ORIGINAL ARTICLES

Chelation Therapy with Desferrioxamine does not Normalize Ferritin Level but Attenuates Oxidative Damage and Improves Total Antioxidant Level in Malaysian Chinese β-thalassaemia Major Patients

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ABSTRACT

Beta-thalassaemia major causes severe anaemia and patients with it may be transfusion-dependent for life. Regular blood transfusions cause iron-overload that leads to oxidative damage which can hasten mortality. The objective of this research was to study the oxidant-antioxidant indices in β-thalassaemia major patients at the University of Malaya Medical Centre (UMMC) who were on desferrioxamine-chelation or without chelation therapy. Blood was collected from 39 Chinese patients and 20 controls. Plasma and peripheral blood mononuclear cell lysates (PBMC) were extracted and biochemical tests to evaluate oxidative stress were performed. Oxidative stress was evident in these patients as advanced oxidized protein products (AOPP) and lipid hydroperoxides were elevated, whereas glutathione peroxidase activity and the ferric reducing antioxidant power (FRAP) were reduced. The catalase activity in the patients' PBMC was elevated, possibly as a compensatory mechanism for the reduced glutathione peroxidase activity in both red blood cells and PBMC. The lower FRAP and higher AOPP levels in the non-chelated patients compared with the chelated patients were indicative of a lower oxidative stress level in the chelated patients. The ferritin levels in the chelated and non-chelated patients were high and the mean levels of liver enzyme activities in the majority of patients were elevated regardless of chelation therapy. In conclusion, this study indicates that desferrioxamine chelation therapy does not normalize ferritin level but attenuates oxidative damage and improves total antioxidant level in Malaysian Chinese β-thalassaemia major patients.

Keywords: Beta-thalassaemia major, desferrioxamine, Malaysian Chinese, non-chelated, oxidative stress

La Terapia de Quelación con Deferoxamina no Normaliza los Niveles de Ferritinaa pero Atenúa el daño Oxidativo y Mejora el Nivel Antioxidante Total en los Pacientes Sinomalayos que Padecen de Talasemia ß

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RESUMEN

La beta-talasemia mayor causa anemia severa, y los pacientes con este padecimiento pueden hacerse dependientes de las transfusiones de sangre por el resto de sus vidas. Las transfusiones regulares de sangre dan lugar a una sobrecarga de hierro que conduce al daño oxidativo, el cual a su vez puede acelerar la mortalidad. El objetivo de esta investigación fue estudiar las tasas de oxidantes-antioxidantes en pacientes de beta-talasemia mayor en el Centro Médico de la Universidad de Malaya, tanto aquellos bajo tratamiento de quelación con deferoxamina, como aquellos sin terapia de quelación alguna. Se recogieron muestras de sangre de 39 pacientes chinos y 20 controles. Se extrajeron plasma

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y lisados de células mononucleares periféricas (CMSP), y se realizaron pruebas bioquímicas para evaluar el estrés oxidativo. El estrés oxidativo era evidente en estos pacientes en forma de productos avanzados de oxidación de proteínas (PAOP), y los hidroperóxidos de lípidos eran elevados, en tanto que la actividad de glutatión peroxidasa y el poder reductor férrico/antioxidante (FRAP) era reducida. La actividad de la catalasa en los pacientes de CMSP era elevada, posiblemente como un mecanismo compensatorio frente a la actividad de glutatión peroxidasa reducida tanto en los glóbulos rojos como en las CMSP. Los niveles más bajos de FRAP y los más altos de PAOP en los pacientes no quelados en comparación con los pacientes quelados, indicaban un bajo nivel de estrés oxidativo en los pacientes quelados. Los niveles de ferritina tanto en los pacientes quelados como en los no quelados, eran altos, y los niveles promedio de actividades enzimáticas del hígado fueron elevados en la mayoría de los pacientes, independientemente de la terapia de quelación. En conclusión, este estudio indica que la terapia de quelación con deferoxamina no normaliza el nivel de ferritina, pero en cambio atenúa el daño oxidativo, y mejora el nivel antioxidante total en los pacientes sinomalayos afectados por la beta-talasemia mayor.

Keywords: Beta-thalassaemia major, desferrioxamine, Malaysian Chinese, non-chelated, oxidative stress

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INTRODUCTION

Beta-thalassaemia is a common single gene disorder which causes a decrease or absence in the production of β-globin chains, and the excess α-globin chains form insoluble tetramers that precipitate within the red blood cells (RBC) leading to their increased fragility (1). Beta-thalassaemia major patients present with life-long transfusion-dependent haemolytic anaemia. Regular blood transfusions and intensive iron-chelation therapies are the mainstay of treatment and although successful bone marrow transplantation can offer a permanent cure, the HLA-matched sibling donor is a limiting factor.

Beta-thalassaemia is a public health problem in Malaysia where the carrier rate is about 4.5% (2). Children with β-thalassaemia major become iron-overloaded even with sporadic blood transfusions. Accumulation of excess iron in major organs leads to increase morbidity and mortality.

Physiologically, iron exists in the form of ferrous (Fe^{2+}) and ferric (Fe^{3+}), and it is kept thermodynamically stable through binding to plasma transferrin. During an iron-overload state, non-transferrin bound iron catalyses free radical (superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide) production through the Fenton and Haber-Weiss reactions. The free radicals induce progressive damage *via* oxidative breakdown of a wide range of macromolecules, including DNA, proteins and lipids (3). The endogenous antioxidants such as superoxide dismutase, glutathione peroxidase and catalase, as well as the non-enzymic antioxidants (uric acid, bilirubin and albumin) provide protection against oxidative insults (4). However, it is not totally effective unless the source of oxidant, free iron, is removed by intensive iron-chelation therapy (5). Desferrioxamine is regarded as the mainstay of iron-chelation therapy because it is believed to remove effectively excess iron from the system through increased iron excretion, causing a negative iron balance. It has been shown to be paramount in extending the life span in β-thalassaemia major patients (5).

The influence of desferrioxamine administration on the oxidative-antioxidant parameters in the blood of Malaysian Chinese β-thalassaemia major patients was determined in this study. Desferrioxamine has been known to remove excess iron but the question of how it confers protection *in vivo* is unknown as most parameters reported were based on measurements carried out in the *in vitro* system. In this study, the differences between oxidative damage and antioxidants (both enzymic and non-enzymic antioxidants) in blood components – plasma, red blood cells (RBC) and peripheral blood mononuclear cells (PBMC) of desferrioxamine-chelated and non-chelated patients were determined. The results obtained will contribute greatly to information on the efficacy of iron-chelation therapy in reducing oxidative stress (oxidative damage) in β-thalassaemia major patients, and in turn allow better management of such patients.

SUBJECTS AND METHODS

A total of 39 β-thalassaemia major Chinese patients were recruited from the Paediatric Day Care of the University of Malaya Medical Centre (UMMC). Their disease status was confirmed by molecular characterisation of the β-globin gene complex where the β-globin gene mutations were identified. (6). Ethics approval was obtained from the Medical Ethics Committee of UMMC in accordance with the Declaration of Helsinki and informed consent was obtained from the patients or their guardians. All patients received monthly transfusions in order to maintain haemoglobin levels above 10 g/dL. In this study group, 33 patients received regular chelation therapy with desferrioxamine (chelated group) and six patients were not on any chelation therapy (non-chelated group). Serum ferritin and iron in the desferrioxamine-

chelated group were high, suggesting inadequate iron depletion. The control group comprised 20 healthy individuals aged between 8−20 years old. Blood (4 ml) was drawn from patients into EDTA tubes just before blood transfusions were carried out.

Plasma was separated *via* centrifugation at 3500 x g for 10 minutes and aliquots of plasma were stored at –80°C immediately after collection. The remaining RBC (2 ml of packed volume) were washed twice with five volumes of phosphate-buffered saline (PBS) pH 7.4 and were haemolysed by suspension in ice-cold reverse osmosis water. The haemolysate was centrifuged at 3500 x g for 30 minutes and the resulting supernatant was stored in 500 µl aliquots at −80°C until enzyme assays were performed. Assays were carried out in quadruplicates using samples that were only thawed once.

Peripheral Blood Mononuclear Cell (PBMC) lysates were isolated using Histopaque gradient centrifugation (7). Prediluted blood (1 part whole blood: 1 part PBS, pH 7.4) was carefully layered onto Histopaque®-1077 and centrifuged at 1800 x g for 30 minutes. The PBMC-containing layer was pipetted into a clean tube, washed twice in PBS and centrifuged at low speed to remove the platelets. The PBMC was resuspended to yield a final concentration of 10^6 cells/ml and were lysed using an ultrasonic homogenizer (UP50H Dr Hielscher, GmbH) on ice and was centrifuged at 3500 x g for 30 minutes. The resulting supernatant was used for the various enzyme analyses.

All the methods used were according to known established protocols. Lipid hydroperoxide (LH) concentration was measured using ferrous ion oxidation xylenol orange (FOX-1) assay (8). The absorbance difference at 560 nm between the samples containing butylated hydroxytoluene, or triphenylphosphine and butylated hydroxytoluene in the presence of FOX-1 reagent were calculated and the hydroperoxide concentration was quantified using *t*-butyl hydroperoxide as the standard.

Advanced oxidized protein product (AOPP) concentration was determined in plasma based on spectrophotometric detection in an adapted microassay system (9) using chloramine T as a standard. The results were expressed as chloramine-T equivalents.

Glutathione peroxidase (GPx) activity in the haemolysate and PBMC lysates were based on coupled reaction that oxidizes glutathione and reduces H_2O_2 and the absorbance changes at 340 nm were measured using a kinetic mode for 30 seconds with a microplate reader (10). Catalase (CAT) activity was measured based on the decomposition of H_2O_2 at 240 nm (11).

Enzymic activity (GPx and CAT) measured in red blood cell and PBMC lysates were expressed as µmol/g Hb and µmol/g protein respectively.

Xanthine oxidase (XO) activity determination was based on the oxidation of the chromogen ABTS [2, 2′-azinodi (3-ethylbenzthiazoline-6-sulphonate)] which absorbs at 410 nm, through coupled reactions catalysed by uricase and peroxidase (12). The absorbance change was measured at 410 nm and the XO activity was calculated from the standard curve obtained using a commercial purified XO (SIGMA, USA).

The total non-enzymic antioxidant was estimated in plasma using the Ferric Reducing Antioxidant Power (FRAP) microassay based on the reduction of a ferric 2, 4, 6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form [Fe^{2+}-TPTZ] (13). Plasma reducing capacity was calculated from the standard curve obtained using ferrous sulfate heptahydrate.

Protein concentration was determined using the standard Bradford method (14) with bovine serum albumin as a standard.

Haemoglobin was estimated using the colorimetric cyanmethaemoglobin method (15). The concentration of haemoglobin (g/L) was calculated using the extinction coefficient (E) = 44 000$M^{-1}cm^{-1}$ (absorbance measured at 540 nm).

The results were expressed as a mean ± SD (standard deviation). Statistical analysis was performed using one-way analysis of variance. When the analyses indicated statistical differences, post-hoc Tukey's honestly significant difference test was performed to determine significant differences between the variables.

RESULTS

Demographic and biochemical data is depicted in Table 1. The serum ferritin levels in the Malaysian Chinese β-thalassaemia major patients who were on desferrioxamine chelation therapy were approximately 10-fold higher than the control range. This level was comparable to those patients who were not on any chelation therapy. The levels of iron, bilirubin and liver enzymes in the chelated group were not significantly different from the control subjects although the mean values were generally higher. Approximately 60% of the chelated patients had iron, bilirubin and liver enzyme levels that were within the control range. The liver enzyme levels in both chelated and non-chelated patients were comparable. Chelation therapy with desferrioxamine was able to reduce the level of serum iron in the β-thalassaemia major patients. The non-chelated patients had higher serum iron and bilirubin levels than the control range. However, only six non-chelated thalassaemic patients could be recruited in this study. The reason for this is that all β-thalassaemia major patients in UMMC are encouraged to undergo chelation therapy to prevent iron overload.

The oxidative indices levels in plasma, haemolysate and PBMC lysate from β-thalassaemia major patients and controls are summarised in Table 2. Lipid hydroperoxide concentrations in both patient groups showed a 4-fold increase compared to the control subjects, while the level of oxidised plasma protein (measured as increased AOPP concentration) in the chelated and non-chelated thalassaemic

Table 1: Demographic and Biochemical data

Characteristic	β-Thalassaemia major patients (n = 39)		Control
	Chelated (n = 33)	Non-chelated (n = 6)	(n = 20)
Male	15	2	8
Female	18	4	12
Age	10 ± 5	8 ± 2	15 ± 6
Ferritin level (μg/L)	*4762 ± 2200	*4835 ± 2457	123 ± 62
Serum Iron (μmol/L)	33 ±11	*41 ± 10	17 ± 6
Total Bilirubin (μmol/L)	26 ± 16	*44 ± 22	8 ± 3
Alkaline phosphatase	150 ± 56	151 ± 60	84 ± 22
ALT (IU/L)	92 ± 59	85 ± 23	42 ± 9
AST (IU/L)	56 ± 37	63 ± 31	24 ± 8

The levels of ferritin, alkaline phosphatase, alanine aminotransferease (ALT), and AST in control subjects was not determined thus the known normal range has been provided for comparison. *$p < 0.05$ compared with control subjects. The comparison between chelated and non-chelated patients did not show a significant difference, ($p > 0.05$) based on Student's t-test as well as one way ANOVA.

Table 2: Oxidant–antioxidant indices in Chinese β-thalassemia major patients with or without chelation therapy

Characteristics	Control subjects	β-Thalassaemia major patients (n = 39)	
	(n = 20)	Chelated (n = 33)	Non-chelated (n = 6)
FRAP (μmol/l)	845.0 ± 192.7	874.2 ± 188.4	670.4 ± 149.5*#
AOPP (μmol/l)	80.1 ± 28.4	131.5 ± 104.5*	334.5 ± 281.4*#
Lipid hydroperoxide (μmol/l)	1.00 ± 0.82	4.02 ± 3.22*	3.94 ± 1.38*
Cat-RBC (U/g Hb)	195.0 ± 42.3	212.8 ± 136.6	160.9 ± 56.8
Cat-PBMC (U/g protein)	38.5 ± 16.0	72.0 ± 55.1*	83.1 ± 76.4*
GPx-RBC (U/g Hb)	101.8 ± 24.2	46.0 ± 43.1*	49.9 ± 53.3*
GPx-PBMC (U/g protein)	36.5 ± 32.2	15.0 ± 19.9*	27.5 ± 33.1
XO-PBMC (U/l)	1.33 ± 1.10	1.36 ± 1.02	1.722 ± 1.089

Differences in means based on ethnic groups were analysed using ANOVA one-way with subsequent post hoc analysis using Tukey's honestly significant difference. *$p < 0.05$ compared with control subjects; # $p < 0.05$ compared between chelated and non-chelated β-thalassaemia major patients.

patients were increased by 64.2% and 317.6% respectively, compared to the controls. The total non-enzymic antioxidant concentration in the non-chelated group was significantly lower than the chelated group as well as in the controls. Glutathione peroxidase activity in red blood cells and catalase activity in PBMC were significantly reduced in both chelated and non-chelated patients when compared with the control levels ($p < 0.05$). However, the catalase activity in the red blood cells did not show any significant changes in both groups. The GPx activity in PBMC of chelated patients was significantly lower than the control level whereas in non-chelated patients, the level was comparable to the control. Xanthine oxidase activities in both patient groups were comparable to the control group.

DISCUSSION

Beta-thalassaemia major individuals are under enhanced oxidative stress, where oxidant-antioxidant imbalance is in favour of the oxidant. β-thalassaemia major patients in India were reported to have higher levels of malondialdehyde production compared to normal subjects (16). Similarly, augmented plasmatic thiobarbituric acid reactive substance concentrations were reported in Tunisian patients, but with decreased plasmatic peroxyl radical trapping potential, vitamin E and zinc concentrations (17).

Lipid peroxidation in both desferrioxamine-chelated and non-chelated β-thalassaemia major patients was enhanced as indicated by their high LH level. Advanced oxidized protein product that mainly consists of dityrosines, disulfide bridges and carbonyl groups (18) is a more sensitive oxidative marker than lipid peroxidation, in view of the fact that there was a significant difference between the chelated and the non-chelated β-thlassaemia major patients. Lower oxidized plasma protein levels in chelated patients reflect the suppression of the oxidation of proteins by desferrioxamine, the *in vitro* study of Gebicki and Gebicki 1999 supports this finding as the extent of protein cross-linking on peroxidized bovine serum albumin was reduced in the presence of desferrioxamine in their study (19). This protective effect of desferrioxamine is also partly influenced by its chain-breaking antioxidant action as it donates an electron or hydrogen atom from its hydroxamate centre to suppress the oxidative

damage produced by the reactive species in the Fenton reaction [in Fe^{2+}/H_2O_2 system] (20). The expected higher free radicals in the non-chelated patients depleted the total non-enzymic antioxidants of the plasma, as indicated by the significantly lowered FRAP value when compared to the chelated patients and normal subjects.

The present study showed that GPx activity in the β-thalassaemia major patients was suppressed and this could be due to the depletion of reduced glutathione (GSH), which is a potent antioxidant by itself (21). It is also possible that GSH becomes dysfunctional as a result of protein glycosylation, or due to interaction with protein cysteinyl thiols during bouts of oxidative stress (22). Since both CAT and GPx decompose hydrogen peroxide (H_2O_2) into water and oxygen, the suppressed GPx activity can probably be compensated with the stimulation of CAT activity. It was also observed that in normal and β-thalassaemia major patients, changes in antioxidant activity, particularly CAT in the red blood cells are less apparent than in the PBMCs. This could be due to the higher concentration of antioxidants in red blood cells, which ameliorates the actual condition that is reflected in the PBMCs. This reduction in GPx (enzymic antioxidant status) in chelated patients was possibly compensated for by FRAP levels (indicates total non-enzymic antioxidant status) which were comparable to normal controls. However, the significantly reduced FRAP levels in the non-chelated patients, accompanied by the reduced GPx in both RBC and PBMC indicate that the patients are under greater oxidative stress compared to the chelated patients.

The exact role of XO in the pathophysiology of β-thalassaemia major patients has not been elucidated as yet. However, several studies have implicated XO in various disease states such as diabetes mellitus (22), cardiovascular disease (23) and acute lung injury (24). The mechanism of action of XO is *via* its ability to generate superoxide anions (25). In conditions of iron-overload, these superoxide anions are converted into highly reactive hydroxyl radicals in the presence of iron by the Haber-Weiss and Fenton reactions (20). It is possible that superoxide anions and hydroxyl radicals are not adequately detoxified due to suppressed GPx activity, and this thus favours the oxidants in the formation of hydroperoxides (25).

In chronic renal failure patients, a suppressed GPx activity has been shown to co-exist with increased AOPP level as well as monocyte activation markers such as neopterin, interleukin (IL)-1R antagonist, tumour necrosis factor (TNF)-α and TNF soluble receptors (TNF-sR55 and TNF-sR75) (18). The levels of inflammatory cytokines such as TNF-α and IL-1β have been shown to be elevated in β-thalassaemia major patients (26). Superoxide anion has been shown to activate the key inflammatory signal, NF*kappa* B that has been implicated in most disease states (27). Thus, the mechanism of the activation of inflammatory signals which play key roles in the development of complications in β-thalassaemia major patients, *via* superoxide generation, requires further investigation.

Desferrioxamine is regarded as the mainstay of iron-chelation therapy because it is believed to remove excess iron effectively from the system and this is reflected by reduced ferritin levels (5). However, the finding of the present study showed otherwise. One possible cause of this contradictory finding is that the majority of patients only started the chelation therapy much later instead of the recommended period (usually after the tenth to twelfth transfusion by which time the ferritin level had reached a level above 1000 μg/L). The current chelation-therapy history was based on patients' medical reports and questionnares filled-up by the patients or their guardians. One should not rule out the possibility that the patients could have been non-compliant in their routine chelation therapy and did not reveal the truth to the doctor. A limitation of this study was also the relatively small sample size as only a small number of patients consented to participation in this study. Therefore, one should not generalize these findings to the broader community based on this study alone.

Nevertheless, this study provides some evidence that iron-chelation therapy with dexferioxamine in the Malaysian Chinese β-thalassaemia major patients in UMMC is not effective in reducing the ferritin level, but is able to reduce serum iron, maintain non-enzymic antioxidant status and reduce oxidative damage in these patients.

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