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Study of oxidative stress and endogenous antioxidant defense in patients with beta thalassemia major

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Abstract

Background: Beta-thalassemia major is an autosomal recessive disease causing severe and hemolytic anemia, which begins about 2-6 months after birth. Iron overload, which arises from recurrent transfusion and ineffective erythropoiesis, can enhance oxidative stress in thalassemic patients. The aim of present study was to assess the oxidative stress and endogenous antioxidants in beta thalassemia major patients.
Method: The study comprises of 49 diagnosed beta thalassemia major patients receiving regular blood transfusion and chelation treatment and 49 age and sex matched normal healthy controls. 5mL blood was collected from patients and controls in plain bulb. The serum was separated and analyzed as per procedure of biochemical parameters such as Iron, ferritin, uric acid, ceruloplasmin, bilirubin and serum malondialdehyde (MDA) in patients and compared with normal healthy controls.
Results: The MDA level increased in β thalassemia major patients (3.01±0.38) as compared to controls (1.68±0.2). Serum free iron, serum ferritin and ceruloplasmin concentration as well as total bilirubin level was high in study group. Uric Acid, an endogenous antioxidant was significantly increased while calculated value of transferrin was decreased in cases than controls.
Conclusion: We conclude that in patients with β thalassemia, the body generates and promotes endogenous antioxidants to defend the oxidative stress. For beneficial effects along with iron chelators, dietary supplementation of antioxidants should be given.
Keywords: Beta-thalassemia major; Oxidative stress; Endogenous Antioxidants; Malondialdehyde; Ferritin; Ceruloplasmin

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1. Introduction

β Thalassemia syndromes are the most common hereditary chronic hemolytic anemias due to impaired globin chain synthesis. The severity of the imbalance of the globin chains generates the different thalassemia phenotypes. Decreased or impaired β- globin biosynthesis results in the accumulation of unpaired α globin chains in erythroblasts, and this leads to ineffective erythropoiesis [1]. Recent studies have demonstrated that any imbalance between the α and β chains plays a crucial role in producing oxidative stress [2] and oxidative stress is an important mechanism in the progression of β thalassemia major [3]. Moreover, the repeated blood transfusion and chelation has been the mainstay of treatment in thalassemia major patients [4]. This severe iron overload leads to imbalance between antioxidant defense and generation of free radicals. A serum low molecular weight iron fraction, not bound to transferrin

which is known to generate oxygen free radicals and peroxidative tissue injury is detectable in these patients [5]. This process is characterized by metabolic hyperproduction of reactive oxygen species (ROS) and induced lipid peroxidation (LPO). Oxidative stress exceeds the capacity of the endogenous antioxidant defenses (content of antioxidants and activity of antioxidant enzymes). It activates diverse damaging processes in cells, including oxidation of intracellular and surface components of the red blood cells in β thalassemia major patients [6].

Malondialdehyde (MDA), a terminal compound of lipid peroxidation, is used widely as an index of oxidative status [7]. Urate protects erythrocyte membrane ghosts from peroxidation. Study shows the protection by urate against peroxidation of erythrocyte membrane ghosts induced by t-butylhydroperoxide. Urate and ascorbate synergistically were effective in preventing lipid peroxidation at concentrations considerably below those normally found in plasma i.e. first line of defense [8]. Ceruloplasmin (CP), an acute phase reactant protein that can convert ferrous iron to its less reactive ferric form facilitating binding to apotransferrin, has ferroxidase activity that is important to iron handling. Genetic absence of CP decreases iron export resulting in iron accumulation in many organs [9].

To prevent mortality and morbidity of β thalassemia major patients treatment consists of repeated blood transfusions, a complication of which is iron overload. Early introduction of chelating agents control and combat iron overload, inhibit ROS- generation by iron and regulate LPO-processes leading to improved life expectancy. Direct measurement of ROS is not reliable due to short half-life of ROS and none of the methods are sensitive enough for quantification of ROS [10]. The indirect methods for estimation of ROS or oxidative stress include estimation of oxidized end products of lipid thiobarbituric acid reacting substances (TBARS) i.e. MDA and status of endogenous antioxidants [11]. The aim of this work was to study the level of oxidative stress as a central pathological process in the blood of β thalassemia major patients with transfusional iron overload and chelation therapy by registering concentration of endogenous antioxidants such as ceruloplasmin, transferring uric acid, bilirubin and pro oxidants like iron and ferritin.

2. Method

After obtaining Institutional Ethical Committee approval and written informed consent from the patients/relatives, this cross sectional, analytical study was conducted in total 98 patients in the Department of Biochemistry in a Tertiary Care Centre during the study period of 1 year from September 2017 to August 2018. They were divided in to two equal groups. Study group: 49 patients of either sex, age 5 to 25 years, diagnosed with beta thalassemia major with blood transfusion and on iron chelating agents. Control group: 49 non thalassemic, normal healthy age and sex matched controls. Patients having Beta Thalassemia minor, thalassemia intermedia, sickle beta thalassemia, sickle cell anemia, patients with any other hematological disorders like leukemia, lymphoma, hemophilia, aplastic anaemia, etc and also with splenectomy were excluded from the study.

A detailed history and examination as well as results of the investigations were documented in a predesigned proforma. 5mL blood was collected from patients and controls in plain bulb. All these samples were collected from patients attending the Thalassemia OPD at a tertiary care centre. The blood in plain bulbs were kept for 15 minutes to allow clotting and to prevent hemolysis and then subjected to centrifugation at 3000 to 4000 rpm for 15 to 20 minutes and serum was collected. The serum samples were analyzed as per procedure of biochemical parameters. The reports thus obtained after estimating the biochemical parameters from serum such as Iron, ferritin, uric acid, ceruloplasmin, bilirubin and serum MDA in patients and compared with normal healthy controls.

Table 1: Method used for analysis of biochemical parameters

SN	Parameters	Method used
1	Iron	Ferrozine Method [12]
2	Ferritin	Immunoturbidimetric Method [13]
3	Uric acid	Uricase Method [14]
4	Ceruloplasmin	Ravin and Henry etal [15]
5	MDA	Modified Sadasivadu *et al* [16]
6	Total bilirubin	Vandate Oxidation [17]

2.1 Statistical analysis

The data was entered in Microsoft excel. Mean, standard deviation was calculated to estimate the significance. Student't' test was analyzed using Open epi info version 2.3, year 2009. P values less than 0.05 were considered as statistically significant.

3. Result

A total of 98 patients were included in the study and divided into two equal groups. There was no statistically significant difference between two groups in regards to demographic profile of the patients with p value greater than 0.05 as shown in table 2.

Table 3 show the comparison of biochemical parameters between two groups and all the values (Serum free iron, ferritin, transferring, uric acid, ceruloplasmin, MDA and total bilirubin level) were highly significant with $p < 0.001$ after applying Student t test.

Level of lipid peroxidation expressed in serum MDA levels which were increased in β thalassemia major patients as compared to controls as depicted in figure 1.

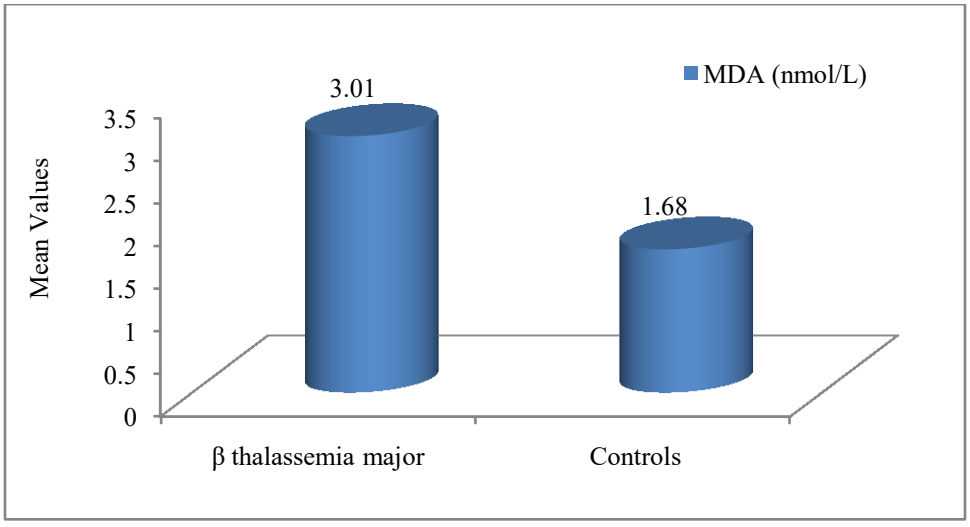
Table 2: Demographic profile of the patients

Demographic data		Cases	Controls	P value
Age (Years)	5 to 10	34 (69.38%)	41 (83.67%)	0.594
	11 to 15	12 (24.48%)	06 (12.24%)	
	16 to 20	03 (6.12%)	02 (4.08%)	
	Mean age	8.72 ± 4.30	7.52 ± 3.98	
Sex	Male	24 (48.97%)	22 (44.89%)	0.3428
	Female	25 (51.02%)	27 (55.10%)	

Table 3: Comparison of serum levels of biochemical parameters between two groups

Biochemical Parameters	β thalassemia major	Normal controls	P value
Iron (µg/dl)	185.65 ± 58.75	79.12 ± 28.73	<0.001
Ferritin (ng/ml)	3431.65 ± 1567.7	128.57 ± 26.8	<0.001
Transferrin (mg/dl)	179.96 ± 17.36	253.29 ±28.42	<0.001
Uric acid (mg/dl)	4.83 ± 1.37	3.11 ± 1.23	<0.001
Ceruloplasmin (µmol/L)	32.17 ± 15.85	27.04 ± 7.29	<0.001
MDA (nmol/L)	3.01 ± 0.38	1.68 ± 0.2	<0.001
Total bilirubin (mg/dl)	1.51 ± 0.58	0.43 ± 0.11	<0.001

Figure 1: Serum MDA levels in β thalassemia cases and normal healthy controls

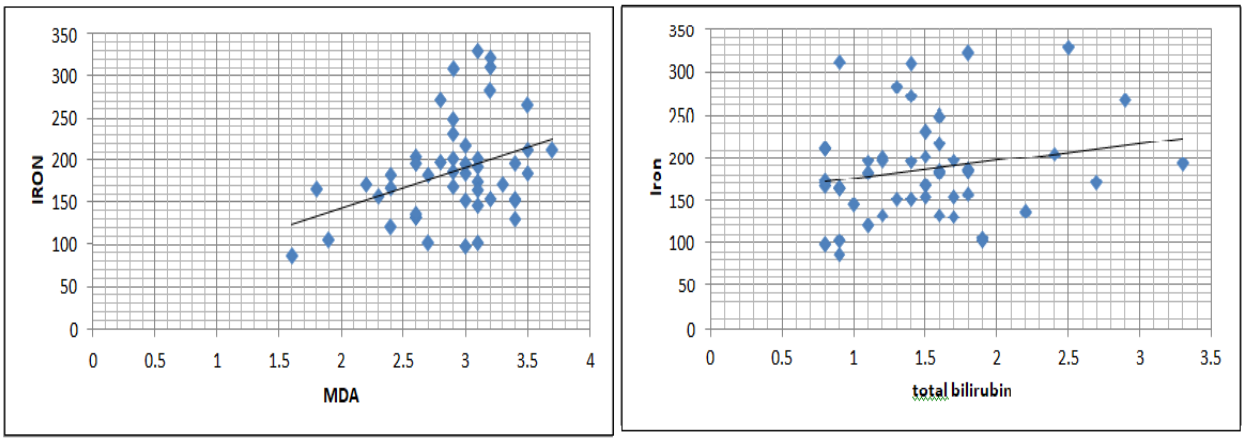


We found significant correlation of ferritin with Iron and MDA. Ferritin has a positive correlation with MDA, Iron, Uric Acid, and Total Bilirubin (Table 4). It was found that the ferritin has a negative correlation with Ceruloplasmin and Transferrin. The degree of correlation with ceruloplasmin and transferrin WAS not significant.

Table 4: Pearson Correlation: Serum Ferritin with other variables in cases

Variables	Pearson Correlation factor	P value (two tailed)
Ferritin with Iron	0.631	< 0.001**
Ferritin with MDA	0.0.488	<0.001**
Ferritin with Uric Acid	0.201	0.166
Ferritin with Transferrin	-0.119	0.415
Ferritin with Total Bilirubin	0.117	0.423
Ferritin with Cerulopasmin	-0.043	0.774
MDA with Iron	0.345	0.015*
Iron with Bilirubin	0.076	0.602

Figure 2: Correlation of iron with MDA and total bilirubin



4. Discussion

Iron induced oxidative stress is known to be one of the most important factors determining cell injury in thalassemic patients. Studies have confirmed the progression of oxidative stress in patients with β-thalassemia major, activation of free radical processes and lipid peroxidation. But our body tries to defend this oxidative stress via antioxidants [18]. There is limited literature available on endogenous antioxidant defense against oxidative stress. In view of present evidences, 49 β thalassemia major cases and 49 normal healthy controls were studied. In Thalassemia major, the mortality rate was found to be 1.5%. The 5, 10, and 20 year's survival rate among the studied patients was 80%, 50%, 20%, respectively [19]. So in current study maximum patients lie within 5 to 10 years of age as compared to patients in 16 to 20 years. The mean age in cases (8.72 ± 4.30) were compared to that of the controls (7.52 ± 3.98). Out of 49 cases 24 (48.97%) were males, 25 (51.02%) were females. In control group, 22(44.89%) were male and 27(55.10%) were females, thus both the groups were comparable in regards to demographic profile of the patients. Similar findings are reported by Nafady *et al* [20].

Malondialdehyde (MDA), the end product of lipid peroxidation is a well known marker of oxidative stress. There are increasing reports indicating imbalance between free radical generation and antioxidant defense mechanism in β thalassemia major patients. In the present study, MDA is increased from 1.68±0.2 to 3.01±0.38 in β thalassemia major when we compared with control group which is correlated with the previous studies [21, 22]. We got positive correlation of MDA with serum ferritin and serum iron which was statistically significant. Similarly other studies [21-23] exhibited positive correlation of MDA with ferritin.

Markers of free radical injury such as MDA and antioxidant enzyme SOD and NOx levels were significantly elevated in thalassemic children while mean GPx levels were decreased in patients compared to controls. All these markers significantly correlated with serum ferritin levels. It has been already known that oxidative stress is increased in patients with iron overload. We know that during the course of metabolism, superoxide anion is converted to H_2O_2 by ubiquitous enzyme superoxide dismutase. Normally H_2O_2 is converted to innocuous compounds by the action of catalase and peroxidase [24]. But if free iron is available, it reacts with H_2O_2 to form hydroxyl radicals. This is a result of mounted concentration of highly reactive Fe 2^+ ions which catalyze the Fenton and Haber weiss reaction and leads to generation of ROS. Moreover our body does not excrete iron in any form hence it remains in circulation; it is stored in the body with the help of ferritin in liver. Only in case of females iron loss is noted during menstruation. Our study results harmonises with the above fact as free iron was found to be increased in males and females inspite of daily iron chelation therapy. These results are supported by various studies [25, 26].

Ferritin is the intracellular protein responsible for the sequestration, storage and release of iron. Ferritin protects the cell against insoluble ferric oxide and oxyhydroxide formation, as well as against the production of oxygen radicals. In present study, serum ferritin levels were 3431.65±1567.7 in β thalassemia major and 128.57±26.8 in controls which was highly significant. The increase of ferritin creates ideal conditions for radical oxygen species formation to damage erythrocytes. The study by Ford *et al* suggests that in beta- thalassemia, the first target organ dysfunction of diminished antioxidant reserves is the liver, enhanced by an iron overload leading to an acute free radicals action [27]. Previous studies demonstrating similar results [28, 29]. Transferrin is an iron transporting protein. In current study calculated value of Serum trasferrin was estimated 180.38±17.44 mg/dl and 252.96±28.22mg/dl in cases and controls respectively. Transferrin level is lower than the normal controls. This result is controversial to the previous studies [30]. Evidence

is that transferrin being a principal (Lung Epithelial Lining Fluid) ELF component is responsible for AOA (anti oxidant activity). Although the present study found that ELF concentrations of both ceruloplasmin and transferrin inhibit lipid peroxidation, additional experiments suggest that transferrin was more important antioxidant protein in ELF i.e. 20 times higher to that of ceruloplasmin by Pacht *et al* [31].

In the present study transferrin came out to be lower than ceruloplasmin level. Also study showed decrease in transferrin level in cases than in controls. The probable reason for this may be estimation of transferrin based on the formula and not the direct analysis. Therefore calculated value of transferrin does not give clear status of transferrin in patients with β thalassemia major on iron chelation therapy. Ceruloplasmin, a chromoprotein in the plasma, transports 6 copper ions. It has ferroxidase activity i.e. it converts ferrous to ferric ion. Fe3+ binds to apoferritin, thus act as an antioxidant. In the present study serum level of ceruloplasmin was significantly (p=<0.001) increased. Several studies have demonstrated ceruloplasmin as an antioxidant and confirm antioxidant activity of ceruloplasmin [9, 32]. All these studies show increases in ceruloplasmin level and in the present study also it has increased significantly. The reason for rise in CP may be due to increased defense against oxidative stress.

Uric acid, the end product of purine metabolism also acts as an antioxidant, urate oxidation in a classical antioxidant scavenger reaction. Reduction of oxidized ascorbate by urate takes place. Fe3+ reduction to Fe2+ occurs by urate. In present study also uric acid was found to be elevated having significant p value of <0.001. It showed positive correlation with serum ferritin but was not significant. The present study shows elevation of uric acid level supporting body's defense to increased oxidative stress in beta thalassemia patients.

Bilirubin is considered to be a waste product but also act as antioxidant by oxidizing itself to biliverdin (BVR). BVR is an abundant and ubiquitous enzyme with a high turnover rate. Hence, endogenous BVR should suffice to reduce newly formed biliverdin back to bilirubin. The intrinsic amplification properties of enzymes could readily augment the antioxidant effects of bilirubin 10000-fold. Total bilirubin was increased in cases than the healthy controls. It correlates positively with ferritin and iron but was not significant. Cause for increase could be the oxidative stress that causes hemolysis of erythrocyte leading to increased hemoglobin catabolism and rise in bilirubin concentration. It might also be due to hepatic insult due to oxidative stress. Ziberna *et al* determined intracellular antioxidant activity by bilirubin. Biliverdin showed similar antioxidant properties as bilirubin. We infer from these observations that intra-endothelial bilirubin oscillates, and may thus be a dynamic factor of the endothelial function [33]. Our study on transfusion dependent β thalassemia major patients on iron chelator therapy gave evidences of increased free radical generation. Decrease in Total Antioxidant Defense could be the reason for stimulating endogenous antioxidants such as Uric acid, Ceruloplasmin and Total Bilirubin.

5. Conclusion

From results of present study, it can be concluded that the presence of lipid peroxidation by remarkable increase in serum MDA level suggests oxidative stress. Secondly it has positive correlation with Serum Iron and Ferritin level indicating iron overload is the prime cause of this pathophysiology. The imbalance between oxidative stress and antioxidant activity may be the cause of hemolysis of RBCs, leading to rise in bilirubin level and free iron. Again, if free iron remains in the body, it will contribute to free radical generation and the vicious cycle will continue.

The introduction of iron chelation is indispensable and vital for regularly transfused β thalassemia major. Inspite of daily chelation therapy, oxidative stress persists. Studies should be conducted on efficacies of various iron chelating agents to minimize the iron overload. Antioxidant namely uric acid, ceruloplasmin and total bilirubin levels have elevated with respect to the underlying oxidative stress. Thus, we conclude that in patients with β thalassemia, the body generates and promotes endogenous antioxidants to defend the oxidative stress. For beneficial effects along with iron chelators, dietary supplementation of antioxidants should be given.

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