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OXIDATIVE DAMAGE IN THE RED CELLS OF VITAMIN E-DEFICIENT RATS

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One month-old male Sprague–Dawley rats were maintained on a basal vitamin E-deficient diet supplemented with either 0 or 50 ppm vitamin E for 5 months. Washed red blood cells were resuspended in phosphate buffered-saline, pH 7.4, that contained 0–50 mM glucose and 0–20 mM ethylenediamine tetraacetic acid (EDTA), and were incubated at 37°C for up to 22 h. Contrary to expectations, glucose in the incubation medium accelerated, rather than retarded, the rates of hemolysis, lipid peroxidation and methemoglobin formation in the vitamin E-deficient cells. EDTA, on the other hand, partially inhibited the extent of oxidative damage. Vitamin E-supplemented cells were resistant to oxidative damage in the presence or absence of glucose and/or EDTA. The levels of reduced glutathione (GSH) and activity of catalase were decreased faster in the vitamin E-deficient cells than the supplemented cells, and the rates of their decline were slowed down by either glucose or EDTA. The activities of GSH peroxidase and superoxide dismutase were not significantly altered in the red cells of either group during incubation. The results obtained suggest that reactive oxygen species and reduced metal ions play important roles in initiating oxidative damage to the red cells of vitamin E-deficient rats. However, the agent responsible for initiating the hemolytic event has yet to be established.

KEY WORDS: Vitamin E, red blood cells, rats, oxidative damage, glucose, EDTA.

INTRODUCTION

In addition to containing high concentrations of polyunsaturated fatty acids and transitional metals, red blood cells are constantly being subjected to various types of oxidative stress, such as inhaled oxidants and ingested chemicals. Red blood cells, however, are protected by a variety of antioxidant systems which are capable of preventing most of the adverse effects under normal conditions.[1,2] Among the antioxidant systems in the red cells, vitamin E possesses an important and unique role.[2,3] Vitamin E may protect the red cells from oxidative damage via a free radical scavenging mechanism or as a structural component of the cell membrane.[2–4]

While vitamin E has long been recognized as an essential agent against hemolysis,[4–8] the mechanism by which the vitamin protects red cells against hemolytic stress has yet to be delineated. It has been suggested that membrane damage resulting from lipid peroxidation is the cause of hemolysis in vitamin E-deficiency,[9–11] and some reports have shown that the event of hemolysis occurs following lipid peroxidation.[5–8] Other studies, however, do not support a cause–effect relationship between lipid peroxidation and hemolysis.[12–14] Also, the role of various antioxidant systems and their relationships during the development of hemolysis are not clear. In this research, therefore, the relationship between the extent of oxidative damage and status of important antioxidant systems was studied using a spontaneous hemolysis test procedure for

vitamin E deficiency.[15] In addition, the effect of glucose and EDTA on hemolytic stress was investigated.

MATERIALS AND METHODS

Thirty-two weanling male Sprague–Dawley rats received from the supplier (Charles River Breeding Laboratory, Wilmington, MA) were maintained on Purina laboratory chow (St. Louis, MO) for one week. Animals were then divided randomly into two groups and were fed a casein-based vitamin E-deficient diet[16] supplement with either 0 or 50 ppm (mg/kg diet) vitamin E (as *d,l*-alpha-tocopheryl acetate). Diets and water were provided *ad libitum*. The basal diet contained less than 0.2 ppm vitamin E,[17] and approximately 0.05 ppm selenium.[18] During the feeding period blood samples were taken from tails periodically to determine the status of vitamin E.[15]

After 5 months on the respective diets, animals from each group were killed by exsanguination via heart puncture following anesthetization. Heparinized blood samples were centrifuged to separate plasma from the blood cells. After 3 washings with phosphate-saline buffer, pH 7.4,[15] the red cells were adjusted to 6–8% hematocrit with the same buffer. Seven ml of the red cell suspension from each animal were then pipetted into 50-ml round bottom centrifuge tubes, and mixed with either nothing, glucose (10, 20 or 50 mM) and/or disodium EDTA (20 mM). The content was then covered with parafilm and incubated at 37°C with constant shaking. Aliquots of the content from each tube were pipetted at 0, 1, 2, 4, 6, 8 and/or 22 h intervals.

For each time interval, aliquots were pipetted for measuring the degree of hemolysis[15] and levels of GSH,[19] hemoglobin and methemoglobin[20] and lipid peroxidation products, mainly malonaldehyde.[21] To avoid artifact formation of malonaldehyde-like compounds, the red cell suspension was reacted with 0.4% thiobarbituric acid (TBA) and 0.01% BHT at 60°C for 1 h. A portion of the incubation mixture was lysed with 0.016 M phosphate buffer, pH 6.6, and was assayed for the activities of GSH peroxidase, using hydrogen peroxide as substrate,[22] catalase[23] and superoxide dismutase.[24] The concentration of vitamin E in the red cells was measured by a high pressure chromatographic method.[17]

The data obtained was analyzed using analysis of variance followed by Honesty's multiple comparison test. Student's "*t*" test was employed to test the difference between two sample means. A 95% level ($p < 0.05$) of confidence was used to determine statistical significance.

RESULTS

Vitamin E

The concentration of vitamin E in the red cells of vitamin E deficient rats averaged 0.1 μg/ml as compared to 4.1 μg/ml in the supplemented group.

Hemolysis

The effect of incubation at 37°C on the rate of hemolysis is shown in Figure 1. As expected, the red cells of vitamin E-deficient rats were susceptible to spontaneous hemolysis and those of the vitamin E-supplemented animals were not. Significant

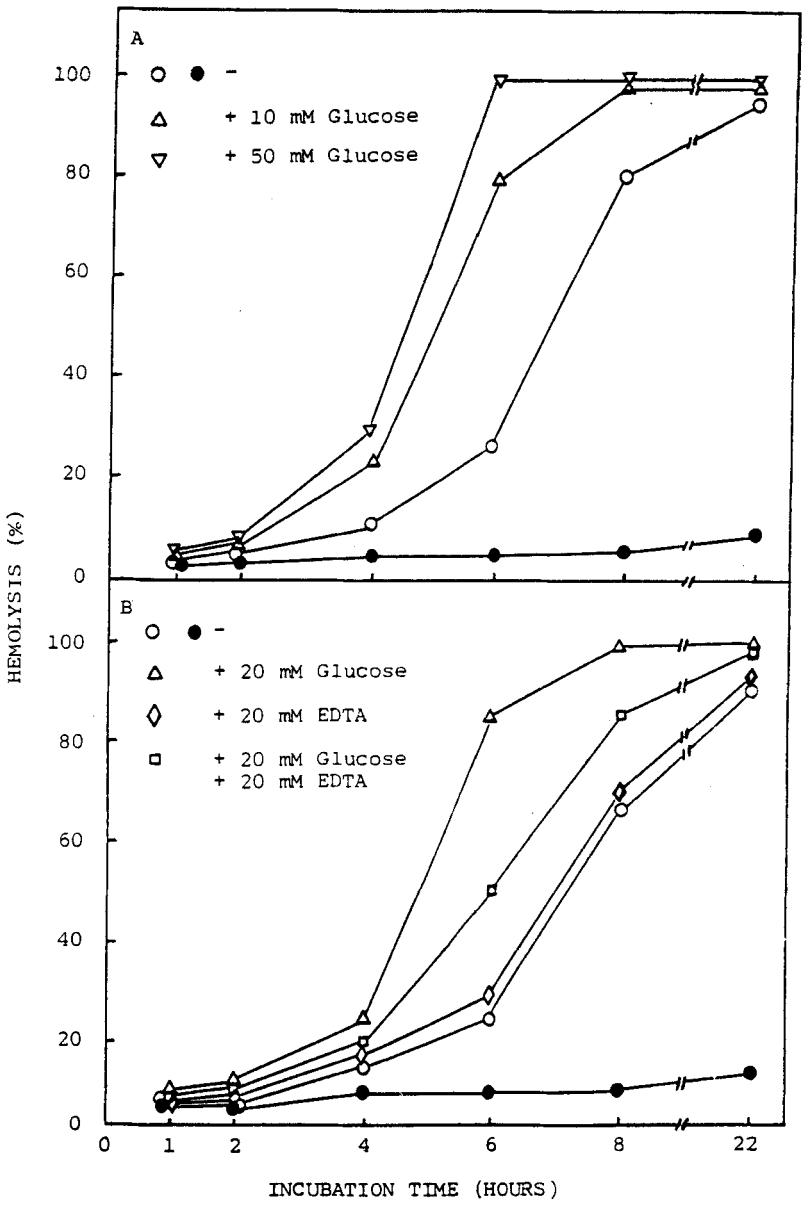

FIGURE 1 Effect of glucose and EDTA on rates of hemolysis. Six to 8% red cells in phosphate-buffered saline, pH 7.4, were incubated with glucose (0, 10, 20 or 50 mM) and EDTA (0 or 20 mM) at 37°C. Open symbols represent vitamin E-deficient cells, and solid symbols represent supplemented cells. Since glucose and EDTA had no significant effect on the rates of hemolysis in vitamin E-supplemented cells, only the data obtained from the subgroup without adding glucose or EDTA is shown.

differences between the two groups were detected after approximately four hours incubation at 37°C. Under the experimental conditions, most of the cells in the vitamin E-deficient group were hemolyzed after 8 h incubation, and less than 10% hemolysis was found in the supplemented cells after 22 h. Contrary to expectations, glucose in the incubation medium accelerated, rather than retarded, the rate of hemolysis in the red cells of vitamin E-deficient rats (Figure 1). The effects of glucose were more profound at 50 mM than at 10 mM. Glucose had no significant effect on hemolysis in the vitamin E-supplemented red cells (data not shown).

The effect of EDTA and glucose on the rate of hemolysis is shown in Figure 1B. EDTA had no significant effect on the rate of hemolysis in the red cells of vitamin E-deficient rats. However, EDTA partially retarded the aggravating effect of glucose on the rate of hemolysis of vitamin E-deficient cells. EDTA alone or in combination with glucose, had no significant effect on the hemolysis of vitamin E-supplemented cells.

Lipid Peroxidation

The effect of incubation at 37°C on the formation of lipid peroxidation products, TBA reactants, is shown in Figure 2. Significantly larger amounts of TBA reactants were detected in the vitamin E-deficient cells than the supplemented cells after incubation for 4 h. Similar to hemolysis, glucose accelerated the rate of TBA reactant formation in vitamin E-deficient cells. Also, EDTA, partially reduced the rate of lipid peroxidation and retarded the accelerating effect of glucose on lipid peroxidation in vitamin E-deficient red cells. Only a small amount of TBA reactants was formed in the vitamin E-supplemented cells, and the levels were not significantly altered by glucose and/or EDTA (data not shown).

GSH

The levels of GSH in the vitamin E-supplemented cells averaged 4.7 μmole/mg hemoglobin as compared to 3.9 in deficient cells ($p < 0.05$). During incubation the levels of GSH gradually decreased and the rate of decline was relatively faster in the vitamin E-deficient cells than the supplemented group (Figure 3). Averaged 42% and 65% of GSH remained, respectively, in the vitamin E-deficient and supplemented-cells at the end of 6 h, and the values were 15% and 40% after 8 h.

The effects of glucose and EDTA on the levels of GSH are also shown in Figure 3. Glucose in the medium retarded the rate of GSH loss in both vitamin E-deficient and supplemented cells. Glucose at 50 mM exerted a greater effect on GSH than at 10 mM (Figure 3A). EDTA had an effect on GSH levels similar to that of glucose. A greater effect was found when both glucose and EDTA were added to the incubation medium (Figure 3B). This effect was observed in the red cells of both vitamin E-deficient and supplemented animals.

Methemoglobin

Less than 2% MetHb was found in the red cells of either animal group 4 h after incubation (Figure 4). Significantly higher levels of MetHb in the vitamin E-deficient cells were detected only after incubation at 37°C for 8 h. Glucose accelerated the formation of MetHb in the vitamin E-deficient cells. EDTA alone had no significant

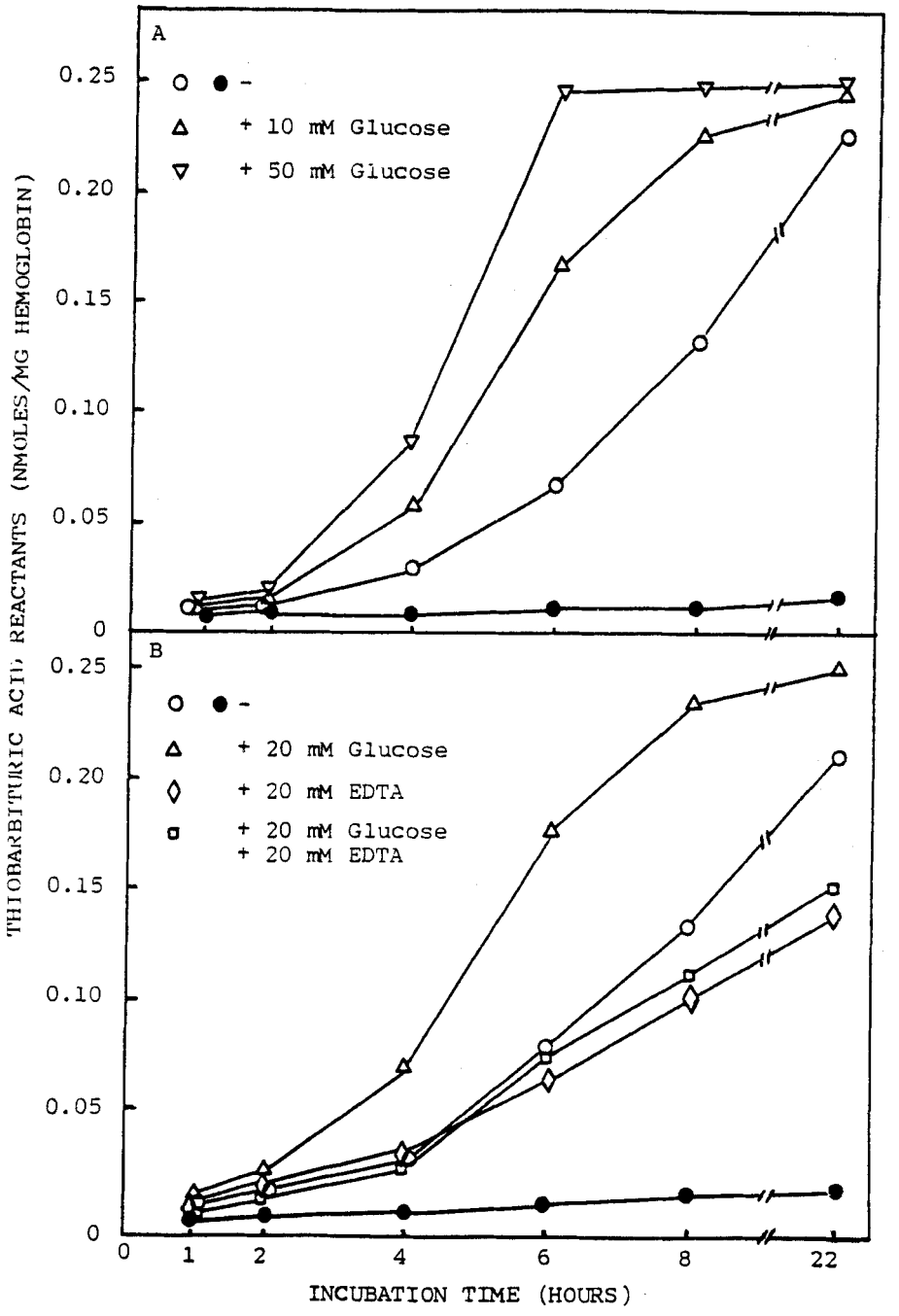

FIGURE 2 Effect of glucose and EDTA on rates of lipid peroxidation. See legends of Figure 1 for detail.

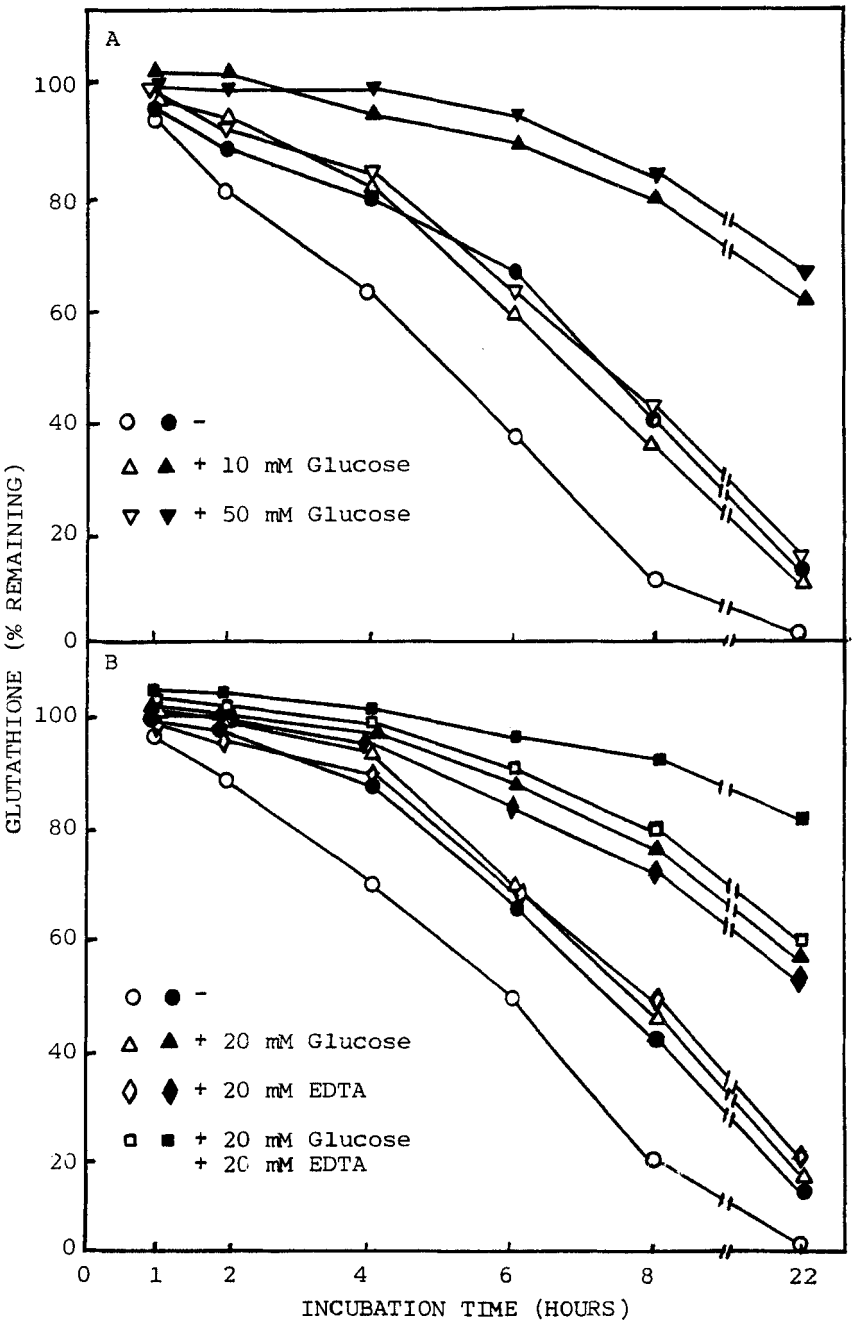

FIGURE 3 Effect of glucose and EDTA on the levels of glutathione. See legends of Figure 1 for detail. Data from all groups are shown. The levels of glutathione in the vitamin E-supplemented cells averaged 4.7 μmoles/mg hemoglobin and 3.9 μmoles mg hemoglobin in deficient cells.

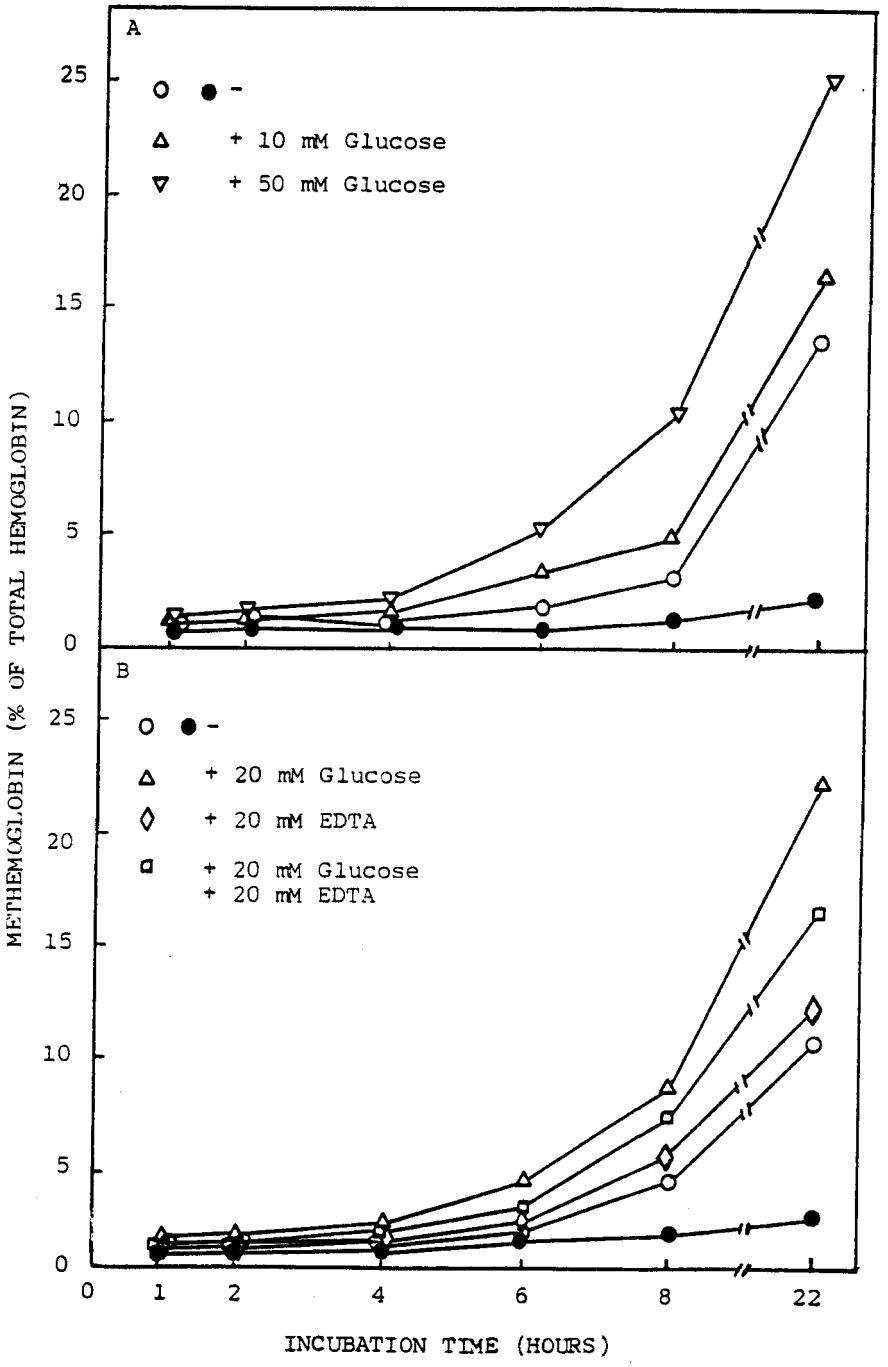

FIGURE 4 Effect of glucose and EDTA on rates of methemoglobin formation. See Figure 1 for detail.

effect on MetHb, but partially retarded the enhancing effect of glucose on the vitamin E-deficient cells. Throughout the incubation period, less than 3% MetHb was found in vitamin E-supplemented cells. Neither glucose nor EDTA exhibited any significant effect on the MetHb levels of vitamin E-supplemented cells.

Catalase

The activity of catalase in the red cells is shown in Table I. The activity of catalase in the red cells was significantly decreased after 22 h of incubation. A relatively smaller decrease was observed in vitamin E-supplemented cells than in deficient cells. Glucose significantly increased the activity of catalase during the early hours of incubation, and partially retarded the loss of catalase activity during the later hours. EDTA not only increased the activity of catalase during the early hours of incubation but also prevented the loss of the enzyme activity during incubation. Glucose and EDTA together had an effect on enzyme activity similar to that of EDTA alone.

GSH Peroxidase and Superoxide Dismutase

The activities of GSH peroxidase and superoxide dismutase in the cells of vitamin E-deficient and supplemented rats are also shown in Table I. The activities of these two enzymes were not significantly altered during the incubation period, nor by the presence or absence of glucose and EDTA in the incubation medium.

DISCUSSION

It has been well-recognized that vitamin E is essential for the integrity of red blood cells.[1-8] As expected, red cells deficient in vitamin E were more susceptible to hemolysis than the supplemented cells. The degree of hemolysis was closely related to the amount of lipid peroxidation products formed. It could not be determined whether the event of hemolysis occurred prior to lipid peroxidation or vice versa. The findings agree with the view that hemolysis and lipid peroxidation in the red cells of vitamin E-deficient rats may be concurrent rather than consecutive events.[12]

While the hemolysis test is considered a measurement of membrane damage, increased levels of MetHb are regarded as an index of intracellular damage to the red cells.[12] In this research, increased amounts of MetHb were detected in vitamin E-deficient red cells only after most of the cells were lysed. It is possible that MetHb reduction systems[25] may be impaired following hemolysis.

Glucose is the principal source of energy and precursor of reducing equivalents necessary for maintaining the integrity of red blood cells. Rotruck *et al.*[26] has shown that addition of glucose to the incubation medium protected both the membrane and hemoglobin from oxidation. The accelerating effect of glucose on the rates of hemolysis and lipid peroxidation, as well as MetHb formation in vitamin E-deficient cells observed in this study was, therefore, unexpected. The reason for this discrepancy is not known. The ability of EDTA to partially counteract the prooxidative effect of glucose suggests that this action of glucose may partly be due to its ability to generate reducing equivalents, which in turn maintain transition metal ions in a reduced or catalytic state. Jain[27] has shown an increase in lipid peroxidation when human red blood cells were incubated with elevated levels of glucose.

TABLE I
Effect of glucose and EDTA on enzyme activities in the red cells

Incubation time (h)	Treatment[1]	Catalase (μmole/min/mg Hb)			GSH peroxidase (nmole/min/mg Hb)			Superoxide dismutase (unit/mg Hb)		
		$-E$[2]	$+E$	p[3]	$-E$	$+E$	p	$-E$	$+E$	p
0	–	41 ± 4[4]	42 ± 5	NS	46 ± 4	47 ± 5	NS	2.3 ± 0.3	2.2 ± 0.4	NS
4	–	40 ± 3[a]	43 ± 4[a]	NS	45 ± 3[a]	49 ± 4[a]	NS	2.4 ± 0.4[a]	2.2 ± 0.5[a]	NS
	+Glucose	55 ± 6[b]	58 ± 7[b]	NS	46 ± 5[a]	48 ± 5[a]	NS	2.2 ± 0.4[a]	2.4 ± 0.4[a]	NS
	+EDTA	63 ± 6[b,c]	65 ± 6[b,c]	NS	47 ± 4[a]	48 ± 6[a]	NS	2.3 ± 0.3[a]	2.5 ± 0.4[a]	NS
	+Glucose +EDTA	68 ± 7[c]	70 ± 6[c]	NS	45 ± 5[a]	47 ± 5[a]	NS	2.4 ± 0.3[a]	2.4 ± 0.4[a]	NS
8	–	38 ± 5[a]	41 ± 5[a]	NS	44 ± 5[a]	46 ± 4[a]	NS	2.2 ± 0.3[a]	2.3 ± 0.4[a]	NS
	+Glucose	49 ± 6[b]	54 ± 5[b]	NS	46 ± 4[a]	42 ± 5[a]	NS	2.2 ± 0.2[a]	2.1 ± 0.3[a]	NS
	+EDTA	64 ± 7[c]	69 ± 6[c]	NS	42 ± 5[a]	43 ± 6[a]	NS	2.4 ± 0.3[a]	2.3 ± 0.2[a]	NS
	+Glucose +EDTA	66 ± 6[c]	72 ± 6[c]	NS	45 ± 4[a]	44 ± 5[a]	NS	2.3 ± 0.2[a]	2.4 ± 0.3[a]	NS
22	–	24 ± 4[a,#]	35 ± 5[a]	< 0.01	43 ± 3[a]	44 ± 4[a]	NS	2.1 ± 0.2[a]	2.0 ± 0.3[a]	NS
	+Glucose	28 ± 4[a,#]	48 ± 5[b]	< 0.001	41 ± 4[a]	42 ± 3[a]	NS	2.1 ± 0.3[a]	2.2 ± 0.3[a]	NS
	+EDTA	61 ± 5[b]	64 ± 6[c]	NS	43 ± 3[a]	44 ± 4[a]	NS	2.2 ± 0.3[a]	2.1 ± 0.2[a]	NS
	+Glucose	63 ± 6[b]	67 ± 5[c]	NS	42 ± 5[a]	41 ± 5[a]	NS	2.1 ± 0.2[a]	2.2 ± 0.2[a]	NS

[1] Addition of 0 (–), 20 mM glucose and/or 20 mM EDTA to the medium.
[2] Animals received 0 (– E) or 50 ppm vitamin E (+ E) in the diet for 5 months.
[3] Based on Student's "t" test. NS represents not significant ($p > 0.05$).
[4] Mean ± standard deviation; 6 animals in each group. Means that do not share the same superscript are different significantly ($p < 0.05$). The symbol # represents significantly different from other time points of the same treatment group.

In addition to being the substrate for GSH peroxidase, GSH is an important reducing agent and possesses many specific functions.[28] Along with ascorbic acid, GSH may also be involved in the regeneration of vitamin E.[2,29,30] However, higher GSH levels due to the addition of glucose accelerated, rather than retarded, the rates of hemolysis (Figure 1) and lipid peroxidation (Figure 2). GSH has been employed as an agent for inducing hemolysis *in vitro*.[5,12,31] This prooxidative action of GSH is linked to the generation of free radicals.[12,31]

While essentially all the activity of GSH peroxidase was retained throughout the incubation period, hemolysis and lipid peroxidation still proceeded in the vitamin E-deficient cells. The failure of GSH peroxidase in the vitamin E-deficient cells to reduce or prevent lipid peroxidation suggests that lipid hydroperoxides formed are membrane-bound and thus can not be reduced by GSH peroxidase,[32] and that the function of phospholipase and/or phospholipid GSH peroxidase[33] in the red cells may be inadequate or impaired during hemolysis.

Metal chelators may prevent the generation of free radicals via the metal ion-catalyzed Fenton reactions, or homolytic fission mechanism.[34-36] EDTA, indeed, partially decreased the rates of hemolysis and lipid peroxidation, as well as MetHb formation, in vitamin E-deficient cells. The findings suggest that free radical generation catalyzed by metal ions may play a role in initiating hemolytic events in vitamin E-deficient cells. As the activities of catalase and GSH peroxidase in vitamin E-deficient cells remained essentially intact prior to hemolysis, hydrogen peroxide *per se* is unlikely to be the primary hemolytic agent. The reason for the apparent increase in activity of catalase during the early hours of treatment with glucose and/or EDTA is not known. Similarly, the lack of effect on superoxide dismutase suggests that superoxide radicals are not the key damaging species. Brownlee *et al.*[12] suggested that intracellular hydroxyl radicals generated from hydrogen peroxide, but not extracellular superoxide or hydroxyl radical, are the hemolytic agents. Whether hydroxyl radicals are, indeed, the damaging species responsible for the hemolytic event remain to be demonstrated.

The precise sequence of events occurring during the development of hemolysis has yet to be delineated. It appears that scavenging of free radicals by vitamin E is the first and most critical step in defending against oxidative damage to the red cells. When vitamin E is adequate, GSH and ascorbic acid may complement the antioxidant functions of vitamin E by providing reducing equivalents necessary for its recycling/regeneration.[2,29,30] On the other hand, when vitamin E is absent, GSH and ascorbic acid may act to release transitional metals from the bound forms and/or maintain metal ions in a catalytic state. Free radical generation catalyzed by transition metal ions can in turn initiate oxidative damage to cell membranes. Membrane damage can then lead to release of heme compounds and lysis of the red cells. The heme compounds released may further promote oxidative damage especially when reducing compounds are present. This phenomenon may partially explain why it is difficult to distinguish the sequence of events between lipid peroxidation and hemolysis.

The results obtained from this research support the view that vitamin E is the most important antioxidant in the red cells and suggest that reactive oxygen species and reduced transition metal ions may be responsible for the onset of oxidative damage in vitamin E-deficient red cells. However, the agent responsible has yet to be established.

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