

## Comparison of Effective $\alpha$ -Tocopherol Concentrations Ameliorating Reduced Glucose Transport and Counteracting t-Butyl hydroperoxide-Induced Lipid Peroxidation in Isolated Erythrocytes from $\beta$ -Thalassemic Patients

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### Abstract

In thalassemia patients Red Blood Cell (RBC) membrane is the major target of oxidative stress caused by hemichrome radicals. Moreover, RBC membranes from thalassemic patients are more susceptible to oxidative stress, which causes lipid peroxidation, membrane rigidity, and dysfunction of membrane proteins. We investigated hemoglobin-free isolated RBC membranes (ghosts) from 28  $\beta$ -thalassemia major patients and from 24 normal blood samples. Our research aimed to determine the effective concentrations of racemic  $\alpha$ -tocopherol i) counteracting experimental oxidative stress induced by 2 mM tertiary butylhydroperoxide (t-BHP) and ii) ameliorating impaired glucose transport in RBC ghosts from thalassemia patients. Reduced glucose transport in thalassemic ghosts was ameliorated significantly by tocopherol with a maximum effect at 75 ppm concentration. Furthermore, ghosts were pre-incubated with/without  $\alpha$ -tocopherol up to 200 ppm before treated with t-BHP as the oxidative agent and reactive membrane thiols determined. As a parameter of lipid peroxidation (LPO) thiobarbiturate-reactive substances were measured and expressed as malondialdehyde levels. Tocopherol counteracted LPO continuously in a concentration-dependent way up to 200 ppm. Hence, it is concluded that tocopherol acts at different concentrations as an antioxidant and on glucose transport by GLUT1 in RBC membranes.

**Keywords:** Thalassemia; Malondialdehyde; Erythrocyte; Glucose transport; GLUT1; Tocopherol

**Abbreviations:** EDTA: Ethylene Diamine Tetra Acetic Acid; GLUT1: Glucose Transporter 1; HPLC: High Performance Liquid Chromatography; LPO: Lipid Peroxidation; MDA: Malondialdehyde; RBC: Red Blood Cells; TBARS: Thiobarbituric Acid-Reactive Substances; t-BHP: Tertiary Butyl Hydroperoxide

### Introduction

Formerly, we reported that vitamin E levels in thalassemic patients are decreased, only slightly in non-transfused thalassemia minor patients, but less than 30% of normal values in regularly transfused thalassemia major patients [1]. Thus, the varying spectrum of clinical pictures in thalassemia mirrors the wide range of reduced vitamin E levels.

Hence, it is recommended to thalassemic patients to replenish their vitamin E levels. The major target of vitamin E supplementation is considered the red cell membrane, because it is the biological structure, which is primarily attacked by free radicals in thalassemic patients and may thus be sensitive to depletion and supplementation.

It was shown that in thalassemic RBC membranes, proteins and lipids are impaired [2]. Proteins are impaired in two ways: first, they are the primary aim of hemoglobin radicals [3] and second they can hardly fulfil their physiological task in a pathologically modified lipid environment in which they are embedded.

Apart from its antioxidant effect vitamin E is known to exert various functions in membranes [4] including also a cholesterol-like structural function [5], in which its phytanyl tail may be involved [6,7].

In this study, we compare the concentrations of vitamin E, which are effective on lipid peroxidation induced by incubation with tertiary butylhydroperoxide (t-BHP) [8] representing its function as an antioxidant and on impaired glucose transport in thalassemic erythrocytes, which may rather represent its structural function.

### Materials and Methods

As vitamin E, DL- $\alpha$ -tocopherol (Merck, Darmstadt, Germany, cat. 613420) was used.

### Hemoglobin determination

For hemoglobin determination, routine HPLC data were used on one hand as provided with blood samples. On the other hand, Hb had to be determined in the frame of our own experiments. In these cases, Hb Kits Merck (cat. 1.03298) or Enzo Life Sciences (cat.nr. ADI-907-

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034) were used: Into the test tube, 5 mL of the respective Hemoglobin Kit containing potassium hexacyanoferrat (II) and potassium cyanide were added to 20  $\mu$ L of blood. Incubation followed at room temperature for 5 minutes; subsequently, absorption was read at wavelength  $\lambda = 540$  nm in a Shimadzu UV-vis spectrophotometer.

### Sampling

Twenty-eight blood samples from thalassemia patients obtained from Thalassemia Center Jakarta and twenty-four normal blood samples from the Indonesian Red Cross were used in this study. All donors from the Thalassemia Center had signed their informed consent. In all experiments more than 20 blood samples were evaluated ( $n > 20$ ).

### Preparation of red blood cells

Blood samples were collected in vacutainer tubes with EDTA as anticoagulant. To separate red blood cells and serum the samples were centrifuged at 2000xg for 5 min. Red blood cells were then washed using physiological saline solution (phosphate buffered saline, PBS). Subsequently, red blood cells were suspended at 20% (w/v) in PBS. Preparation procedures followed Dodge et al. [9] and Ling et al. [10]. Protein concentration was measured by Lowry et al. [11].

### Testing the effect of $\alpha$ -tocopherol on experimentally induced oxidative stress

The experimentation followed the method of Trotta et al. [8];  $\alpha$ -tocopherol was added at final concentrations of 10 to 200 ppm to the suspension of red blood cells in 500  $\mu$ L of PBS. The suspension was incubated for 30 min at 37°C, washed and centrifuged as above. Then, the red blood cell sediment was incubated with 2 mM t-BHP at 37°C for 30 min. The suspension was washed and centrifuged as above, before the supernatant was tested for TBARS [12].

### Testing the effect of $\alpha$ -tocopherol on glucose transport of isolated red blood cell ghosts

We used the method developed by Sahib [13] for glucose transport experiments in red blood cell ghost. Glucose levels were determined photometrically by the Nelson-Somogyi reaction [14]. Testing the effect of tocopherol, we followed the same experimental steps after incubation with the respective concentrations of tocopherol.

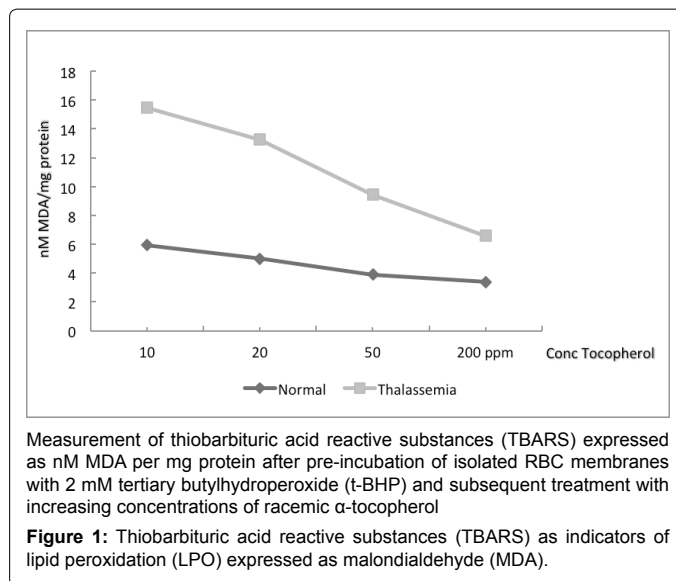
### Results

For all data evaluation 21 samples from thalassemia patients and 21 controls were used ( $n = 21$ ), except for the experiments of glucose transport, where 24 samples from patients and controls were used ( $n = 24$ ).

Table 1 demonstrates that the basic level of TBARS is almost twice as high in thalassemic than in normal erythrocyte membranes. Incubation with t-BHP increases TBARS roughly ten-fold both in normal and in thalassemic RBC membranes. Thus, we had a start point of serial tocopherol treatment of 19.59 nM in thalassemic vs. 11.42 nM in normal RBC membranes (Table 1 and Figure 1).

No	Normal RBC nM MDA / mg prot.	Thalassemic RBC nM MDA / mg prot.	Treatment
1	1.02 $\pm$ 0.64	1.85 $\pm$ 1.37 <sup>s</sup>	-
2	11.42 $\pm$ 3.39	19.59 $\pm$ 6.82 <sup>s</sup>	t-BHP 2mM

**Table 1:** Basic data, thiobarbituric acid reactive substances (TBARS) expressed as malondialdehyde (MDA) levels with tertiary butylhydroperoxide (t-BHP); significance: \$ normal vs thalassemic RBC samples.



Measurement of thiobarbituric acid reactive substances (TBARS) expressed as nM MDA per mg protein after pre-incubation of isolated RBC membranes with 2 mM tertiary butylhydroperoxide (t-BHP) and subsequent treatment with increasing concentrations of racemic  $\alpha$ -tocopherol

**Figure 1:** Thiobarbituric acid reactive substances (TBARS) as indicators of lipid peroxidation (LPO) expressed as malondialdehyde (MDA).

After pre-incubation of isolated RBC membranes with 2 mM t-BHP ghosts were subsequently treated with increasing concentrations of racemic  $\alpha$ -tocopherol and thiobarbituric acid reactive substances (TBARS) were measured (Figure 1). Serial tocopherol treatment started at 10 ppm, which immediately reduced TBARS 15.5 nM in thalassemic vs. 6 nM in normal RBC membranes.

Levels of TBARS further decrease both in normal and thalassemic RBC membranes up to the final concentration used, 200 ppm. The slope of decrease is steeper in thalassemic than in normal membranes demonstrating that thalassemic RBC membranes are more sensitive both to pro-oxidative processes (Table 1) and to antioxidative treatment with tocopherol (Figure 1).

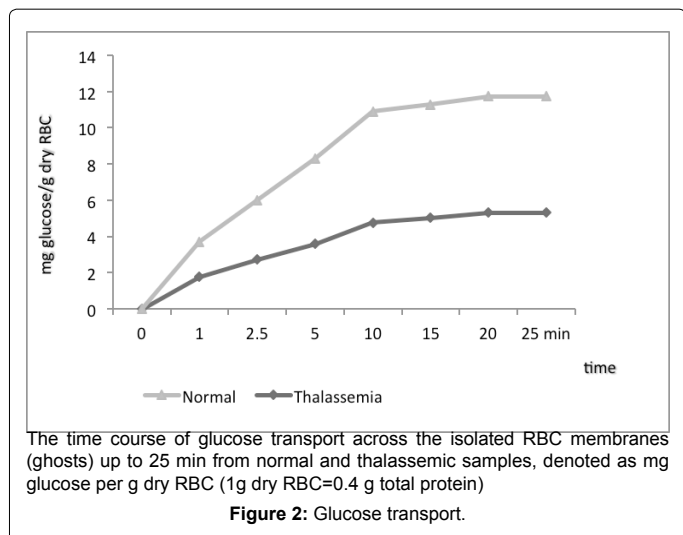
The thiol status of the isolated RBC membranes (Table 2) was 3.9  $\pm$  1.4 nmol per mg of protein in normal blood samples and 70% of this value in membranes from thalassemic patients (2.7  $\pm$  1.1 nmol;  $p < 0.05$  vs. controls). Treatment with 2mM t-BHP diminished reactive thiols by about 70% to 1.2  $\pm$  0.5 nmol in normal controls ( $p < 0.05$ ) and by about 85% to 0.4  $\pm$  0.3 nmol in thalassemic samples ( $p < 0.05$ ). After pre-treatment with 2mM t-BHP racemic  $\alpha$ -tocopherol at concentrations between 50 and 100 ppm increased reactive membrane thiols about two-fold to 60% of the original value in normal controls and 4- to 5-fold to 70% of the original value in thalassemic samples. Table 2 presents the representative values obtained with 60 ppm racemic  $\alpha$ -tocopherol.

The time course of glucose transport across the isolated RBC membranes (ghosts) was measured up to 25 min from normal and thalassemic samples. Between 1 and 10 min, a linear time-dependent increase was observed and a subsequent plateau between 10 and 25 min. Glucose transport has a much lower time-dependent rate in thalassemic than in normal RBC membranes (Figure 2). It was decided to carry out experiments with racemic  $\alpha$ -tocopherol at 5 min.

If measured at 5 min, glucose transport in thalassemic membranes is 76% of normal controls. With 50 ppm and 120 ppm of tocopherol, glucose transport does not improve (it is even slightly lower than without tocopherol), but it improves with 75 and 100 ppm by roughly 10%. This difference is statistically significant ( $p < 0.05$ ) with 75 ppm tocopherol (Table 3).

No	Normal RBC nmol/mg prot	Thalassemic RBC nM MDA /mg prot.	Treatment
1	3.9 ± 1.4 nmol	2.7 ± 1.1 nmol <sup>s</sup>	-
2	1.2 ± 0.5 nmol	0.4 ± 0.3 nmol <sup>s</sup>	t-BHP 2mM
3	2.3 ± 1.4 nmol	1.9 ± 1.0 nmol <sup>s</sup>	toc 60 ppm

**Table 2:** Thiol levels with tertiary butylhydroperoxide (t-BHP); significance: <sup>s</sup> normal vs thalassemic RBC samples; toc=tocopherol (representative concentration of 60 ppm) after pre-incubation of isolated RBC membranes with 2mM tertiary butylhydroperoxide (t-BHP) and subsequent treatment with varying concentrations between 50 and 100 ppm of racemic  $\alpha$ -tocopherol.



Glucose (n=24)	Normal RBC mg glucose/g dry RBC	Thalassemic RBC mg glucose/g dry RBC	Treatment tocopherol ppm
mg glucose/g dry RBC;	6.08 ± 0.65 <sup>s</sup> (100%)	4.76 ± 0.82 <sup>s*</sup> (76%)	0
	-	4.45 ± 0.87 <sup>s</sup>	50
(1 g dry RBC=0.4 g total protein)	-	5.17 ± 0.68 <sup>*</sup> (85%)	75 (* p<0.05)
	-	5.12 ± 0.67	100
	-	4.35 ± 0.88 <sup>s</sup>	120

**Table 3:** Glucose transport at 5 min with racemic  $\alpha$ -tocopherol; significance: <sup>s</sup> normal vs thalassemic RBC samples; <sup>\*</sup>thalassemic samples without treatment vs. treatment with 75 ppm racemic  $\alpha$ -tocopherol.

## Discussion

Lipid peroxidation (LPO) is widely used as parameter of oxidative membrane damage; in our case we use it in experimental oxidation with tertiary butylhydroperoxide (t-BHP). Thiobarbiturate reactive substances (TBARS) are indicators of LPO, in the first place malondialdehyde (MDA) as one of the major by-products of LPO, especially in our *in vitro* model with unambiguous experimental conditions [15,16].

Oxidative stress is basically higher in thalassemic than in normal erythrocyte membranes and incubation with t-BHP further increases LPO about ten-fold. Serial treatment with tocopherol up to 200 ppm reduces TBARS in thalassemic and in normal RBC membranes, the slope being steeper in thalassemic than in normal membranes. Our results demonstrate that thalassemic RBC membranes are more sensitive to pro-oxidative processes but also to antioxidative treatment with vitamin E.

For comparison, we measured the thiol status of our RBC preparation. In normal RBC, thiol status decreased with t-BHP to

roughly one third and in thalassemia to one sixth of the original value. The latter was already much lower in RBC membranes from thalassemic patients (less than 70%) than in normal RBC. Vitamin E increased the thiol status in normal and thalassemic RBC to similar levels. This picture reflects the pro-and antioxidant balance of our RBC preparation.

Concerning the effect of tocopherol concentrations, the picture differs between antioxidant activity and glucose transport across the RBC membrane. In the latter, we do not have a continuous effect from 10 to 200 ppm but a bell shaped effect with its peak at 75 ppm.

Time-dependent glucose transport rate is lower in thalassemic than in normal RBC ghosts and only improves significantly with 75 ppm of tocopherol. Thus, the optimum of tocopherol concentration under our experimental conditions is at 75 ppm (or possibly slightly higher, but certainly below 100 ppm). This bell-shaped effect on glucose transport mirrors a similar bell-shaped structural effect of anti-diabetic type II metformin normalizing the fluidity of rigidified isolated RBC membranes [17,18]. With respect to reactive thiols in membrane-integral proteins, maintenance of redox potential is essential to keep active cysteine thiols in a reduced state, but also membrane-structural effects to stabilize the functional protein conformation via dithiol bonds in cystine (=di-cysteine).

In erythrocytes GLUT1 is responsible for the transport of glucose across the membrane. Its structure was widely investigated and a conformation with 8 of its 12 transmembrane helices forming inner water channel with hydrophilic amino acid residues responsible for the glucose transport across the hydrophobic membrane moiety. These hydrophilic amino acid residues, i.e., hydroxyls and amides, were suggested to form hydrogen bonds with the hydroxyls of glucose [19]. In GLUT1, thiols appear to be more important in stabilizing protein conformation as cystines than actively participating in glucose transport as reduced cysteines, which may explain the concentration-limited effect of tocopherol as a stabilizer of membrane structure and protein conformation rather than functioning as an antioxidant.

Vitamin E is discussed to have a broad spectrum of biological effects apart from its action as antioxidant [20-24]. No wonder, that also structurizing membrane components like tocopherol have differing concentration-dependent effects [25] if compared to the antioxidant action.

Another aspect is the difference between synthetic all-rac (DL) tocopherol and natural (RRR) vitamin E. However, it appears more relevant to compare these differences *in vivo* since in the body bio-kinetic or pharmacokinetic effects differ considerably between synthetic and natural tocopherols [26,27].

In conclusion, the antioxidant effect of vitamin E on experimental lipid peroxidation in thalassemic RBC membranes as measured by TBARS shows a continuous reduction up to a concentration of 200 ppm. On the contrary, glucose transport across thalassemic RBC membranes shows a bell-shaped effect with a peak at a concentration of 75 ppm indicative of a primarily non-antioxidant action but possibly rather a structural membrane-effect of tocopherol.

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