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RELATIONSHIP BETWEEN OXIDATIVE STRESS AND ANTIOXIDANT STATUS IN BETA THALASSEMIA MAJOR PATIENTS

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ABSTRACT

In beta thalassemia, decreased or impaired biosynthesis of β-globin leads to accumulation of unpaired α-globin chains. Excess presence of the α-globin in chains is the primary reason for the cellular oxidative damage in thalassemia and also iron overloading. As a result of both, high plasma iron and intracellular non-hemoglobin iron in beta thalassemia leads to enhanced generation of reactive oxygen species and oxidative stress. Blood samples were collected from (100) subjects (50 beta thalassemia major patients and 50 healthy controls) in the range of age 4-18 years. The goal of the present investigations was to study the relationship between oxidative stress by measuring the malonyldialdehyde level, which is the marker of oxidative stress in thalassemia major patients, serum trace elements (Fe, Cu, Zn, Se) and the role of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH), glutlathione peroxidase (GPX) in thalassemia blood samples. From result obtained, it was clear that plasma (MDA) levels was higher whereas erythrocyte SOD, CAT, GSH and GPX levels decreased in thalassemic patients as compared to the normal controls, There were significant changes in the values of (Se, Zn, Cu and Fe) between two groups, increase levels of (Fe) and (Cu) were observed, whereas decrease in levels of (Zn) and (Se) were there in group of beta thalassemia, when compared with group of controls. The administration of selective antioxidant along with essential trace elements and minerals to reduce the extent of oxidative damage and related complications in β thalassemia major still need further evaluation.

Key words: Oxidative stress, Antioxidant, Beta thalassemia.

INTRODUCTION

β-thalassaemia major (βTM), is a type of chronic, inherited and microcytic anemia that is characterized by impaired biosynthesis of the β-globin leading to accumulation of unpaired α-globin chain. leading to red blood cells damage by oxidative means, which may be further potentiated by the heme[1]. All the physiological changes result in ineffective erythropoiesis, hemolysis and anemia[2]. In addition to the direct effects of reduced β-globin synthesis, many of the symptoms of this disorder appear to be consequences of the cytotoxic buildup of free α-globin. Free α-globin is highly unstable and readily precipitates, and release iron in reactive form[3,4]. In addition to this, repeated blood transfusions and increased gastrointestinal iron absorption lead to iron overload in the body[5]. Humans are unable to eliminate the iron, and the excess iron is deposited as hemosiderin and ferritin in the liver, spleen, endocrine organs and myocardium. The deposited iron is responsible for the formation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radical (OH^-), singlet oxygen and hydrogen peroxide (H_2O_2), which induces oxidative stress in thalassemia major patients via Fenton reaction. On the other hand, super oxide is

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the main reactive oxygen species, which react with nitric oxide radical and forms peroxynitrate, therapy causing oxidative stress and cellular damage[5,6] is shown in scheme below:

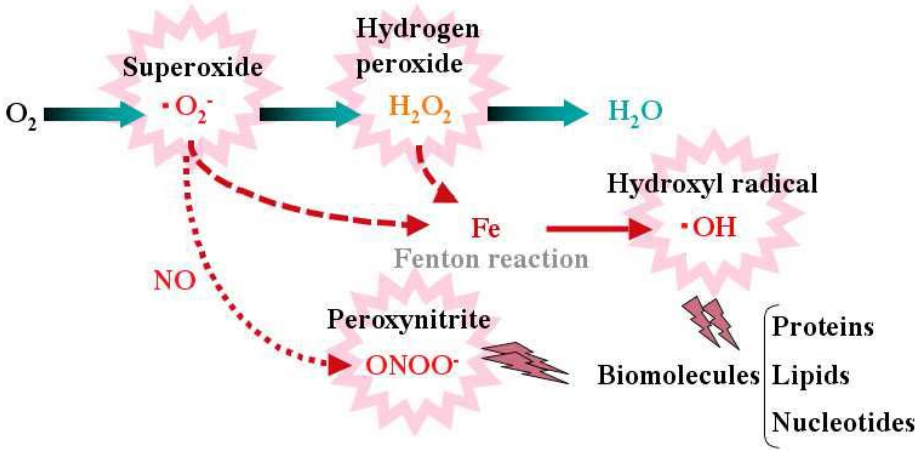

Fig. 1: Scheme elucidate the Fenton reaction

If the production of ROS exceeds the capacity of enzymatic and non-enzymatic antioxidants systems to scavenge these species, then oxidative stress occurs. This oxidative stress may contribute to shortened life span of erythrocytes, primary or secondary amenorrhoea, hypogonadism, osteoporosis, heart failure, endocrine abnormalities like diabetes, hypothyroidism, liver failure and ultimately, early death[7,8]. Biomarkers of oxidative stress included plasma malonyldialdehyde (a marker of lipid peroxidation)[9,10].

Antioxidants are protective agents that inactivate ROS and play an essential role in protection of the cells from oxidative damage[11]. They include several agents such as enzymes (glutathione peroxidase, superoxide dismutase, catalase), large molecules (ferritin, albumin), small molecules (uric acid, glutathione, bilirubin, ascorbic acid, α-tocopherol, and vitamin E) and some essential trace elements such zinc, copper and selenium[12,13]. The activities of enzymatic antioxidants viz, catalase, glutathione peroxidase and glutathione reductase were found to be drastically reduced in untreated β-thalassemic patients when compared to normal subjects. However, the activity of superoxide dismutase was found to be increased in untreated thalassemic patients when compared to normal individuals. An increase in superoxide dismutase and a decrease in catalase activity reflect the presence of a severe oxidative stress situation in the erythrocytes of the untreated transfusion dependent β-thalassemia patients[14]. Changes in erythrocyte membrane protein pattern in untreated β-thalassemia patients when compared to normal erythrocyte further confirm the presence of continued oxidative stress in the ailing thalassemic erythrocytes[15]. It is clear that patients with β-thalassemia suffer from chronic oxidative stress and have an altered redox state characterized by gross depletion of antioxidant nutrients[16].

Most of the clinical events in patients with this disorder were precipitated directly by severe antioxidant depletion resulting in inadequate protection. Therefore, systematic large scale clinical trials involving the supplementation of combined antioxidant nutrients may generate useful information from which antioxidant replacement therapy may be used as adjunct treatment for this disabling condition[17].

The aim of the present investigation was to study the relationship between oxidative stress by measuring the MDA level, which is the marker of oxidative stress in thalassemia major patients and study also measures the enzymatic and non-enzymatic antioxidant level in thalassemic blood sample.

EXPERIMENTAL

Materials and methods

This study was conducted in the lab of Biochemistry of Al-Sader medical city. The duration of the study was 12 months. The study has been performed on total of 100 subjects which included 50 age and sex matched (25 males and 25 females) healthy controls and 50 patients (25 males and 25 females). The patients were blood transfusion dependent, and aged between 4-18 years. All the patients having history of cardiovascular diseases, hypertension, thyroid dysfunction and diabetes mellitus, which induce oxidative stress, were excluded from the study. After obtaining a written consent, total of 5 mL blood was withdrawn aseptically from the anticubital vein from each subject, out of this approximately 2 mL blood in EDTA (0.47 mol/L K3-EDTA) container and 3 mL blood in plain blub. The samples were centrifuged at 3000 rpm for 10 min to separate serum and RBCs, respectively. The separated serum was collected for further analysis in polythene tube with cork and stored at –20 ^{0}C. Serum lipid peroxide was measured by precipitating lipoproteins with trichloroacetic acid and boiling with thiobarbituric acid, which reacts with malonyldialdehyde to get pink colour as per the method of Kei Satho[18]. Superoxide dismutase (SOD) was measured by using RANSOD kit and Glutathione peroxidase (GPx) was measured by using RANSEL kit (Randox Laboratories Ltd. Crumlin, United Kingdom) This method is based on Paglia DE and Valentine WN[19]. Catalase activity in RBCs was determined as Goth method[20]. Serum ferritin concentration was performed by Assay Max Human Ferritin ELISA kit. For serum iron estimations, Ramsay's Dipyridyl method was used[21,22]. The parameters were run on UV visible Spectrophotometer (Systronix). For zinc and copper measurements, standard zinc and copper solutions of 0.1%, 0.2%, 0.3% and 0.4% were prepared and then defreezed the serum. One cc of defreeze serum sample was put into a Balloon Jujeh and 5% glycerol solution was added into it. In the standard solutions and the serum sample solution, zinc and copper levels was then measured using Flame Atomic Absorption Spectrometry (Perking Elmer Analyst 100). Using 213.9 nanometer wavelength for Zn and 324.8 nanometer wavelength for Cu, the standard solution curves and equation line Serum zinc and copper levels drawings were calculated. Selinuim was measured by the direct graphite furnace AAS (AA 220, GTA 110, Varian, Australia) equipped with pyrolytically coated graphite tubes and deuterium background correction after a further dilution of serum with Triton X-100 (0.1% v/v). Direct determination of Se in body fluids by graphite furnace AAS may suffer from problems like severe background, matrix effects, preatomization losses, and spectral interferences. So, the mixture of Pd+Mg $(NO_3)_2$ was used as matrix modifier in graphite furnace AAS for the direct determination of Se in the serum[23].

Statistical analysis

The statistical analysis was made by the SPSS statistics software, version 17 for Windows. The Student 't' test was applied to assess the difference between means of intervention and controls. The results were expressed in mean ± SD. P values ($P < 0.001$) and ($P < 0.005$) were considered as highly significant.

RESULTS AND DISCUSSION

Our results referred to significantly elevated ($P < 0.001$) levels of serum iron and copper while the means value of selenium and zinc significantly reduced ($P < 0.001$) in cases than healthy controls (Table 1). Patients with thalassemia are known to have poor growth, altered puberty, and immune function as well as reduced bone mineral acquisition[24]. The etiology of these comorbidities is typically ascribed to the toxic effects of transfusion related iron-overload in β-thalassemia major patients compared to controls. People with severe forms of thalassemia often suffer from anemia, so they often require blood transfusions. The transfusion therapy can cause iron overload in patients, but they also suffer from iron overload independent

of blood transfusions. Thalassemia patients overproduce a protein called GDF15, which suppresses the production of a liver protein, which in turn leads to an increase in the uptake of dietary iron in the gut[25].

The iron overload can generate oxygen-free radicals and promote peroxidative damage to cell and organelle membranes in organs that accumulate the excess iron including liver, pituitary gland, pancreas and heart. In such condition, depletion of endogenous antioxidants may be expected. In addition, iron overload in β-thalassemia major could greatly decrease selenium and zinc absorption via the gastrointestinal tract[26].

Zn is the second most abundant trace element in the body. Our data showed that in patients with beta thalassemia, there was a significant decrease in the levels of serum Zn. These results are in agreement with other studies published elsewhere[27-29]. Zn deficiency in thaladssemia may not only be due to high iron levels, but may also be due to multi-factorial causes such as, hyperzincuria, hepaticdysfunction and impaired Zn absorption. It is possible that these trace metals are also chelated with iron and are removed by urine. So if the trace metals are measured in the urine of the patients, they may be increased[30].

Cu is present largely in the form of organic complexes, many of which are metalloproteins acting as enzyme. In present study, serum Cu levels was found to be significantly increased in patients when compared with controls. The increased level of Cu could be explained by the antagonistic effect of the Zn, as Zn deficiency in β-thalassemia major could greatly increase the Cu absorption via the gastrointestinal tract[31].

One of the essential trace elements in human plasma is selenium. Selenium was first discovered as a byproduct of sulfuric acid production. It is a well-known electrometalloid and is mostly famous due to its anti-cancerous properties. It is an essential constituent of the enzyme glutathione peroxidase and also incorporates in various important proteins such as hemoglobin and myoglobin. It helps in preventing free radical damage caused by ferrous chloride, and heme compounds. Its deficiency may affect the iron binding capacity of transferrin which leads to iron stores and subsequent tissue damage[32]. The study indicates a significant decrease in plasma concentrations of the essential element selenium as well as decrease in plasma activity of selenium-dependent antioxidant enzyme glutathione peroxidase (GPx).

Table 1: Biochemical data for elements (mean ± SD) in children with β-thalassemia and the control group

Elements	Thalassemia major group N = 50	Control group N = 50	P value
Selenium µg/L	44.87 ± 9.84	112.63 ± 27.8	< 0.001
Zinc µg/dl	65.70 ± 12.25	122.72 ± 23.40	< 0.001
Copper µg/dl	182.10 ± 28.71	94.48 ± 24.10	< 0.001
Iron µg/dl	158.70 ± 20.62	108.98 ± 33.15	< 0.001

Table 2: Hematological data of β-thalassemia major patients and the control group

Parameters	Thalassemia major group N = 50	Control group N = 50	P value
Hematocrite (%)	26% ± 4.2%	36% ± 3.9%	< 0.005
Ferritin µg/L	2424.55 ± 1389.75	60.87 ± 20.12	< 0.001
Hemoglobin g/dl	8.6 ± 1.4	13.4 ± 0.8	< 0.005

A significant increase of serum ferritin in the patients (Table 2) indicated an existing iron overload. The acute iron overload found in β-thalassemia can lead to the accumulation of an abnormal molecular iron form (non-transferrin-bound: NTBI) NTBI has hepatic and cardio-cytotoxic properties. Furthermore NTBI contributes to the formation of free radicals and increases hemolytic process[33]. The released iron could play essential role in the oxidation of membrane cells and senescent cell antigen formation of the major pathways for erythrocyte removal.

Malonyldialdehyde MDA levels were higher in the study group than control group (Figure 2); these results agree with previous studies, which reported increased plasma malonyldialdehyde MDA level, as measured by thiobarbituric acid reaction substance (TBARS) method, in β-thalassemia patients[34-36]. MDA is a good indicator of oxidative damage. In addition, malondialdehyde (MDA), a product of lipid peroxidation, is generated in excess amounts in β-thalassemia.

MDA is a bifunctional reagent and has been reported to crosslink several cell constituents including membrane components. A cross-linked erythrocyte membrane is expected to be rigid and this could probably explain the rigidity of thalassemic erythrocytes when compared to normal ones. Further, erythrocyte deformability is a major determinant of anemia in thalassemia[35]. In one of the previous studies, free and total MDA was found to be higher in regularly transfused thalassemia major patients than in the thalassemia intermedia patients[37]. As a result of continuous blood transfusions, the patients might be subjected to peroxidative tissue injury by the secondary iron overload. These findings might support the idea that iron overload in β-thalassemia leads to an enhanced generation of reactive oxygen species and oxidative stress.

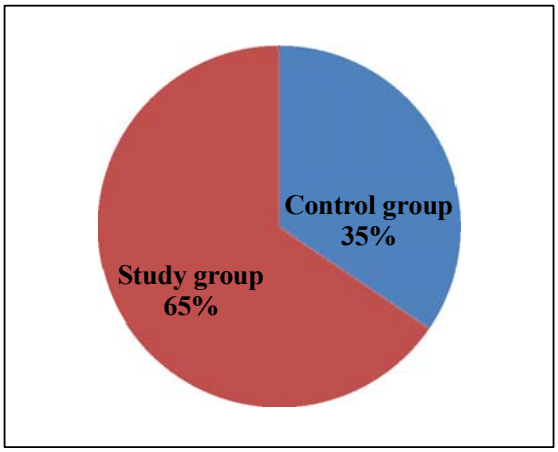

Fig. 1: The percentage of MDA levels in β-thalassemia major patients and control group

Table 3: Activities of multi enzymes and malonyldialdehyde MDA levels in patients with β-thalassemia major and controls

Enzymes	β-Thalassemia major patients N = 50	Controls N = 50	P value
Superoxide dismutase (SOD) U/mL	77.5 ± 22.2	106.4 ± 35.2	P < 0.001
Catalase (CAT) U/L	44.88 ± 19.87	120.29 ± 25.2	P < 0.001
Glutathione peroxidase (GPX) U/L	5.35 ± 1.2	15.45 ± 5.87	P < 0.001
Glutathione reductase (GSH) U/L	5.26 ± 1.9	14.64 ± 4.83	P < 0.001
Malonyldialdehyde (MDA) nmol/mL	17.8 ± 6.5	9.08 ± 2.30	P < 0.001

Thalassemic major children suffer from low levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSH), (Table 3) when compared with those contols. The p value of these enzymes are $P < 0.001$.

Superoxide dismutases are the proteins co-factor with copper, zinc, manganese, iron or nickel. In humans, it exists in three different forms including SOD1 found in cytoplasm, SOD2 present in cytoplasm, and SOD3 is extracellular. Superoxide is the main reactive oxygen species, which react with nitric oxide radical and forms peroxynitrite; thereby, causing oxidative stress and cellular damage. SOD is the essential antioxidant that decreases the formation of ROS and oxidative stress; thus, protecting the cells from damage. Erythrocyte SOD protects the erythrocyte from being damaged during oxidative stress[38]. SOD activity in patients with thalassemia major is decreased, resulting in pronounced inhibition of the blood antioxidant. Our results are in agreement with those of Dhawan et al.[12], who found that the mean SOD enzyme activity was at least 1.5 times lower in the thalassaemic than in controls. The findings pertaining to erythrocytic SOD enzyme activity reported by other investigators are varied. They ranged from high SOD activity to no difference in patients and controls[38,39].

Catalase (CAT), widely distributed in all cells, is present in high amounts in erythrocytes. It is an intracellular enzyme made up of four polypeptide chains with four porphyrin heme groups. Catalase is responsible for detoxification of hydrogen peroxide in the cells[40]. In present study, the enzymetic antioxidants CAT in the hemolysate were significantly lower in thalassemia patients (44.88 ± 19.87) as compared with healthy controls (120.29 ± 25.2). Decrease in the activity of CAT could be due to increase in the lipid peroxidation product malonyldialdyhyde, which can form crosslinks, therapy inactivating several membrane bound enzymes[41,42].

Glutathion peroxidase (GPX) belongs to group of antioxidant selenoenzymes that protects the cell damage by catalyzing the reduction of lipid hydroperoxides. This action requires the presence of glutathione. Glutathione peroxidase levels in the body are in close relation with the glutathione, which is the most important antioxidant present in the cytoplasm of the cells[43]. The present study demonstrated significant reduction in red cell GPX in thalassemic patients (5.35 ± 1.2), as compared with healthy volunteers (15.45 ± 5.87). Decreased levels of GPX is due to inactivation by the increased super oxide anion production leading to an increase in oxidative stress[44]. Our findings are in confirmation with the study of Garelnabi et al.[43] Low level of GPX seems to result from the enzyme inhibition or reduced activity due to excessive production of hydrogen peroxide. This study showed significantly lower levels of all the antioxidants vitamin E, GPX and SOD in thalassaemic children compared with the matched healthy controls.

The present study reported a deficiency in levels of reduced glutathione, which is 2.3 times lower than in healthy controls. These results go hand in hand with previous study[45], which suggested that glutathione (GSH) is a major intracellular reducing agent, which is very sensitive to oxidative pressure and has several important function such as: protection against oxidative stress, regulation of gene expression, induction of a poptosis activation and proliferation in T lymphocytes.

In conclusion, the oxidative stress in patients with β-thalassemia major is mainly caused by peroxidative injury due to secondary iron overload. Production of free radicals by iron overload, alteration in serum trace elements and antioxidant enzymes status play an important role in the pathogenesis of the β-thalassemia major. Impairment of the antioxidant status is associated with elevated plasma levels of lipid peroxidation. The administration of selective antioxidant along with essential trace elements and minerals in order to reduce the extent of oxidative damage and the related complications in β-thalassemia major still need further evaluation.

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