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Biochemistry Section

A Study on the Biomarkers of Oxidative Stress: the Effects of Oral Therapeutic Supplementation on the Iron Concentration and the Product of Lipid Peroxidation in Beta Thalassemia Major

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ABSTRACT

Background: β - thalassemia major is genetic disorder which is caused by mutations in the HBB gene which is on chromosome 11. It is associated with a profound anaemia which is characterized by extreme pallor, jaundice or a failure to thrive, which is accompanied by poor feeding, irritability, decreased activity or an increased somnolence. Hepatosplenomegaly, an expanded bone marrow, siderosis, cardiomegaly, an impaired erythropoiesis, haemolysis in the peripheral circulation and the deposition of excess iron in the tissues are usually present.

It has been proposed that the iron overload in the patients with beta thalassaemia major is associated with enhanced free radical formation and defects in the antioxidant defense system. Excess iron facilitates the generation of free radicals, which is the main cause of the tissue damage in the biological system.

Aim: The aim of this study was to examine the impact of the iron overload in the patients with beta thalassaemia major on the markers of oxidative stress, which included Malondialdehyde (MDA), which was a breakdown product of lipid peroxidation, Erythrocytic Superoxide Dismutase (ESOD) and vitamin E.

Material and Methods: A total of 120 subjects were assessed, who included 60 beta thalassaemia major patients before and after they were supplemented with antioxidants for one month, and their status was compared with those of 60 age and sex matched healthy controls.

The levels of serum MDA was analyzed by the Kei Satoh method, serum iron was analyzed by the dipyridyl method, ESOD was analyzed by the Kajari Das method and the vitamin E concentration was measured by the Baker and Frank method.

Results: The serum levels of MDA, ESOD and iron were found to be significantly increased ($p<0.001$) and the serum vitamin E level was significantly decreased ($p< 0.001$) in the beta thalassaemia major patients as compared to those in the healthy controls.

After the supplementation of the antioxidants, we found significantly lower ($p<0.001$) mean values of serum MDA, iron and ESOD, while the activity of the serum level of vitamin E was significantly higher ($p<0.001$) as compared to those in the healthy controls.

Conclusion: Repeated blood transfusions result in excessive free iron concentration in the blood, which causes increased oxidative stress and the generation of free radicals. The increased oxidative damage in thalassaemia major may be due to the depletion of lipid soluble antioxidants such as vitamin E. These effects may be minimized with the supplementation of antioxidants.

Key Words: β-thalassemia major, Iron overload, Oxidative stress, Antioxidant status

INTRODUCTION

The term "thalassaemia" is derived from the greek words "thalassa" (sea) and "haema" (blood). This disorder is associated with a defective synthesis of the α or β- globin subunits of haemoglobin -HbA ($\alpha_2\beta_2$). It is inherited as the pathologic alleles of one or more of the globin genes which are located on the chromosomes 11 (β) and 16(α) [1].

Fifteen million people have clinically apparent thalassaemia disorders worldwide. Reportedly, there are about 240 million carriers of beta thalassaemia globally, and in India alone, the number is approximately 30 million, with a mean prevalence of 3.3% [2]. The pathophysiology of β-thalassaemia is characterized by a decreased haemoglobin production and the survival of red blood cells (RBCs), which result from the excess of the unaffected globin chains, which form the α- homotetramer which is more unstable than the β- homotetramer, that precipitates as inclusion bodies [1]. The free α- homotetramer readily precipitates which can damage the cell membrane structure and trigger the apoptotic cell death of the erythroid precursors [3,4]. The symptoms appear after about 2-4 months of age [5]. Iron Homeostasis in human body as shown in [Table/Fig-1].

During the past few years, there has been an exaltation of interest in the role of antioxidants in health and diseases. Antioxidants act as free radical scavengers and they play a significant protective roles in many diseases [6]. Repeated blood transfusions and an increased gastrointestinal iron absorption lead to an iron over-

load in the body, which induces a vicious circle, which results in chronic oxidative stress [3,4,7]. The induced oxygen free radicals and a peroxidative tissue injury accompany the severe anaemia (range 2-7gm/dlHb) and the unavoidable complications, that accelerate the multi-organ abnormalities, especially the organs that accumulate excess iron, which include the liver, spleen, pancreas, heart, etc [4]. The excessive iron burden leads to congestive heart failure, which is the most prevalent and the important causes of death in the β thalassemia major patients [4,8]. In the recent years, the effect of antioxidants on the oxidative stress and the heart failure has been emphasized. In the present study, we intended to investigate the status of the increased oxidative stress and the iron overload in relation to the antioxidant supplementation in children with β-thalassaemia major.

MATERIALS AND METHODS

The present research was carried out in the Department of Biochemistry, NSCB Govt. Medical College, Jabalpur, (M.P.),India. and in the Department of Biochemistry, PDVVPF Medical College, Ahmednagar, Maharashtra,India. Prior to start the study, a local institutional ethical clearance was obtained from the above mentioned institutes and utmost care was taken during the experimental procedures according to the Declaration of Helsinki, 1964.

This study was performed on a total of 120 subjects, who included 60 age and sex matched (35 males and 25 females) healthy controls and 60 (37 males and 23 females) β- thalassemia major children, who were previously diagnosed by High Performance Liquid Chromatography (HPLC) and electrophoretic patterns. All the patients were blood transfusion dependent and under the strict supervision of medical professionals during this period. The patients were aged between 3-12 years and the average haemoglobin concentration ranged between 3 - 7.7 gm/dl. All the patients who had a history of cardiovascular diseases, hypertension, thyroid dysfunction, taking antioxidants and Diabetes mellitus which induced oxidative stress, were excluded from the study.

After obtaining a written consent from all the subjects who were included in the study, a total of 5ml of blood was withdrawn aseptically from the antecubital vein from each subject. Out of this, approximately 2 ml blood was collected into an EDTA (0.47mol/L K3-EDTA) container and 3 ml blood was collected in a plain container.

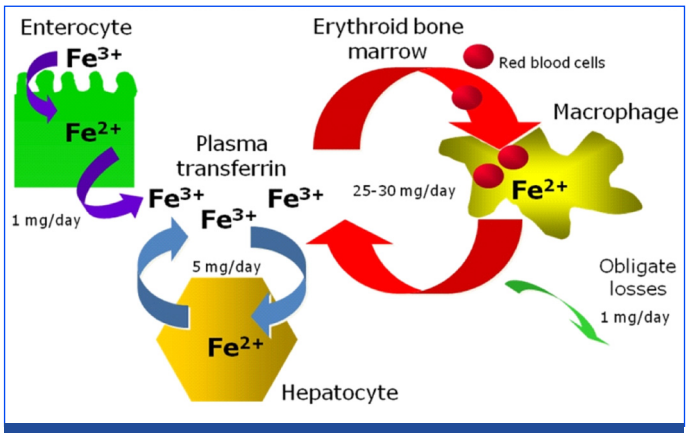
The samples were centrifuged at 3000 rpm for 15 min to separate the RBCs and the sera respectively. The separated sera were collected in polythene tubes with corks and they were stored at -200C. The serum levels of malondialdehyde, a product of lipid peroxidation, were measured by a thiobarbituric reaction which was described by Kei Sathoh [9]. For the estimation of iron, 2-2' dipyridyl in acetic acid was added into deproteinized serum which reacted with the ferrous ions to give a pink colour, as was described in Ramsay's Dipyridyl Method [10]. The erythrocyte SOD activity was measured by Kajari Das' method which was based on the superoxide radicals which were generated by the photoreduction of riboflavin which reacted with hydroxylamine hydrochloride to produce nitrite. The nitrite in turn reacted with sulphanilic acid to produce a diazonium compound, which subsequently reacted with napthylethylenediamine, a red azo-compound [11]. The serum levels of vitamin-E was determined by the Baker and Frank Method which was based on the reduction of the ferrous ions which formed a red coloured complex with α-α dipyridyl [12]. The analysis of all the parameters was done manually by using the chemicals from Qualigens Fine Chemicals Co., Mumbai, India. The parameters were run on a UV visible spectrophotometer (Systronics).

The assessment of the above parameters, except the controls, was conducted before and on the 30th day of the antioxidant supplementation, in the form of an antioxidant tablet A-Z b.i.d. which was composed of predominantly antioxidant vitamins and trace elements.

The statistical analysis was carried out by using the SPSS (Statistical Package for Social Sciences) software, version 16.0 for Windows. The Student's 'z' test was applied for the statistical analysis and the results were expressed in mean ± SD. The p values which were <0.001 were considered as highly significant.

RESULTS

[Table/Fig-2] shows that the significantly elevated (p<0.001) baseline characteristics of serum MDA, iron and ESOD and that the mean value of serum vitamin E were lower (p<0.001) than those in the healthy controls. After one month of the antioxidant supplementation, it was observed in the patients, that there were a significant decrease in the levels of MDA, iron and ESOD, as compared



[Table/Fig-1]: Iron Homeostasis in Human Body

to the results before the supplementation. Similarly, the serum vitamin E level was determined to be higher on the 30th day of the antioxidant supplementation as compared to the level before the supplementation.

DISCUSSION

Iron overload is major complication in β-thalassaemia major, which increases the free radical production, the peroxidative damage to the tissues and the depletion of the endogenous antioxidants 7, 13. Our findings strongly supported those of MA Livrea et al, that the peroxidative damage to the lipids and the proteins were indicated by an increase of about twofold in the serum MDA level because of the membrane bound iron which was present in the thalassaemic erythrocytes [3,7,13]. The plasma level of MDA was studied as a marker of the tissue injury and the oxidative stress. Our findings implied that in the thalassaemic cells, the lipids were more susceptible to autooxidation.

In the human body, the iron storage is maintained normally within the range of 200-1500mg by an adequate adjustment of the intestinal iron absorption, since no excretory mechanism exists [14,15]. From the quantitative point of view, the most important pathway of the iron metabolism is the unidirectional recycling of iron from the senescent red blood cells to the erythroid bone marrow through the macrophages [14]. The secondary surplus iron

Parameters	Controls (n=60)	Group I Beta thalassemia major patients (n=60) Before supplementation of antioxidants		Group II Beta thalassemia major patients After 30th day supplementation of antioxidants	
	(Mean± SD)	(Mean ± SD)	'p' value	(Mean ± SD)	'p' value
Serum MDA (nmols/ml)	1.12 ± 0.72	2.38±0.65	p<0.001	1.71±0.34	p<0.001
Serum Iron (mg/dl)	106.1 ± 32.2	169±0.57	p<0.001	135.7±10.7	p<0.001
ESOD (unit/gm Hb)	1249±254	3094±724	p<0.001	2613±269	p<0.001
Serum vit. E (mg/dl)	1.48±0.87	0.74±0.22	p<0.001	0.91±0.66	p<0.001

[Table/Fig-2]: Indices of oxidative stress and antioxidant status in blood samples of control and beta thalassemia major children before and after treatment

and the continuous blood transfusion in β- thalassaemia major lead to an enhanced generation of the reactive oxygen species and oxidative stress. In our study, it was found that there was a significantly increased iron burden in β-thalassaemia major.

Vitamin E, particularly α-tocopherol, functions in vivo as a lipid soluble, chain breaking antioxidant and it is also a potent peroxyl radical scavenger [6,7,13,16]. When the lipid peroxides are oxidized to peroxyl radicals (ROO•), they react 1000 times faster with β-tocopherol (TOH) than with the polyunsaturated fatty acids (RH). The phenolic hydroxyl group of the chromanol ring reacts with an organic peroxyl radical to form the corresponding organic hydroperoxide and the vitamin E radical (TO) [18].

In the presence of vitamin E,

$$ROO + TOH \longrightarrow ROOH + TO \quad (1)$$

In absence of vitamin E.

$$ROO + RH \longrightarrow ROOH + R \quad (2)$$

$$R + O_2 \longrightarrow ROO \quad (3)$$

In this way, α-tocopherol acts as a chain breaking antioxidant, which prevents the further auto oxidation of PUFA.

Previous studies [16-18] and our study strongly supported Livrea and Lisboa's findings 7 that the increased production of free radicals by ferritin and iron was responsible for the decreased concentration of vitamin E in beta thalassaemia major.

The erythrocytes are protected from oxidative stress by the intracellular preventive antioxidant enzyme, ESOD and they are the 1st line of defense against the oxidative stress. Earlier studies and our findings suggested that an increased ESOD activity was found in the patients with β-thalassaemia major.

The repeated blood transfusions increased the proportion of the younger erythrocytes, which led to the in vivo lipid peroxidation and the compensatory increases in the levels of ESOD [3,13].

The elevated activity of erythrocyte dismutase is essential for the dismutation of the superoxide radical to H_2O_2, which is subsequently detoxified by glutathione peroxidase [6,15].

Our study was supported by Kooshki A, Atia MMA, Rachmilewitz EA, who observed that the decreased intravascular haemolysis with elevated erythropoietin and the haemoglobin concentration by the trial of the antioxidant supplementation in children with beta thalassaemia major [8,16,17].

CONCLUSION

The beta thalassaemia major patients need regular blood transfusions for survival, resulting in the increase of the free iron concentration in the blood, which causes oxidative stress, leading to the development of other abnormalities in the body. Till date, the techniques which are available for the treatment of this genetic disorder are bone marrow transplantation and stem cell therapy, but these treatments are very expensive and hence, people depend on repeated blood transfusions throughout their lives. Minimizing the effects of the oxidative stress is one of methods for improving the survival rate in such patients.

Our findings indicated an increased oxidative stress in the form of serum MDA, a secondary iron overload, ESOD and decreased serum vitamin E levels before the treatment. The therapeutic trial of the beta thalassaemia major patients with an antioxidant supplementation improves the antioxidant status of these patients, which protects the erythrocytes from the damage which results from oxidative stress. It also reduces the rate of haemolysis and it recovers the haemoglobin concentration.

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