

PERIPHERAL HAEMOLYSIS, LIPID PEROXIDATION, IRON STATUS, AND VITAMIN E IN HAEMOGLOBIN H SYNDROMES IN WEST MALAYSIA

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ABSTRACT

Following complete DNA characterisation patients with Hb H disease were assigned into two groups: deletional (α^0/α^0) and non-deletional (HbCS/ α^0). Earlier studies have indicated that the group with (HbCS/ α^0) has more severe clinical problems. The serum malonyldialdehyde (MDA) levels, a secondary product of lipid peroxidation were within the normal range, though significantly higher levels of MDA were seen in the non-deletional type of Hb H disease when compared with the deletional type. Markedly low vitamin E levels were also seen in the former group. There were no significant differences in clinical severity may be attributed to an interplay of the accelerated destruction of damaged mature red blood cells secondary to the oxidative denaturation of Hb H and inclusion precipitation; higher levels of Hb H and more inclusion precipitation were seen in the group with (HbCS/ α^0). Low levels of vitamin E in the (HbCS/ α^0) group being due to its consumption in the neutralisation of free radicals formed with the oxidation of globin chains.

Keywords: Hb H disease, West Malaysia, peripheral haemolysis

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INTRODUCTION

A marked genetic and clinical variability of haemoglobin H (Hb H) syndrome occurs because of the molecular heterogeneity of alpha (α) - thalassaemia. The hallmark is the presence of excess beta (β) chains forming Hb H (β^4 , beta tetramer)⁽¹⁾. In West Malaysia, classical Hb H disease presents as "alpha - thalassaemia intermedia" and at a molecular level two types of defects are seen. The first, is due to a double heterozygosity for two deletional forms of alpha-thalassaemia (α^0/α^0)^(2,3). The majority of cases with an α^0 -thalassaemia defect have a deletional defect of at least 18.1 kb Starting 3', to the $\alpha 1$ gene which includes the $\alpha 2$ and the $\alpha 1$ genes; it is similar to that described in the Thai⁽⁴⁾ and the Chinese⁽⁵⁾. Deletions of the α globin complex that cause α^0 -thalassaemia remove one alpha gene. Two types of α^0 -thalassaemia genes have been observed, one involving a deletion 4.2 kb DNA (leftward deletion, $\alpha^{4,2}$), and another removing 3.7 kb (rightward deletion, $\alpha^{3,7}$). Cer-

tain haemoglobin variance with remarkable reduction of the α gene product lead to α^0 -thalassaemia syndromes upon interacting with the α^0 gene. Studies of the molecular aspects of Hb H syndromes in West Malaysia describe Hb Constant Spring (HbCS) as the most common haemoglobin variant in this group^(2,3). Comparison of the clinical parameters (the necessity of blood transfusions, thalassaemia facies), haemoglobin and bilirubin levels show that the non-deletional type of Hb H disease in West Malaysia is associated with a more severe clinical state than the deletional type⁽³⁾. The mechanism leading to the difference in the severity between the two genotypes is not clear. Patients with thalassaemia intermedia can show evidence of increasing iron overload secondary to ineffective erythropoiesis and increased gastrointestinal iron absorption in the absence of blood transfusions⁽⁶⁾. Studies have shown that Chinese adult patients with Hb H disease in Taiwan without multiple blood transfusions and prolonged iron therapy frequently have iron overload⁽⁷⁾. Thalassaemic red blood cells have more lipid per cell and data have accumulated that suggested increased lipid peroxidation took place in the thalassaemic red blood cells⁽⁸⁻¹⁰⁾. Biologic peroxidation of membrane lipid is accompanied by the formation of various, carbonyl compounds, one of which is malonyldialdehyde (MDA), a compound that promotes erythrophagocytosis⁽¹¹⁻¹³⁾. The mechanisms facilitating oxidative damage of thalassaemic red blood cells are multifactorial⁽¹⁴⁾. Traces of iron salts and the oxidised excess globin chains are known to generate in vitro free oxygen radicals such as superoxide and the hydroxyl radical⁽¹¹⁾. There are innumerable endogenous antioxidants in the red blood cell, including uric acid, ascorbate, glutathione (GSH), and vitamin E to scavenge free-floating radicals⁽¹⁵⁾. The lack of vitamin E (alpha tocopherol) increases the rate of hydrogen peroxide - induced haemolysis of red blood cells in vitro⁽¹⁶⁾.

The red cell creatine of human red blood cell decreases significantly with the increase in age of the red cells. Measurements of erythrocyte creatine is therefore a sensitive index of the mean age of a mixed erythrocyte population and therefore erythropoiesis activity. Hence, ⁵¹Cr red cell survival studies and data of the red cell creatine in patients with Hb H disease would provide better information of the degree of red cell destruction⁽¹⁷⁾. The aim of this study was to establish the relationship of red cell survival, serum vitamin E, serum ferritin, and serum malonyldialdehyde in patients with Hb H disease, taking into account their genotype and clinical expression.

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Table I - Haematological, serum ferritin, serum bilirubin, serum vitamin E, serum malonyldialdehyde, ⁵¹Cr red cell survival and red cell creatine data for Hb H patients

Mean ± 1SD	Normal (n=20)	Deletional (n=4)	Non-deletional (n=4)
Hb g/L	M 140 ± 25 F 130 ± 25	85 ± 19	80 ± 15
RBC x 10 ¹² /L	M 4.5 ± 1.8 F 4.2 ± 1.2	4.9 ± 1.1	3.9 ± 0.8
MCV fl	86 ± 10	61.5 ± 11.0	74.7 ± 6.5 *
MCH pg	29 ± 10	17.4 ± 1.3	20.1 ± 1.3 *
MCHC %	32 ± 2.5	28.4 ± 1.6	26.9 ± 1.4
S. ferritin µg/L	140 ± 120	173.3 ± 89.7	243.8 ± 176.8
S. Bilirubin µmol/L	0 ± 18	21.5 ± 19.5	64.7 ± 23.5 *
MDA n mol/gm protein	6.4 ± 1.6	3.3 ± 0.4	5.7 ± 0.8 *
Vitamin E µg/ml	7.2 ± 1.0	8 ± 3.8	1.0 ± 0.4 *
⁵¹ Cr (days)	29 ± 4	20.6 ± 2.5	13.7 ± 3.5 *
Red cell creatine mg/ml	118 ± 59	233.5 ± 72.9	579.9 ± 132 *

*p<0.01, non-deletional versus deletional type

Table II - Haematological and Biochemical data for Hb H patients

	CODE	Age	Sex	Race	Hb	MCV	MCH	MCHC	H	MDA	VE	SF	RCS
Non - deletional n=4	H11 NMD	34	F	M	75	70.6	20.2	28.6	25.0	5.5	0.43	500	16.2
	H10 MS	32	M	M	83	78.3	20.7	26.4	18.0	4.8	1.1	197	17.4
	H6 CCY	20	F	C	87	76.1	19.5	25.7	16.5	5.7	1.7	168	11.3
	H5 CCT	19	F	C	76	73.7	19.8	26.9	15.4	4.5	1.3	110	10.0
Deletional n=4	D11 PA	36	F	M	82	58.5	16.3	27.8	4.5	3.5	7.7	38	23.4
	D12 YFO	37	M	C	99	57.9	17.1	29.6	3.7	3.7	12.2	210	18.0
	D14 CFC	40	M	C	82	60.6	16.9	27.9	3.9	3.2	9.1	170	23.0
	D4ZR	19	F	M	76	68.9	19.4	28.1	7.2	2.8	3.1	275	18.0

M = Malay; C = Chinese; Hb 75 G/L; MCH pg; MCHC = %; H = % Hb H; MDA, malonyldialdehyde = nmol/gm protein; VE, vitamin E = µg/ml; SF = serum ferritin = µg/l; RCS, red cell survival = days.

PATIENTS AND METHODS

Patients

Eight patients with Hb H disease from the specialist clinic, National University of Malaysia were studied. The study group consisted of four Malays and four Chinese who were divided into two groups following DNA analysis (Table I). Four patients (females 2, males 2; aged 19 to 40 years) had the deletional type of Hb H (α^+ / α^0 ; three had the defect $\alpha^{3,7}$, and one had the defect $\alpha^{4,2}$). In the group with the non-deletional type of Hb H (HbCS/ α^0) there were four patients (females 3, and male 1; aged 19 to 34 years). The female patient H11NMD (Table II) had in addition Hereditary Ovalocytosis. The control group consisted of 20 adults in good health with no family history of thalassaemia.

Haematological

Red cell indices were collected on a Coulter counter M530. Electrophoresis of the haemoglobins (Hb) was carried out on cellulose acetate pH 8.6 and 6.5, and by isoelectric focusing

(IEF) pH 5-8^(18,19). The quantitative determination of serum ferritin (SF) was by an enzyme linked immunoassay (Spectro ferritin, Ramco Laboratories, Texas, USA) done in duplicate for each sample.

Molecular

DNA was prepared from 10 ml of blood samples collected in vacutainers with EDTA for detailed molecular studies⁽²⁰⁾. The alpha globin genotype was assessed by digestion of the DNA with restriction enzymes BAM HI, Bg I and 11, and by hybridisation with alpha and zeta probes, respectively⁽²¹⁾.

Vitamin E

Vitamin E (alpha tocopherol) concentration was measured by high performance liquid chromatography (HPLC) according to the method of De Leenher et al⁽²²⁾. All results were expressed in µg/ml and samples were done in duplicate.

Serum malonyldialdehyde (MDA)

Serum MDA was measured fluorometrically as thiobarbituric

reactive materials according to Yagi⁽²³⁾. All results were expressed as nmol/gm protein and samples were done in duplicate.

Red cell creatine

The red creatine was estimated by the diacetyl alpha naphthol reaction with each blood sample done in duplicate⁽²⁴⁾.

⁵¹Cr - survival of erythrocytes

The red cell survival was done using Chromium radiolabelled red cells as described in detail by Dacie and Lewis⁽¹⁸⁾.

Oral vitamin E therapy

Patients with reduced vitamin E (less than 1.5 µgm/ml) were given an oral dose of natural vitamin E d-alpha tocopherol 400 i.u. daily for two months. A repeat study of the serum vitamin E and MDA were done in the last week of therapy.

Statistical Analysis

To compare the features between the deletional and non-deletional types of Hb H disease, non parametric analysis used the Wilcoxon rank-sum test. The Spearman's rank correlation coefficient was used to evaluate the relationship between the red cell survival to the levels of MDA, Hb H, and vitamin E. All data were expressed as means and SDs. Statistical significance was assessed at the $p < 0.05$ level.

RESULTS

Haematological, serum ferritin and serum bilirubin data are described in Table I. There were no significant differences in the haemoglobins, red blood cell counts (RBC) and mean cell haemoglobin concentration (MCHC) of the patients with the deletional and non-deletional types of Hb H. Significant differences were seen in the mean cell volume (MCV), Hb H and serum bilirubin levels. Patients with the non-deletional type of Hb H have significantly higher serum bilirubin levels and higher MCV (Table I). The mean levels of Hb H in the non-deletional and deletional types were 18.7% and 4.8% respectively (Table II).

Serum Ferritin

There was no significant difference for the serum ferritin (SF) of the grouped data (Table I) in patients with both forms of Hb H. Serum ferritin levels were all < 600 µg/L. Six (75%) of the patients had serum ferritin levels within the normal range of the control group. The highest serum ferritin level was 500 µg/L which was seen in a female patient with the non-deletional type of Hb H disease, who had in addition hereditary ovalocytosis (Table II).

Lipid-peroxidation/vitamin E

Serum MDA levels were within the normal range in patients with Hb H disease studied though higher levels of MDA were seen in the patients with the non-deletional type of Hb H disease when compared with the deletional type ($p < 0.01$). The vitamin E levels were within the normal range with the deletional type (α^+/α^0) but markedly reduced with the Hb H disease of the non deletional type (HbCS / α^0). Following vitamin E therapy all four patients in the non-deletional group showed raised serum vitamin E levels, which were two to three times that of normal values (Table III).

Red cell survival/red cell age

In this study patients with Hb H disease had reduced red cell survival and raised red cell creatine levels: a feature compatible with a young population of red cells in the circulation. A significant difference was found in the red cell survival and red cell creatine in the HbCS/ α^0 group when compared to the α^+/α^0 group (Table I); the red cell survival was significantly

reduced but the red cell creatine was significantly raised in the former group. The red cell survival showed a negative correlation to MDA ($p = 0.01$, $r_s = -0.73$) and to Hb H ($p = 0.01$, $r_s = -0.66$) but a positive correlation to vitamin E levels ($p = 0.01$, $r_s = 0.68$).

DISCUSSION

Severe iron overload has been recently reported in two Chinese patients with Hb H disease in Taiwan⁽²⁵⁾. In 90% of Italian patients less than 45 years of age with Hb H disease, the serum ferritin levels were normal⁽²⁶⁾ and in Thailand studies have suggested that iron overload is not an important feature⁽²⁷⁾. In this present study, our results indicate that the mean serum ferritin levels in both types of Hb H (deletional and non deletional) were higher than that in the normal control groups and beta thalassaemia carriers⁽²⁸⁾. However severe iron overload was not a frequent finding in Malaysian Hb H patients, with no patient having serum ferritin levels equivalent to that seen in homozygous beta-thalassaemia with associated transfusion siderosis. Excessive alcohol consumption, a feature which was not present in our patients has been described to exacerbate iron absorption in Hb H patients⁽⁷⁾. Adult female patients because of regular monthly loss of iron through menstruation and pregnancy might be expected to have lower serum ferritin levels. Studies of patients with Hb H disease described progressive iron overloading with increasing age in males with the non-deletional type of Hb H disease, though the serum ferritin levels were all < 500 µg/L⁽³⁾. In this same study in the deletional type of Hb H disease there was a negative correlation of serum ferritin with age in the females and a weak positive correlation in the males. In the present study, the highest serum ferritin level was seen in a female patient who had both the non-deletional type of Hb H disease and hereditary ovalocytosis (Table III). Further studies are needed to study the synergistic effect of hereditary ovalocytosis in inducing iron overload in patients with Hb H.

The red cell survival as indicated by the ⁵¹Cr red cell survival studies in Hb H disease was decreased when compared with the normal control groups (Table I). This was more significant with the genotype HbCS / α^0 where the moderate reduction in the red cell survival was associated with high levels of red cell creatine, a feature in keeping with the presence of a population of young rather than mature red blood cells. The ⁵¹Cr red cell survival also showed a negative correlation with the levels of MDA and Hb H but a positive correlation to the level of vitamin E free radicals can involve many cellular components of the thalassaemic red blood cells (lipids, proteins, and nucleic acids). The sources of activated oxygen are multifactorial and may result from the low concentration of normal haemoglobin in the cells, high intracellular content of non haemoglobin iron, and the presence of excess unpaired globin chains⁽²⁹⁾. Haemoglobin has been described to act as a protective buffer for the red cell membrane^(9,29). The reduced haemoglobin levels and the presence of hypochromia in the thalassaemic red cells of Hb H may facilitate the oxidation of the red cell membrane components. In this study the levels of serum malonyldialdehyde (MDA), a secondary product of lipid peroxidation were within the normal range but significantly higher levels of MDA were seen in the patients with non-deletional type of Hb H disease. Malonyldialdehyde induced membrane rigidity has been associated with a shortened ⁵¹Cr red cell survival, which suggests a biochemical basis for the decreased survival of erythrocytes undergoing oxidative damage of the membrane⁽³⁰⁾. Excess iron is known to induce peroxidation of various red blood cell membrane components⁽³¹⁾. However, there was no significant difference in the serum ferritin for both genotypes of Hb H in this study.

Table III - Outcome of Vitamin E treatment (n=8)

Mean + 1 SD	Prior to treatment		Following treatment	
	Deletional	Non-deletional	Deletional	Non-deletional
MDA 'nmol/gm protein	3.3 ± 0.4	5.7 ± 0.8	3.2 ± 0.3	5.5 ± 0.6
Vitamin E µg/ml	8.0 ± 3.8	1.0 ± 0.40	9 ± 1.4	4.9 ± 0.3

The red blood cells of patients with Hb H disease are known to contain excess beta globin chains which are unstable and polymerise to form beta inclusions bodies. Considerable evidence has recently been reported to indicate oxidative haemoglobin denaturation plays an important role in red blood cell survival⁽³²⁾. The autooxidation of these chains releases toxic free radicals at a greater rate than normal haemoglobin which then reacts with cellular constituents especially haemoglobin and the red cell membrane⁽¹⁴⁾. The globin chain synthetic ratios in both these genotypes of Hb H are known to be similar⁽³³⁾. The differences in the severity and the pattern of oxidative damage may be related to the type and quantity of the precipitated globin chains⁽³⁴⁾. In addition globin chains have been found to interact and disrupt the red cell membrane, damaging the cytoskeleton. Acute infections which are common in patients with HbCS / α^0 may induce the formation of erythrocyte inclusions⁽²⁹⁾. In our study patients with HbCS / α^0 have high levels of Hb H and this group has been shown to have greater number of cells containing inclusion bodies⁽³²⁾. Red blood cells of patients with Hb H disease containing these inclusions have been described to have decreased deformability and hence would be trapped in the splenic sinuses^(35,36). This latter feature is consistent with the negative correlation of the red cell survival to the level of Hb H ($\alpha=0.01$, $r_s=-0.66$).

The final defence against oxidation is the presence of antioxidants in the red cell membrane, of which the most important is vitamin E^(12,15). The serum vitamin E was significantly lower in the genotype HbCS / α^0 (Table I) when compared with those from α^+ / α^0 Hb H disease. Vitamin E therapy in patients with Hb H with low levels of vitamin E will most likely fail as vitamin E most probably can correct the peroxidative damage to the membrane lipids, which appears to be a limited feature in Hb H disease, but not the other membrane components⁽¹²⁾.

We conclude that the clinical severity of these two genotypes are not linked to differences in their iron status. The present observation suggest that the shorter red cell survival makes the disease more severe in HbCS / α^0 when compared to α^+ / α^0 . Premature red cell destruction in the former could be explained by the higher amount of Hb H that precipitated as inclusions in the red blood cell. Red cell damage secondary to lipid peroxidation appears to play a minor role in the survival of Hb H red blood cells. Hence, it would be prudent to prevent oxidative stress in the genotype HbCS / α^0 by selective use of drugs and by early treatment of infections so as to prevent precipitation of excess globin chains.

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