethanol plus internal standard (dl-α-tocopheryl acetate), and the remaining lipid was extracted with petroleum ether. An aliquot of the ether extract was evaporated, reconstituted in methanol, and analyzed. Chromatographic separation was accomplished using a Novapak C18 column (4 μm, 3.9 × 150 mm) (Waters, Mississauga, Ontario, Canada) and a mobile phase of methanol:water (97:3) pumped at 1.5 mL/min. The HPLC system consisted of a pump (Model 6000A, Waters), injector (Model 7125, Rheodyne, Cotati, California, USA), UV-visible spectrophotometric detector (290 nm) (Model SPD-6AV, Shimadzu), and electronic integrator (Model 3390, Hewlett-Packard). Under these conditions, retention times for α-tocopherol and α-tocopheryl acetate were 4.2 and 5.9 minutes, respectively. The standard curve (α-tocopherol/α-tocopheryl acetate peak area ratio) was linear up to ≥21 μg/mL (r = 0.997).

In vitro hydrogen peroxide erythrocyte hemolysis test

The in vitro hydrogen peroxide erythrocyte hemolysis test was performed using minor modifications of the procedure of Gordon et al.[16] The modifications included washing the packed red cells two times with phosphate-buffered saline initially and altering the incubation time and temperature to 2.5 hours and 37° C, respectively.

Hematological assessment

A complete blood count was measured by routine Coulter Counter techniques (Coulter Counter Model S+IV, Coulter Electronics Incorporated, Hialeah, FL USA) in the Veterinary Clinical Pathology Laboratory at the University of Saskatchewan.

Measurements of erythrocyte deformability

Erythrocyte deformability was measured using an ektacytometer (Technicon Instruments Corporation, Tarrytown, NY USA). The principle of the instrument has been previously described.[17] The erythrocytes suspended in a fluid medium are deformed in a rotational shearing device. The elongated cells diffract a helium-neon laser beam, and the pattern of diffracted light is measured by an image analyzer to give a value for cell elongation (elongation index or EI).

Whole blood (100 μL) was suspended in a solution containing 3.1% polyvinylpyrrolidone (average molecular weight = 360,000), 6.3 mmol/L Na_2HPO_4, 2 mmol/L NaH_2PO_4, and 0.04% NaN_3 (pH 7.35 ± 0.05) with varying NaCl concentration. The erythrocytes were subjected to constant shear stress (160 dynes/cm^2) in the instrument while the osmolality of the suspending medium was progressively increased from 50 to 500 mOsm/kg. The elongation index of the cells was recorded continuously on a linear X-Y recorder. Parameters of the osmotic deformability profile described previously by Clark et al.[18] were used to characterize cell deformability.

The osmolality of solutions used in the ektacytometer was measured by a freezing point osmometer (Model 3MO, Advanced Instrument Inc., Needham Hts., MA USA).

Statistical analysis

The data were analyzed using an unpaired t test.

Source of chemicals

All reagents and solvents were analytical or HPLC grade. Deionized double distilled water was used for all reagent preparation. NaCl,

Na_2HPO_4, NaH_2PO_4, methanol, and H_2O_2 were purchased from BDH Inc., Edmonton, Canada. KH_2PO_4 and NaOH were supplied by J.T. Baker Chemical Company (Phillipsburg, NJ USA). Polyvinylpyrrolidone, d-α-tocopheryl acetate (internal standard) and bovine serum albumin were obtained from Sigma Chemical Company (St. Louis, MO USA). Eastman Kodak Co. (Rochester, NY) supplied the d-α-tocopherol standard. Sodium azide was from Aldrich Chemical Company (St. Louis, MO USA), and petroleum ether was from Baxter Diagnostics Corporation, Canlab Division (Mississauga, Canada). Absolute ethanol was obtained from Stanchem Incorporated (Winnipeg, Canada), and methoxyflurane was from MTC Pharmaceuticals (Mississauga, Canada).

Results

There were no overt clinical signs of vitamin E deficiency in the −E group. Feed intake was similar in the experimental groups. Mean (±SEM) weight gain over the 8 weeks was 370 ± 9 g for the −E group and was not significantly different from that of the +E group (365 ± 12 g). Evidence for the development of a subclinical vitamin E deficiency in the −E group was demonstrated by a depression in plasma α-tocopherol concentration (mean ± SEM of 0.5 ± 0.1 μmol/L) as compared with the +E group with a mean (±SEM) plasma α-tocopherol concentration of 20.3 ± 1.8 μmol/L. Mean (±SEM) percent hemolysis in the hydrogen peroxide erythrocyte hemolysis test was also dramatically higher with erythrocytes from the −E animals (92.6 ± 2.4%) as compared with that found with red cells from animals in the +E group (4.2 ± 1.6%).

There were no differences in the hematologic profile of the two groups with the exception of reticulocyte count, which was significantly depressed in the deficient group (*Table 1*).

A representative osmotic deformability profile for erythrocytes from the normal 11-week-old control rats (+E) is shown in *Figure 1*. Profiles representative of those obtained for erythrocytes from normal adult humans and 6-week-old control rats from a previous study[17] are also shown for comparison with an explanation of the features of the curve used to characterize cell deformability.

Table 1 The effect of vitamin E deficiency on hematological profile

	Experimental group*	
	−E§	+E¶
RBC (× 10^{12}/L)	6.8 ± 0.2 (8)[a]	7.0 ± 0.3 (4)[a]
Hgb (g/L)	115 ± 2 (8)[a]	112 ± 3 (7)[a]
PCV (L/L)	0.33 ± 0.01 (8)[a]	0.32 ± 0.01 (7)[a]
MCV (fL)	49 ± 1 (8)[a]	47 ± 1 (4)[a]
MCH (pg)	17.0 ± 0.4 (8)[a]	16.5 ± 0.3 (4)[a]
MCHC (g/L)	346 ± 1 (8)[a]	348 ± 3 (7)[a]
RDW (%)	13.7 ± 1.0 (8)[a]	15.8 ± 0.4 (4)[a]
Reticulocytes (× 10^{12}/L)	0.19 ± 0.02 (8)[a]	0.46 ± 0.03 (4)[b]

*Results expressed as mean ± SEM (n). n was reduced in some cases due to clotted samples. Statistical analysis was a *t* test (unpaired). Values in a row not sharing a common superscript letter are significantly different ($P < 0.05$).
§Male Wistar rats fed ad libitum an AIN76-based diet without added vitamin E for 8 weeks ($n = 8$).
¶Male Wistar rats fed ad libitum the AIN76-based diet containing 50 IU/kg dl-α-tocopheryl acetate ($n = 7$).

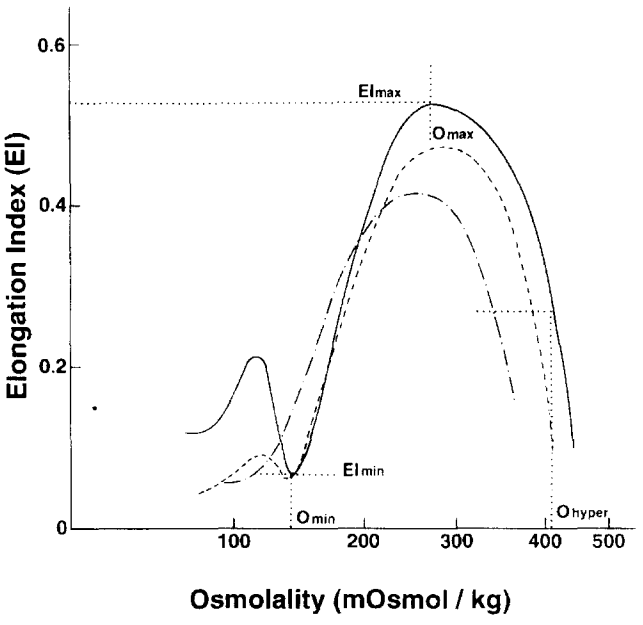

Figure 1 Representative osmotic deformability profiles for normal erythrocytes (- - - -, 6-week-old rat; —·—·—, 11-week-old rat; ——, adult human). Parameters of the curve used to characterize cell deformability are those described by Clark et al.[18]: (1) EI_{max}, the maximum elongation index (EI); (2) O_{max}, the osmolality at which EI_{max} occurs; (3) EI_{min}, the minimum EI obtained under hypotonic conditions; (4) O_{min}, the osmolality at which EI_{min} occurs; and (5) O_{hyper}, the osmolality at which EI is half of EI_{max} on the hypertonic arm.

Table 2 The influence of vitamin E deficiency on erythrocyte deformability

	Experimental group*	
	−E§	+E¶
EI_{max}¥	0.409 ± 0.005[a]	0.393 ± 0.005[b]
O_{max}†	246 ± 3[a]	238 ± 5[a]
O_{hyper}£	347 ± 1[a]	331 ± 4[b]

*Results expressed as mean ± SEM. Statistical analysis was a *t* test (unpaired). Values in a row not sharing a common superscript letter are significantly different ($P < 0.05$).
§Male Wistar rats fed ad libitum an AIN76-based diet without added vitamin E for 8 weeks ($n = 8$).
¶Male Wistar rats fed ad libitum the AIN76-based diet containing 50 IU/kg dl-α-tocopheryl acetate ($n = 7$).
¥The maximum elongation index (EI).
†The osmolality at which EI_{max} occurs.
£The osmolality at which EI is half of EI_{max} on the hypertonic arm of the osmotic deformability profile.

Table 2 shows the influence of vitamin E intake on erythrocyte deformability as measured by parameters of the osmotic deformability profile for the two experimental groups. Vitamin E deficiency had a small but statistically significant effect on erythrocyte deformability. EI_{max} (the maximum elongation index) and O_{hyper} (the osmolality at which the elongation index is half of EI_{max} on the hypertonic arm of the curve) were significantly elevated in the erythrocytes from the −E group.

Discussion

Cellular deformability is critical to normal erythrocyte function, influencing blood viscosity in large vessels, controlling removal of nonfunctional and aged erythrocytes by the reticuloendothelial system, and influencing release of immature erythrocytes into the circulation.[19] As previous studies of the influence of vitamin E status on this important physiological function have given conflicting results,[8-10] the question was readdressed in the present study using a technique that is more sensitive to changes in deformability associated with membrane damage. While the rats developed no overt signs of deficiency after 8 weeks of feeding the vitamin E-deficient diet, the depressed plasma α-tocopherol concentration and increased in vitro hydrogen peroxide-induced hemolysis provide evidence for a subclinical vitamin E deficiency. The absence of a hemolytic anemia was confirmed by the hematologic profile. While a tendency toward decreased cell survival in vivo might be suggested by the increased susceptibility to in vitro hydrogen peroxide-induced hemolysis, there was no increase in circulating reticulocytes as would be expected with a compensatory increase in red cell production. On the contrary, the reticulocyte count in the vitamin E-deficient group was depressed, and this represented the only significant finding in the hematologic profile. This finding suggests the presence of ineffective erythropoiesis, which is usually a less significant contributor to the hematological consequences of vitamin E deficiency in the rat as compared with species such as swine and nonhuman primates.[2-4]

While erythrocyte deformability has been measured by a variety of techniques, we employed the ektacytometer, which measures the deformability of a population of cells under well-defined fluid shear stress conditions using small quantities of blood.[20] We have previously described the application of this instrument for assessing the influence of a nutrient deficiency on red cell deformability in the rat.[17] Deformability under constant high shear stress was recorded as the osmolality of the medium was increased *(Figure 1)*. For the normal human red cell, maximum deformability occurs near the osmolality to which cells are normally exposed. As osmolality is decreased, the elongation index declines and reaches a minimum (EI_{min}) at an osmolality (O_{min}) at which the cells on average would have attained their maximum volume prior to hemolysis. The osmolality associated with minimum elongation coincides with that at which 50% of cells hemolyze in an osmotic fragility assay. Thus, the value for O_{min} provides information about the initial surface area to volume ratio of the cells. The decrease in elongation index with increasing hypertonicity is due to an increase in intracellular viscosity resulting from osmotic water loss and increasing mean corpuscular hemoglobin concentration.[18]

The osmotic deformability profile can be used to assess the influence on whole cell deformability of internal viscosity, surface membrane area to cell volume ratio, and cell membrane deformability.[18] Reduced cellular surface area to volume ratio causes O_{min} to be increased and EI_{max} to be decreased with no change in the hypertonic arm of the osmotic deformability profile. A change in intracellular viscosity (determined by mean corpuscular hemoglobin concentration) shifts the osmotic deformability profile along the x-axis. Values for O_{min}, O_{max}, and O_{hyper} increase with a decline in mean corpuscular hemoglobin concentration, while that for EI_{max} is unchanged. When erythrocytes are treated with agents that decrease membrane deformability, the value for EI_{max} decreases, but there is little shift of the curve along the osmolality axis.

The osmotic deformability profile of red cells from the 6-week-old rat was shown in a previous study to be similar to that of human erythrocytes.[17] In the present study, the profile of erythrocytes from the 11-week-old control rats varied from that reported previously for the younger rat *(Figure 1)*. Differences in both the age of the animals and in the composition of the control diet in the two studies may account for the variation. Although the control diet fed in the previous study was based on the AIN-76 diet,[13,14] modifications included an increase in the fat content, replacement of casein with egg white protein, and the addition of sodium phytate and calcium carbonate as zinc antagonists. The downward shift of the osmotic deformability profile along the x-axis seen with erythrocytes from the older animals is most likely attributable to a higher intracellular viscosity. Their mean ($\pm$ SEM) corpuscular hemoglobin concentration was 348 $\pm$ 3 as compared with 336 $\pm$ 2 in the 6-week-old animals (Paterson, P.G. and Card, R.T. Unpublished observation). In addition, the maximum deformability (EI_{max}) attained was lower with erythrocytes from the older rats. Without further analysis of cell subpopulations, it is not possible to conclude whether this difference was due to reduced membrane deformability, less membrane surface area, or a greater heterogeneity in cell water content. The overall shape of the deformability profile obtained with the red cells from the 11-week-old rats suggests a more heterogeneous cell population than obtained from the human or the younger rat. The former is characterized by the absence of a sharp quantifiable minimum value for deformability under hypotonic conditions (EI_{min}), slightly greater flattening of the maximum value (EI_{max}), and a more gradual decrease in the elongation index with increasing hypertonicity.

We had hypothesized that erythrocytes obtained from vitamin E-deficient rats would be less deformable in the ektacytometer. In contrast, the osmotic deformability profile showed small but statistically significant increases in the values for maximum deformability (EI_{max}) and O_{hyper} (the osmolality at which the elongation index is half of EI_{max} on the hypertonic arm). These findings, in the absence of a shift in the profile along the x-axis, are most consistent with an increase in membrane deformability. Our inability to demonstrate a depression in erythrocyte deformability in the vitamin E-deficient group may be related to the length of our study period. The deficiency may not have been severe enough to cause sufficient oxidative damage to erythrocytes to decrease their deformability properties. However, while the extent of erythrocyte membrane α-tocopherol depletion was not measured in this study, we predict that red blood cell α-tocopherol concentration would have been reduced to less than 10% of control values. This is based on previously published values of plasma and erythrocyte α-tocopherol concentrations measured at varying stages of vitamin E deficiency.[21] Previous investigators were also unable to show a depression in filterability of erythrocytes from the vitamin E-deficient premature infant,[10] rat,[8] or mouse.[9] Only if the cells from premature infants[10] and rats[8] were incubated under

specific conditions was filterability decreased. It would be of interest to assess the influence of a more severe vitamin E deficiency on erythrocyte deformability when the animal model is subjected to greater than normal oxidant stress.

While evidence is still lacking from this and other studies that vitamin E deficiency decreases the property of erythrocyte deformability, there are reports that supplemental vitamin E under a variety of circumstances is helpful in maintaining normal cell deformability. In a group of mountaineers used as a model for individuals at increased risk of oxidative stress, it was demonstrated that vitamin E supplementation prevented the small decrease in erythrocyte filterability that occurred in the placebo group during an ascent.[22] Injections of α-tocopherol in the rat have also been shown to inhibit decreased erythrocyte filterability associated with sepsis[23] and burn trauma.[24] Perhaps the clinical significance of vitamin E to this physiological property can only be demonstrated under situations of unusually elevated free radical generation.

The ektacytometer provides a sensitive tool for detecting changes in erythrocyte deformability. While the increase in maximum deformability of erythrocytes subjected to vitamin E deficiency is too small to be of clinical significance, this finding may be relevant to the hypothesized structural role of α-tocopherol in biologic membranes. In addition to its function in protecting cell membranes from peroxidative damage, a role for α-tocopherol in stabilizing membranes has been hypothesized through a physico-chemical interaction between its phytyl side-chain and the fatty acyl chains of polyunsaturated phospholipids.[25] Studies have reported both increases and decreases in membrane fluidity when α-tocopherol is incorporated into phospholipid bilayers depending on whether model membranes are prepared from unsaturated or saturated lipids,[26] but there are fewer studies of membranes from animals depleted of the vitamin. If the increase in membrane deformability reported here is related to a structural role of α-tocopherol in the membrane, our results indirectly suggest that dietary vitamin E depletion increases membrane fluidity as the latter is known to increase erythrocyte deformability.[27]

In summary, vitamin E deficiency in the rat did not depress erythrocyte deformability when this property was assessed with the ektacytometer. This may be related to the degree of deficiency imposed or may indicate that this physiologic property is not influenced by vitamin E status unless the organism is simultaneously faced with an increased oxidant stress. The small but statistically significant increase in membrane deformability measured in erythrocytes from vitamin E-deficient rats in this study may be related to the structural role that has been suggested for α-tocopherol in biologic membranes.

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