# RED CELL AND PLASMA CALCIUM, COPPER AND ZINC IN β-THALASSEMIA/HEMOGLOBIN E

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**Abstract.** β-thalassemia/Hb E is a genetic disease prevalent in Thailand. This study has used atomic absorption spectroscopy to evaluate red cell and plasma calcium, copper and zinc in patients with β-thalassemia/Hb E, both splenectomized and non-splenectomized. The levels of these trace elements in both red cells and plasma were different between the non-thalassemic controls and the disease patients. The most prominent result was that calcium concentration in red cells increased significantly in thalassemia subjects, particularly in splenectomized cases. These results might reflect the abnormal trace element metabolism and defects in the calcium transport system of the red cell membrane in thalassemia.

## INTRODUCTION

β-thalassemia/Hb E is a genetic disease, with a defect in β-globin chain synthesis, which is prevalent among the Thai population (Flatz *et al,* 1965; Wasi *et al,* 1980; Rowley *et al,* 1987), and, indeed, among peoples of the region. Several previous studies indicated that there can be changes in trace element metabolism in thalassemia patients (Arcasoy and Cavdar, 1975; Dogru *et al,* 1979; Prasad *et al,* 1965; Vatanavicharn *et al,* 1982; Wiley, 1977: Shalev *et al,* 1984). These included reports on zinc deficiency in thalassemia (Arcasoy and Cavdar, 1975; Dogru *et al,* 1979; Prasad *et al,* 1965; Vatanavicharn *et al,* 1982). The Cu:Zu ratios, a more sensitive index for zinc deficiency than the plasma zinc level alone, have been reported to increase in thalassemia (Vatanavicharn *et al,* 1982). Increased uptake of cations in metabolic-depleted red cells was demonstrated in both α - and β-thalassemia (Wiley, 1977). Of particular interest is the report of increased red cell Ca accumulation without changes in Ca-Mg-ATPase activity in β-thalassemia intermedia, in both non-splenectomized and splenectomized cases as compared to non-splenectomized and splenectomized control subjects (Shalev *et al,* 1984). Normaly, the level of Ca in red cells is quite low (Wiley and Shaller, 1977; Schatzmann and Vincenzi, 1969). Increased Ca in red cells would lead to shape transformation, an increase in membrane rigidity, together with a decrease in deformability which would result in a decreased lifespan in the peripheral circulation (Kretchman and Rogers, 1981). It has been suggested that disordered red cell Ca homeostasis might be an important element in oxidant-induced red cell destruction (Eaton and Skelton, 1973; Shalev *et al,* 1981). The natural protection from free radical oxidation depends on superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px). An increase in red cell activity of SOD, the copper/zinc enzyme, has been demonstrated in various thalassemic syndromes including β-thalassemia/Hb E and Hb H disease (Yenchitsomanus and Wasi; 1983; Suthipark *et al,* 1985). These elements, calcium, copper and zinc, are probably, to some extent, involved in red cell integrity, enzyme activity and membrane rigidity. This study was conducted to examine the levels of calcium, copper and zinc in both red cells and plasma of both β-thalassemia/Hb E patients and non-thalassemic controls.

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## MATERIALS AND METHODS

### Subjects

Thirty-one patients (15 male and 16 female, 19-44 years old) with β-thalassemia/Hb E were recruited from the thalassemia patients who regularly attend the Division of Hematology, Siriraj Hospital. None of these patients had received blood transfusions or chelate therapy in the three months prior to this study. Eighteen patients had been previously splenectomized. Twenty-nine non-thalassemic subjects (23 males and 6 females, 19-36 years old) were used as controls. All of the control subjects had normal hematological data with normal hemoglobin typing, with Hb $A_2$ less than 3.5%.

### Glassware

Polypropylene tubes and plastic syringes for collection and analysis were soaked overnight in nitric acid (10%), rinsed (5x) with doubly deionized water (Amicon Milli-Q system) and allowed to drain dry.

### Chemicals

Standard solutions were prepared from commercial (BDH Chemical) solutions: lanthanum chloride (10%) and atomic absorption grade standard (1mg/ml) for calcium chloride, copper chloride, zinc nitrate and iron(III) nitrate.

### Blood Samples

Fifteen milliliters of venous blood were collected into 15 ml heparinized (5,000 IU/ml) tubes. The heparin used was also analysed for Ca, Cu and Zn. The blood samples were centrifuged at 2,500 rpm, 4°C, for 10 minutes to obtain plasma and red cells. The red cells were separated from plasma and washed (3x) with phosphate buffer (5 mM, pH 7.4) containing 10 mM dextrose. Suspensions of red cells (50% in phosphate buffer) were prepared for the determination of the number of red cells present. Plasma and packed red cells were kept frozen in polypropylene tubes and shipped to Murdoch University for trace element analysis. The washing buffer was also analyzed for the three elements under study. Statistical evaluation used Student's *t* test.

### Analysis

Atomic absorption analysis was carried out using a Perkin-Elmer Model 503 double beam spectrophotometer interfaced to a Commodore Microcomputer, Model 4032. Operating conditions were as stated in the manufacturer's Handbook (Anonymous, 1973).

Accuracy and precision of the analysis were validated by Youden's technique (Youden, 1962) using a bulk sample of ovine blood. Standard additions of all elements analysed gave recoveries of between 95 and 110%.

Red cell samples were analysed by the method of Eaton *et al* (1973) with some modification. Trichloroacetic acid (10%, 2.5 ml) and lanthanum chloride (10%, 0.25 ml) were added to 1 ml of packed red cells and the mixture diluted to 5 ml with doubly deionized water. After homogenization using a vortex mixer, the sample was centrifuged at 3,000 rpm for 10 minutes at room temperature. The supernatant was analysed for Ca, Cu and Zn using suitably prepared standards containing trichloroacetic acid.

For analysis of plasma samples for Cu and Zn, 1 ml aliquots of plasma were diluted to 10 ml with doubly deionized water. For Ca analyses, 100 µl aliquots were added to 10% lanthanum chloride (0.5 ml) and made up to a final volume of 10 ml with doubly deionized water.

## RESULTS

The subjects' information is summarized in Table 1. The concentrations of Ca, Cu and Zn in red cells are reported on the basis of red cell volume (ml) because there was no significant difference ($p < .05$) in red cell number of 50% suspensions prepared from different groups of subjects. The mean results are shown in Table 2. A significant difference ($p < 0.0005$) was seen between the red cell Ca levels in non-thalassemic controls and in thalassemic patients. A remarkable difference in red cell Ca levels between the splenectomized (9.52 µg/ml) and non-splenectomized (1.66 µg/ml) β-thalassemia/Hb E subjects ($p < 0.0005$) was clearly apparent. However, red cell concentrations of Zn and Cu showed no differences between normal and thalassemic subjects, either with or without splenectomy.

In the plasma of thalassemic subjects, the concentration of Ca was depressed significantly

Table 1

Number, age, sex, hemoglobin level, and red cell number in 50% suspensions of non-thalassemic controls and β-thalassemia/Hb E subjects.

| Subjects | No. | Age | Sex | | Hb level (g/dl) | Red cells/ml × $10^6$ (in 50% suspension) |
|---|---|---|---|---|---|---|
| | | | Male | Female | | |
| Non-thalassemic controls | 29 | 19-36 | 23 | 6 | 15.4 ± 1.6 | 4.7 ± 0.73 |
| β-thalassemia/Hb E | 31 | 19-44 | 15 | 16 | 7.5 ± 1.6 | 4.88 ± 1.18 |
| - splenectomized | 18 | 19-44 | 9 | 9 | 8.0 ± 1.8 | 4.77 ± 1.18 |
| - non-splenectomized | 13 | 20-42 | 6 | 7 | 7.1 ± 1.3 | 5.06 ± 1.07 |

Table 2

Mean concentrations (± SD) of red cell calcium, copper and zinc in non-thalassemic controls and β-thalassemia/Hb E subjects.

| Subjects | Calcium (μg/ml RC) | Copper (μg/ml RC) | Zinc (μg/ml RC) |
|---|---|---|---|
| Non-thalassemic controls | 0.25 ± 0.14 (n = 27) | 0.78 ± 0.25 (n = 29) | 13.0 ± 3.4 (n = 29) |
| β-thalassemia/Hb E | 6.23 ± 5.09 (n = 31) | 1.01 ± 0.33 (n = 31) | 18.2 ± 4.0 (n = 31) |
| - splenectomized | 9.52 ± 4.10 (n = 18) | 1.03 ± 0.28 (n = 18) | 17.2 ± 4.0 (n = 18) |
| - non-splenectomized | 1.66 ± 1.45 (n = 13) | 0.98 ± 0.40 (n = 13) | 19.5 ± 4.0 (n = 13) |

Table 3

Mean concentrations (± SD) of plasma calcium, copper and zinc in non-thalassemic controls and β-thalassemia/Hb E subjects.

| Subjects | Calcium (μg/ml) | Copper (μg/ml) | Zinc (μg/ml) |
|---|---|---|---|
| Non-thalassemic controls | 98.6 ± 19.8 (n = 29) | 0.76 ± 0.12 (n = 29) | 1.53 ± 0.70 (n = 29) |
| β-thalassemia/Hb E | 84.7 ± 15.0 (n = 31) | 0.92 ± 0.19 (n = 31) | 0.66 ± 0.22 (n = 31) |
| - splenectomized | 84.6 ± 4.04 (n = 18) | 1.01 ± 0.17 (n = 18) | 0.61 ± 0.20 (n = 18) |
| - non-splenectomized | 84.8 ± 3.80 (n = 13) | 0.80 ± 0.15 (n = 13) | 0.74 ± 0.23 (n = 13) |

($p < 0.005$), as was that of Zn, but Cu was elevated. Splenectomy led to no significant difference in these plasma metal concentrations for the thalassemic patients (Table 3).

## DISCUSSION

The plasma concentrations of Ca, Cu and Zn in the study group of Thai patients with β-thalassemia/Hb E are in good agreement with earlier reports (Vatanavicharn *et al,* 1982; Shalev *et al,* 1984). The plasma Zn levels indicate Zn deficiency in the patients, as expected (Vatanavicharn *et al,* 1982).

A striking observation of the present study was the difference in erythrocyte concentrations of the three metals between controls and patients. In particular, erythrocyte Ca levels increased in splenectomized cases, from 1.66 to 9.52 μg/ml. In patients with thalassemia intermedia (Shalev *et al,* 1984), a similar but less dramatic change followed splenectomy: erythrocyte Ca increased from 1.04 to 3.41 μg/ml. The thalassemic erythrocyte membrane has been reported to show increased permeability to cations (Wiley, 1977), consistent with our observations of increased concentrations of Ca, Cu and Zn in these cells. An increase in red cell Ca may adversely affect the circulation and survival of these cells (Shalev *et al,* 1981). Calcium accumulation may reflect the increased age of these cells, which survive for abnormally long periods in the peripheral circulation following the removal of the spleen.

After splenectomy, erythrocytes with other abnormalities can also be detected, including changes in deformability (Shalev *et al,* 1984; Stater *et al,* 1965, Tinman and Schroter, 1979). Of particular interest is the report that disordered erythrocyte Ca homeostasis is an important consequence of oxidant-induced erythrocyte damage. The present study has shown that erythrocyte Cu and Zn, essential components of Cu, Zn-superoxide dismutase, are elevated in cases of β-thalassemia/Hb E, which also exhibit increased levels of erythrocyte activity of this enzyme (Yenchitsomanas and Wasi, 1983; Suthipark *et al,* 1985). Interestingly, Zn levels in heart and pancreas tissue are also elevated (Shuler *et al,* 1990), as are the Se concentrations in these organs, suggesting some association with increased oxidative stress.

The mechanism of accumulation of Ca in the thalassemic erythrocytes remains unclear. In thalassemia intermedia, the calmodulin-dependent component of the calcium pump appeared intact, with normal level of $Ca^{2+}$, $Mg^{2+}$-ATPase (Shalev *et al,* 1984). Membrane alterations and accumulation of Ca are also observed in erythrocytes infected with the malarial parasite, *Plasmodium berghei* (Yuthavong, 1985) suggesting that there may be similarities in the pathophysiology of both infected and thalassemic erythrocytes.

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