Studies on Vitamin E and Selenium Deficiency in Young Pigs
II. The Hydrogen Peroxide Hemolysis Test and the Measure of Red Cell Lipid Peroxides as Indices of Vitamin E and Selenium Status

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ABSTRACT

The usefulness of the hydrogen peroxide hemolysis test and the measure of red cell lipid peroxides as indices of vitamin E and selenium deficiency in swine has been evaluated. Results indicated that although the hydrogen peroxide hemolysis test may be of some indication of the vitamin E status, it is not a reliable index of vitamin E deficiency in swine, at least on an individual basis. In contrast, the measure of red cell lipid peroxides can be considered a reliable test for vitamin E deficiency in swine. The hydrogen peroxide hemolysis test and the red cell lipid peroxides were not significantly affected by selenium deficiency.

RÉSUMÉ

Cette expérience visait à évaluer la fragilité lytique des hématies en présence de peroxyde d'hydrogène et la mesure des peroxydes lipidiques des hématies, comme indices de déficiences en vitamine E et en sélénium, chez le porc. Les résultats révélèrent qu'en dépit de l'utilité relative de l'épreuve d'hémolyse au peroxyde d'hydrogène, dans le cas de la vitamine E, cette épreuve ne constitue pas un moyen fiable pour déceler une déficience de cette vitamine chez le porc, du moins sur une base individuelle, contrairement à la mesure des peroxydes lipidiques des hématies. Par ailleurs, une déficience en sélénium n'affecta pas de façon appréciable l'une ou l'autre de ces deux épreuves.

INTRODUCTION

In early studies, it has been demonstrated that erythrocytes of vitamin E deficient rats were hemolysed by dialuric acid and by hydrogen peroxide (15,30). Subsequently, the *in vitro* lytic sensitivity of red blood cells under oxidant stress has been widely used as an index of vitamin E deficiency in man and animals (1, 7, 11, 12, 13, 18, 19, 21, 22, 25, 29, 31). In man, except unfed neonates, the hydrogen peroxide hemolysis test has been found to be a convenient method for separating patients with plasma tocopherol levels below and above 5 μg/ml; appreciable hemolysis usually being absent with a plasma tocopherol concentration above 5 μg/ml (20, 26). At the present time, literature reports concerning the red cell lytic sensitivity under oxidant stress in vitamin E deficient animals other than laboratory animals, have been equivoqual (2, 10, 14, 16, 17).

An alternate method of evaluating the red cell antioxidant potential (vitamin E) has been to measure *in vitro*, lipid peroxides formation (3, 22, 23, 24, 25, 35, 36). Al-

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though this method is an indirect measurement of the product of lipid peroxidation (malonyldialdehyde) and only a portion of the peroxidized unsaturated fatty acids are measured (6), most investigators agree, that this procedure may be used as a measure of lipid peroxidation (36, 38, 39, 41).

Recently, selenium was found to be a component of the enzyme glutathione peroxidase in erythrocytes of rats (4, 32, 33, 34), chicks (27), cattle (8) and sheep (28). The importance of selenium as a component of glutathione peroxidase is therefore evident for the red cell protective mechanism against oxidative damage, since glutathione peroxidase is reported to be the main protective mechanism against hydrogen peroxide in human erythrocytes (5).

The objective of this study was to evaluate the usefulness of the hydrogen peroxide hemolysis test and the measure of red cell lipid peroxides as indices of vitamin E and selenium deficiency in swine.

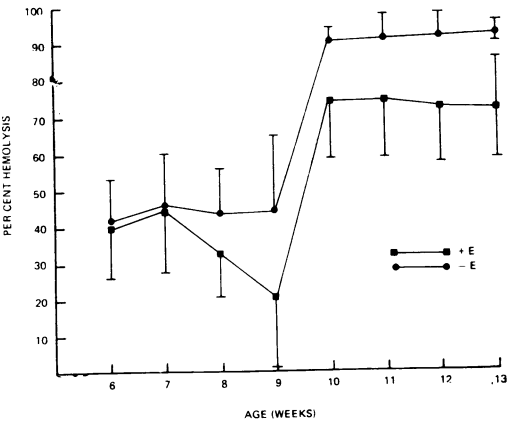

Fig. 1. Effect of vitamin E supplementation on hydrogen peroxide hemolysis of erythrocytes (each point represents the mean value from 12 animals and brackets indicate 95% confidence intervals).

MATERIALS AND METHODS

The first group of 24 pigs previously described (9) was used in the present study where the red cell lytic sensitivity to hydrogen peroxide and the estimation of red cell lipid peroxides were weekly determined from six weeks of age (base level) to 13 weeks of age.

For these determinations, about 0.5 ml of blood was collected in 2 ml saline citrate solution (1% sodium citrate in 0.9% saline) using the orbital sinus bleeding technique as previously described (9). The hydrogen peroxide hemolysis tests were performed according to the technique of Gordon *et al* (13). About 1 ml of the blood saline citrate mixture was transferred into a graduated centrifuge tube and centrifuged at 2000 rpm[1] for ten minutes at 20°C. Supernatant and excess red cells were removed, leaving 0.1 ml packed red cells from which was prepared the 2.5% red cell-saline buffer mixture suspension. The phosphate buffer used by Fisher *et al* (7) was selected and used in the procedure as pilot studies revealed that a significant percentage of swine erythrocytes readily hemolyzed in the presence of the phosphate buffer used by Gordon *et al* (13). The remainder of procedure was exactly the same as described by Gordon *et al* (13) except for the incubation period which was modified in that all the tubes were incubated at 37°C for two and three-quarter hours, being gently shaken every 30 minutes. A fresh dilution from a stock hydrogen peroxide solution[2] was prepared each time just before use and the potency of the stock hydrogen peroxide solution had been verified three times during the period of study by titration with potassium permanganate and not found to be altered.

Red cell lipid peroxides were estimated according to the procedure of Mengel *et al* (25) which measures the chromogen formed by the reaction of 2-thiobarbituric acid with malonyldialdehyde, a product of lipid peroxidation.

About 1.5 ml of the blood saline citrate mixture was transferred into a graduated centrifuge tube and centrifuged at 2000 rpm[3] for ten minutes at 20°C. Supernatant and excess red cells were then removed, leaving 0.2 ml packed red cells which were washed once with 2 ml of 0.9% saline and resuspended in the same amount of 0.9% saline to obtain a 10% red cell suspension. The red cell suspension was then incubated at 37°C for two hours with 2 ml of 1% H_2O_2 solution in saline and the mixture precipitated with 2 ml of 10% trichloro-

[1]R.C.F. of 1120 x gravity.

[2]Hydrogen Peroxide 30%, Fisher Scientific Co., Toronto, Ontario.

[3]R.C.F. of 1120 x gravity.

TABLE I. The Effect of Vitamin E and/or Selenium Deficiency in Swine on the Hydrogen Peroxide Hemolysis and Lipid Peroxides of Erythrocytes, Determined Weekly from Six to 13 Weeks of Age

Parameter	Treatment	Age (weeks) 6	7	8	9	10	11	12	13	F-Values	
H_2O_2 Hemolysis of Erythrocytes (%)	+ E + Se	45.9[a]	61.9	36.5	18.4	78.9	79.6	78.9	82.3	Vitamin E	5.84[c]
	+ E − Se	32.9	27.4	30.0	22.1	70.4	69.8	65.9	61.8	Selenium	0.27[b]
	− E + Se	34.9	38.6	36.6	42.6	88.1	91.8	93.3	90.3		
	− E − Se	48.4	53.5	50.7	45.6	94.1	90.7	90.7	95.5	E x Se	2.77[b]
Erythrocytes Lipid Peroxides (O.D. at 535 nm)	+ E + Se	0.046	0.014	0.022	0.035	0.013	0.022	0.019	0.012	Vitamin E	476.02[d]
	+ E − Se	0.037	0.018	0.027	0.014	0.012	0.020	0.016	0.011	Selenium	1.04[b]
	− E + Se	0.034	0.069	0.123	0.144	0.152	0.145	0.122	0.149		
	− E − Se	0.050	0.101	0.150	0.146	0.158	0.155	0.140	0.143	E x Se	3.04[b]

[a]Mean (n = 6)
[b]Not significant (F 0.05; 1, 15 degrees of freedom = 4.54)
[c]($p < 0.05$)
[d]($p < 0.001$)

acetic acid, followed by filtration through Whatman #1 paper.[4] Two ml of the filtrate was added to 2.5 ml of 0.67% 2-thiobarbituric acid,[5] thoroughly agitated and heated in a boiling water bath for 15 minutes. After cooling to room temperature, optical density readings were taken at 535 nm. A blank of 0.9% saline and H_2O_2 was carried throughout.

The statistical analysis of the data was performed by analysis of variance according to the plan previously described (9).

RESULTS

Both the red cell lytic sensitivity to hydrogen peroxide and the red cell lipid peroxides were found to be significantly increased in association with vitamin E deficiency but not with selenium deficiency, as indicated in Table I where the weekly means for each of the four different treatments (+ E + Se, + E − Se, − E + Se, − E − Se) and the F-values derived from the analysis of variance, are given. Furthermore, the influence of vitamin E supplementation on these two parameters is illustrated in Figs. 1 and 2.

DISCUSSION

The hydrogen peroxide hemolysis of red cells was found to be increased ($p < 0.05$) in vitamin E deficient animals as compared to vitamin E replete animals by analysis of variance but when the weekly means of each group are plotted with their 95% confidence intervals, there is overlap except for the last determinations at 13 weeks of age (Fig. 1). The present results tend to indicate that the use of the hydrogen peroxide hemolysis test as an index of vitamin E status in swine is unreliable, especially on an individual basis, because of the great variability in per cent hemolysis under quite constant serum vitamin E levels which were previously reported (9). The marked increase of the red cell peroxidative hemolysis for both vitamin E deficient and vita-

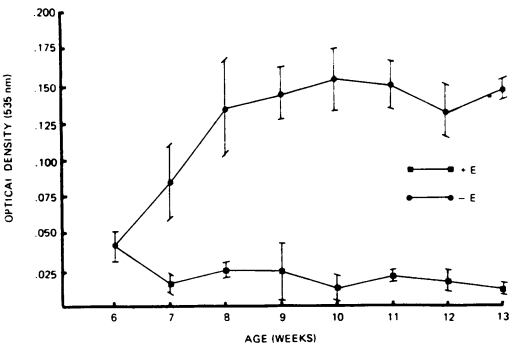

Fig. 2. Effect of vitamin E supplementation on red cell lipid peroxides level (each point represents the mean value from 12 animals and brackets indicate 95% confidence intervals).

[4]Canlab Supplies, Toronto, Ontario.

[5]Sigma Chemical Co., St. Louis, Missouri.

min E supplemented animals, from ten weeks on, is somewhat difficult to explain since the same test procedure and reagents were used all the way through and variations of the serum vitamin E concentrations do not correlate with the sharp increase in hemolysis. Possibly an unidentified age dependent factor, enzymatic or other, is responsible for the increase with age of the erythrocyte lytic sensitivity to hydrogen peroxide in swine. In such a case, however, a gradual increase rather than the sudden increase observed would have been expected. In man, the hydrogen peroxide hemolysis test has been reported to be a convenient method for separating patients with plasma tocopherol levels below and above 5 μg/ml (20, 26). However, in swine the plasma tocopherol levels are about one-tenth the levels found in man and other animal species which may account for the lack of specificity of the hydrogen peroxide hemolysis test in swine. At the present time, the only favorable literature reports for the usefulness of that test as an index of the vitamin E status in swine has been by Hill (17) and Young *et al* (40). It was pointed out that when the dietary polyunsaturated fatty acids were increased to levels over 5% the supplements of tocopherol did not protect against increased peroxidative hemolysis of erythrocytes, even when blood serum showed substantial amounts of tocopherol (17). Furthermore, it is evident from the data given by Young *et al* (40) that under quite constant serum vitamin E levels, the peroxidative hemolysis of erythrocytes varied widely from week to week.

The red cell lipid peroxides were found to be increased ($p < 0.001$) in vitamin E deficient pigs as compared to vitamin E supplemented pigs. This measurement appears to be quite useful for separating vitamin E deficient animals from replete ones as indicated in Fig. 2.

Surprisingly, the red cell lipid peroxides and the red cell peroxidative hemolysis were not found to be increased in selenium deficient pigs which was expected in view of the recently discovered function of selenium as a component of glutathione peroxidase in other animal species (8, 27, 28, 34). The present results do not substantiate such a function for selenium as a component of glutathione peroxidase in swine or glutathione peroxidase is not an important protective mechanism against hydrogen peroxide in pig erythrocytes, as it is in man (5). Interestingly, a very poor correlation between blood selenium level and glutathione peroxidase was recently found in pigs (37).

It is concluded that although the hydrogen peroxide hemolysis of red cells may be of some use as an indication of vitamin E status, is is not a reliable index of vitamin E deficiency in swine, at least on an individual basis. In contrast, the measure of red cell lipid peroxides can be considered a reliable test for vitamin E deficiency in swine.

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