

Non-haem Iron-mediated Oxidative Stress in Haemoglobin E Beta-thalassaemia

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Abstract

Introduction: Haemoglobin (Hb) E beta-thalassaemia is a common thalassaemic disorder in Southeast Asia and is very common in the eastern and north-eastern parts of India. The disease cause rapid erythrocyte destruction due to the free radical mediated injury but factors for the oxidative injury are not clearly known. We investigated the free reactive iron (non-haem) mediated insult in Hb E beta-thalassaemia. **Materials and Methods:** Thirty Hb E beta-thalassaemic patients (age range, 3 to 15 years) who had undergone blood transfusion at least 1 month prior to sampling and 32 normal healthy individuals (age range, 18 to 30 years) were included in this study. We estimated the ferrozine detected intracellular erythrocytic free reactive iron (non-haem iron), reduced glutathione (GSH), glutathione reductase activity, cellular damage marker serum thiobarbituric acid reacting substances (TBARS) and also serum ferritin using standard methods. **Results:** We found that the erythrocytic free reactive iron was significantly higher ($P < 0.001$) in Hb E beta patients and was about 30% more than in controls. The elevated level of erythrocytic non-haem iron was associated with a high level of serum TBARS which was about 86% higher in patients than in controls. The serum ferritin level was also significantly higher ($P < 0.001$) compared to controls. The erythrocytic reduced glutathione level was significantly lower ($P < 0.001$) at about 65% less in the patients' group and the erythrocytic glutathione reductase enzyme was also found to be significantly lower ($P < 0.001$) in Hb E beta-thalassaemia. **Conclusions:** We concluded that a significantly elevated level of erythrocytic free reactive iron and lipid peroxidation end product was associated with low erythrocytic GSH level. This reflects non-haem iron mediated cellular damage in Hb E beta-thalassaemia.

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Introduction

Thalassaemia is a genetic haemoglobinopathy in which the production of normal haemoglobin is partly or completely suppressed because of a defective synthesis of 1 or more of its component globin chains. In haemoglobin (Hb) E beta-thalassaemia, an individual inherits 1 gene for Hb E from 1 parent and 1 beta-thalassaemia gene from the other parent. These 2 genes together lead to a double heterozygous Hb E beta-thalassaemia which causes a moderately severe anaemia similar to beta-thalassaemia major.¹

Hb E beta-thalassaemia is a common haemolytic anaemia in Southeast Asia. It is also most common in the eastern and north-eastern parts of India.² To our knowledge, there have been very few studies relating to the causes of rapid erythrocyte breakdown in Hb E beta-thalassaemia.

Iron overload is the consequence of rapid erythrocyte

breakdown in the beta and Hb E beta-thalassaemia.³ Non-haem ferrous iron is a strong pro-oxidant and is responsible for the production of damaging hydroxyl ($\bullet\text{OH}$) radical by Fenton reaction.⁴ In our study, we investigate the level of ferrozine detected erythrocytic non-haem iron, important cellular redox marker reduced glutathione (GSH), glutathione reductase enzyme within erythrocyte and cellular damage marker thiobarbituric acid reacting substances (TBARS), ferritin level in serum of Hb E beta-thalassaemia and comparing these to the normal controls to understand the mechanism of rapid erythrocyte destruction in Hb E beta-thalassaemia.

Materials and Methods

Sample Collection

The current study included Hb E beta thalassaemic

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patients (n = 30; age range of 3 to 15 years, with mean age of 7.88 ± 3.02 years) determined by the clinical picture and laboratory investigation including high performance liquid chromatography (HPLC) (Biorad) by the Department of Haematology of NRS Medical College and Hospital, Kolkata, India. Blood samples were collected from the patients who have received blood transfusion at least 1 month prior to sampling. Normal control samples (n = 32; age range of 18 to 30 years, with mean age of 23.52 ± 3.56 years) who had no history of haematological abnormality and liver disease were selected. Blood was collected from patients and from healthy normal individuals after getting their prior consent. The study protocol was approved by the ethical committee of NRS Medical College and Hospital (Government of West Bengal), Kolkata, India.

Characteristics of Patients

The clinical features of the patients showed pallor and jaundice (variable); most had hepatosplenomegaly, bony deformities-fragile and frontal bossing. Haematological findings showed microcytic hypochromic red cells with Hb <7 g/dL, low haematocrit (HCT), mean corpuscular volume (MCV) <70 femtolitre (fl), mean corpuscular haemoglobin (MCH) <18 picogram (pg), low mean corpuscular haemoglobin concentration (MCHC) and high red cell distribution width (RDW). Hb analysis with HPLC showed HbA₂ 30% to 65%, HbF 21% to 30% and HbA 4.5% to 11%.

Blood samples (3 mL/subject) from thalassaemic and control subjects were collected aseptically in heparinised condition. Serum samples were obtained by centrifuging the clotted blood samples at 5000 rpm for 5 minutes and processed for estimation. Stroma-free haemolysate was prepared from heparinated blood by removing serum and buffy coat, washing thrice by 0.15 mol/L sodium chloride (NaCl) solution and treating with hypotonic phosphate buffer followed by centrifugation at 10,000 rpm for 30 minutes.

Estimation of Biochemical Parameters

Erythrocytic ferrozine detected free reactive iron was measured using Panter's method.⁵ Intracellular erythrocytic GSH concentration was estimated using 2,2-dithio-bisnitrobenzoic acid (DTNB) method spectrophotometrically at the wave length of 412 nm and expressed against per gram of Hb with slight modifications according to Hu's method.⁶ The enzyme glutathione reductase catalysed reduction of oxidised glutathione to reduce GSH. The red blood cell glutathione reductase was reduced nicotinamide adenine dinucleotide phosphate (NADPH) dependent and has flavin adenine dinucleotide (FAD) as co-factor. The enzyme activity was assayed spectrophotometrically by measuring NADP formed following the method suggested

by Tillotson et al and Bayoumi et al.^{7,8} It has been proposed that the percentage of stimulation of the NADPH dependent erythrocyte glutathione reductase (EGR) in the presence of added FAD be used as a measurement of the status of riboflavin in man. This stimulatory effect is referred to as activity co-efficient (AC). The AC is defined as the reduction of absorbance (oxidation) of NADPH in the presence of FAD divided by the reduction of absorbance of NADPH without FAD over a given period of time, as shown in the equation.⁹

$$\frac{\text{Reduction of absorbance with added FAD/10 minutes}}{\text{Reduction of absorbance without added FAD/10 minutes}} = \text{AC}$$

Serum ferritin was estimated by the enzyme-linked immunosorbent assay (ELISA) technique supplied by the kit from Omega Diagnostic Limited, Scotland, UK. The cellular damage was measured by estimating TBARS. This is a spectrophotometric assay based on thiobarbituric acid (TBA) reaction, read at a wavelength of 532 nm following Ohkawa et al's method.¹⁰

Statistical Analysis

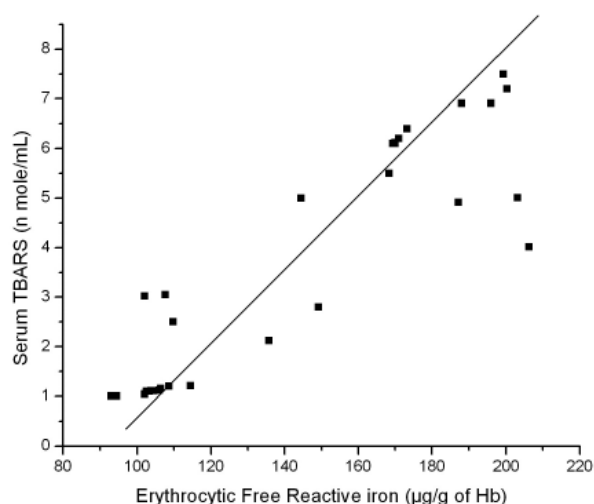
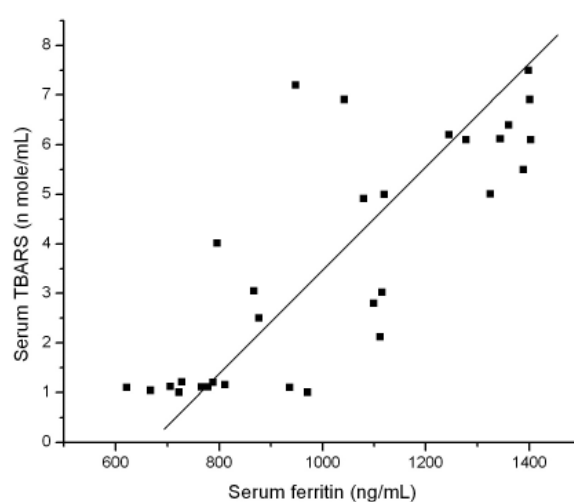
All parameters were expressed as mean \pm standard error (SE). The statistically significant differences between values were obtained using the 2-tailed Student's unpaired *t*-test. Significance level was considered up to *P* value less than 0.05.

Results

Table 1 shows that the erythrocytic free reactive iron was significantly higher in Hb E beta-thalassaemic patients (*P* <0.001) than in controls. It was about 30% more in patients and an elevation was associated with a high level of serum TBARS, which was about 86% more in patients than in controls. Again, we tried to correlate the erythrocytic free reactive iron level and serum TBARS level of Hb E beta patients and found that the correlation co-efficient (*r*) between them was 0.895 (*P* <0.001) (Fig. 1). We observed that the serum ferritin level was also significantly higher in Hb E beta-thalassaemia patients (*P* <0.001) compared to controls. A correlation co-efficient between serum ferritin and serum TBARS of patients was found to be highly significant (*r* = 0.81) (*P* <0.001). Intracellular GSH, an important antioxidant marker, was found to be depleted by >3 times (approx) in thalassaemic patients compared to controls. Strikingly, erythrocytic glutathione reductase (both basal and stimulated) was low in Hb E beta patients. Glutathione reductase (basal) was about 35% less in Hb E beta and glutathione reductase (stimulated) was about 10% less in patients compared to controls. We analysed

Table 1. Comparative Study of Mean Concentration (\pm SE) of Biochemical Parameters (intracellular level and serum) and their Significant Levels of Normal Control and Thalassaemic Patients

Parameters	Control (n = 32)	Hb E (n = 30)	Significance
Erythrocytic free reactive iron (μ g/g of Hb)	109.72 \pm 4.83	142.97 \pm 7.34	<i>P</i> <0.001
Serum ferritin (ng/mL)	50.09 \pm 2.65	1022 \pm 47.03	<i>P</i> <0.001
Serum TBARS (n mole/mL)	1.96 \pm 0.17	3.65 \pm 0.43	<i>P</i> <0.001
Erythrocytic reduced glutathione (μ M/g of Hb)	72.92 \pm 2.55	25.79 \pm 1.35	<i>P</i> <0.001
Erythrocytic glutathione reductase (Basal) (U/mL)	437.56 \pm 6.13	284.89 \pm 6.62	<i>P</i> <0.001
Erythrocytic glutathione reductase (Stimulated) (U/mL)	521.78 \pm 9.43	470.51 \pm 4.79	<i>P</i> <0.001
Erythrocytic glutathione reductase (Activity co-efficient)	1.19	1.65	


 Fig. 1. Correlation between serum TBARS and erythrocytic free reactive iron in Hb E beta thalassaemic patients (n = 30, $r = 0.895$, $y = 0.053x - 3.93$).

 Fig. 2. Correlation between serum TBARS and serum ferritin in Hb E beta thalassaemic patients (n = 30, $r = 0.81$, $y = 0.0075x - 4.02$).

the activity co-efficient by calculating the ratio of the stimulated and the basal erythrocytic glutathione reductase enzyme level. We found that the ratio was high, at 1.65, in thalassaemic patients whereas it was within range in normal individuals.

Discussion

From our findings, it is evident that the level of non-haem iron detected by ferrozine reaction is significantly increased within erythrocytes of Hb E beta-thalassaemia. Iron in its 6th coordination state is tamed within the protoporphyrin cage of haem pocket of haemoglobin. However in Hb E beta-thalassaemia where there is a structural anomaly in the globin chain, iron may be liberated from haem and ligated to other parts of globin moiety possibly with distal histidine. It has been reported that H₂O₂ and super oxides are generated due to the auto oxidation of unstable haemoglobin chain.¹¹

Thus, this free non-haem iron might catalyse Haber Weiss reaction in the presence of H₂O₂ producing ferryl moiety and hydroxyl radical.^{12,13} These further damage erythrocyte membrane lipids and proteins.^{14,15} Elevation of non-haem

iron is further reflected by an increased level of serum ferritin and the cellular damage is indicated by a high level of lipid peroxidation end product TBARS in Hb E beta-thalassaemic patients. Iron loading is a constant feature in transfusion dependent homozygous E beta-thalassaemia which potentiate the increased level of ferritin.¹⁶ We also found a strong correlation between serum TBARS and erythrocytic free reactive iron ($r = 0.895$) of patients and also between serum TBARS and serum ferritin ($r = 0.81$) of patients as shown in Figures 1 and 2. These results indicate that both erythrocytic free reactive iron and serum ferritin levels are important parameters to estimate the cellular damage. The reduced glutathione is an important redox marker of the cell.¹⁷ GSH functions in diverse ways such as regulating antioxidant defence, xenobiotics, cellular communication and redox regulation of signal transduction.¹⁸ The redox imbalance in Hb E beta-thalassaemia is reflected by the lowering level of erythrocytic GSH which is very significantly reduced compared to the normal controls. This clearly depicts that the erythrocyte environment of the Hb E beta-thalassaemia is in oxidative stress and vulnerable

to a free radical attack which is also supported by others.¹⁹ Moreover, glutathione reductase enzyme which converts oxidised glutathione (GSSG) to reduced GSH is also found to be significantly reduced in Hb E beta-thalassaemia. Thus, the regeneration of reduced GSH in non-nucleated mature erythrocyte is not possible. Thus, an oxidised environment of erythrocytes is predominant which also destabilises the lifespan of erythrocytes.

Erythrocyte glutathione reductase has been proposed as an enzymatic index to the riboflavin status in humans.²⁰⁻²³ EGR activity is altered in vivo by riboflavin in diet and in vitro by FAD.²⁴⁻²⁶ The degree of stimulation of the EGR in vitro is dependent on the FAD saturation of the protein which in turn is dependent on the availability of riboflavin. It has been proposed that an AC > 1.2 would be suggestive of possible riboflavin deficiency.⁹ In our study, the ratio of stimulated and basal glutathione reductase enzyme level, that is AC, was found to be 1.65 for Hb E beta-thalassaemia whereas the normal range reported by Glatzle et al⁹ was 0.90 to 1.2. This indicates a significant riboflavin deficiency in Hb E beta-thalassaemia.

Serum TBARS is also found to be significantly elevated in Hb E beta-thalassaemia which reflects a massive cellular damage in the said disorder.

The above observations may help us to conclude that catalytic non-haem iron and low GSH level stimulate the early destruction of erythrocytes. The therapeutic use of GSH precursor such as n-acetyl cystine and riboflavin associated with iron chelator may be taken under consideration for delayed erythrocyte breakdown in Hb E beta-thalassaemia. However, further studies can be undertaken to understand the possible corners of redox imbalance in Hb E beta-thalassaemia.

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REFERENCES

- Weatherall DJ. Haemoglobin E beta-thalassaemia: an increasingly common disease with some diagnostic pitfalls. *J Pediatr* 1998;132:765-7.
- Weatherall DJ, Clegg JB. Thalassaemia—a global public health problem. *Nature* 1996;2:847.
- Inacu TC. Ultrastructural pathology of iron overload. *Clin Haematol* 1989;2:475.
- Gutteridge JMC. Iron promoters of the fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Lett* 1986;201:291-5.
- Panter SS. Release of iron from haemoglobin. *Meth Enzymol* 1994;231:502-14.
- Hu ML. Measurement of protein thiol groups and glutathione in plasma. *Meth Enzymol* 1994;233:382-5.
- Tillotson JA, Sauberlich HE. Effect of riboflavin depletion and replition on the erythrocyte glutathione reductase in rat. *J Nutr* 1971;101:1459-66.
- Bayomi RA, Rosalki SB. Evaluation of methods of coenzyme activation of erythrocyte enzymes for detection of deficiency of vitamins B₁, B₂ and B₆. *Clin Chem* 1976;22:327-35.
- Glatzle D, Korner WF, Christeller S, Wiss O. Method for the detection of a biochemical riboflavin deficiency. *Int J Vitam Res* 1970;40:166.
- Ohkawa H, Ohishi N, Yagi K. Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. *Ann Biochem* 1979;95:351-8.
- Scott MD, Meshnick SR, Eaton JW. Superoxide dismutase—rich bacteria. Paradoxical increase in oxidant toxicity. *J Biol Chem* 1987;262:3640.
- Puppo A, Halliwell B. Formation of hydroxyl radicals from hydrogen peroxide in the presence iron. Is haemoglobin a biological Fenton reagent? *Biochem J* 1988;249:185-90.
- Gutteridge JMC, Smith A. Antioxidant protection by haemopexin of haem—stimulated lipid peroxidation. *Biochem J* 1988;256:861-5.
- Braugher JM, Duncan LA, Chase RL. The involvement of iron in lipid peroxidation. Importance of ferric to ferrous ratios in initiation. *J Biol Chem* 1986;261:10282-9.
- Minotti G, Aust SD. The requirement for iron (III) in the initiation of lipid peroxidation by iron (II) and by hydrogen peroxide. *J Biol Chem* 1987;262:1098-104.
- Warwood M, Cragg SJ, McLaren C, Ricketts C, Economidou J. Binding of serum ferritin to concanavalin A: patients with homozygous thalassaemia and transfusional iron overload. *Br J Haematol* 1980;49:409.
- Moran LK, Gutteridge JMC, Quinlan GJ. Thiols in cellular redox signaling and control. *Curr Med Chem* 2001;8:763-72.
- Gutteridge JMC. Does redox regulation of cell function explain why antioxidants perform so poorly by therapeutic agents? *Red Rep* 1999;4:129.
- Chakraborty D, Bhattacharya M. Antioxidant defense status of red blood cells of patients with beta-thalassaemia and Ebeta. *Clin Chim Acta* 2001;305:123-9.
- Glatzle D. Dependency of the glutathione reductase activity on the riboflavin status. 7th Int Congr Clin Chem, Geneva/Evian *Clin Enzymol* 1969;2:89-94.
- Bamji MS. Glutathione reductase activity in red blood cells and riboflavin nutritional status in humans. *Clin Chim Acta* 1969;26:263-9.
- Tillotson JA, Baker EM. An enzymatic measurement of the riboflavin status in man. *Am J Clin Nutr* 1972;25:426-31.
- Sauberlich HE, Judd Jr JH, Nichoalds GE, Broquist HP, Darby WJ. Application of the erythrocyte glutathione reductase assay in evaluating riboflavin nutritional status in a high school student population. *Am J Clin Nutr* 1972;25:756-62.
- Glatzle D, Weber F, Wiss O. Enzymatic test for the detection of riboflavin deficiency. NADPH-dependent Glutathione reductase of red blood cells and its activation by FAD in vitro. *Experientia* 1968;24:1122.
- Beutler E. Effect of flavin compounds on glutathione reductase activity: in vivo and in vitro studies. *J Clin Invest* 1969;48:1966-7.
- Glatzle D, Korner WF, Christeller S, Wise O. Method for the detection of a biochemical riboflavin deficiency stimulation of NADPH2—dependent glutathione reductase from human erythrocytes by FAD in vitro. Investigations on the vitamin B2 status in healthy people and geriatric patients. *Int J Vitam Res* 1970;40:166-83.