

## Status of oxidant, antioxidant and serum enzymes in thalassaemic children receiving multiple blood transfusions

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### Abstract

**Objective:** To determine the levels of oxidant, antioxidant and serum enzymes in thalassaemic children receiving multiple blood transfusions.

**Methods:** The case-control study was done from February to August 2012, and comprised thalassaemic children receiving multiple blood transfusions at Allied Hospital, Ali Zeb Foundation, and the Thalassaemia Centre in Hilal-e-Ahmar Hospital, Faisalabad, Pakistan. Healthy subjects were also screened for any related disease condition that could prejudice the results. Blood samples were analysed for the values of total oxidant status, total antioxidant capacity, serum malondialdehyde, catalase, paraoxonase, arylesterase, glutathione peroxidase and ceruloplasmin.

**Results:** There were 180 children in the study; 90(50%) cases and 90(50%) controls. Of the cases, 48(53.3%) were under-weight while the weight of 42(46.7%) was in the normal range. The values of total oxidant status and total antioxidant capacity were significantly ( $p < 0.01$ ) higher in thalassaemic children compared to normal values. Serum malondialdehyde and catalase levels were also considerably elevated ( $p < 0.05$ ), suggesting the increased activity of these enzymes. However, the concentrations of serum paraoxonase, arylesterase, glutathione peroxidase were significantly ( $p < 0.01$ ) lower in cases than the controls, displaying diminished activities during multiple blood transfusions in these patients.

**Conclusion:** Multiple blood transfusions disconcert the levels of oxidants, antioxidants and serum enzymes of thalassaemic children. Oxidative damage is seen because of the increased iron overload in these patients. Hence, regular evaluation of oxidant and antioxidant status should be monitored in thalassaemic patients during initial few years of life.

**Keywords:** Thalassaemia, Multiple blood transfusions, Oxidative stress, Serum enzymes, Children. (JPMA 65: 838; 2015)

### Introduction

Thalassaemia refer to a group of blood disorders characterised by decreased synthesis of one of the two types of polypeptide chains (either  $\alpha$  or  $\beta$ ) that constitute the normal adult human haemoglobin molecule (HbA).<sup>1</sup> In general, thalassaemia is categorised into alpha or beta thalassaemia in relation to the involvement of particular polypeptide chain. The frequency of alpha thalassaemia is very uncommon and found periodically in different areas of the world. On the other hand,  $\beta$ -thalassaemia, also called "Cooley's anaemia" or "Mediterranean anaemia", is the most common genetic disorder worldwide.  $\beta$ -thalassaemia is rampant throughout the Mediterranean region, Africa the Middle East, Iran, Indian subcontinent, Burma, southeast Asia, including southern China, the Malay peninsula, and Indonesia.<sup>2</sup> In Pakistan, the gene

frequency of  $\beta$ -thalassaemia has been expected to be 5-8% with 8-10 million carriers.<sup>3</sup>

An alteration in the gene for the beta globin is supposed to be the possible cause of  $\beta$ -thalassaemia. The inability to produce  $\beta$ -globin chains allows the  $\alpha$ -globin chains to accumulate and precipitate within erythroid precursors in the bone marrow.<sup>4</sup> Typically,  $\beta$ -thalassaemia is characterised by moderate to severe anaemia, caused by haemolysis and ineffective erythropoiesis. The other signs, frequently observed in thalassaemic patients, include skeletal and/or endocrine changes and splenomegaly, diarrhoea, irritability, fever, feeding problems and gradual bulging of the abdomen due to spleen and liver enlargement.<sup>5</sup>

The therapy of  $\beta$ -thalassaemia is programmed and frequent blood transfusions along with chelation therapy in order to eliminate the high rise of free iron in the body. Over the past few decades, regular blood transfusions and iron chelation therapies have substantially improved the quality of life, and extended life by an average of 20 years.<sup>6</sup> However, these recurrent transfusions may result in iron overload and a new range of complications

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emerge that could lead to several intracellular damages in adolescents and young adults. Moreover, the non-transferrin-bound iron (NTBI) fraction within plasma may promote the production of reactive oxygen species (ROS), and propagators of oxygen-related disorders via Fenton reactions, leading to increased lipid and protein peroxidation, which eventually disturbs the oxidant and antioxidant profile of the body. In Pakistan, there is paucity of data regarding this aspect in thalassaemic children, hence the present study was planned to determine the possible effects of multiple blood transfusions on oxidant, antioxidant and serum enzyme levels in thalassaemic children.

### Patients and Methods

The case-control study was conducted from February to August 2012, and comprised thalassaemic children receiving multiple blood transfusions at Allied Hospital, Ali Zeb Foundation, and the Thalassaemia Centre in Hilal-e-Ahmar Hospital, Faisalabad, Pakistan. Healthy subjects were also screened for any related disease condition that could prejudice the results. Children from both genders, 1-10 years of age with thalassaemia, receiving multiple blood transfusions for at least 6 months with no other systemic illness or long-term complications were included in the study. Children with known acute infection, and terminally ill were excluded.

The study was approved by the ethics committee of Punjab Medical College, Faisalabad, and verbal consent was obtained from each patient's guardian after explaining the study procedure to each one of them. All patients were subjected to detailed history regarding thalassaemia, start of blood transfusions, number of transfusions/month and chelation therapy.

The sample size was calculated on the basis of the data available at the Paediatric Medicine Unit, Allied Hospital, Faisalabad, during the years (2000-08) according to the following formula:<sup>7,8</sup>

$$\text{sample size} = Z_{1-\alpha/2}^2 P(1-p)/d^2$$

Where  $Z_{1-\alpha/2}$  is standard normal variate at 1% type 1 error ( $p < 0.01$ ) it is 2.32,  $P$  is the expected proportion in population based on previous studies or pilot studies,  $d$  = absolute error or precision.

The proportion of thalassaemic children was 0.169% of the total number of children brought to the hospital during the above mentioned period. Hence, the value ( $P = 0.00169$ ) was used in the above equation for estimating the sample size.

All the patients were recruited by non-probability

consecutive sampling method. In this technique, all accessible patients fulfilling the inclusion criteria were selected. Only those thalassaemic children were selected who had been diagnosed by Haemoglobin Electrophoresis technique. Children without any clinical signs and symptoms were considered healthy. On the basis of sample size, equal number of children was selected in both groups because of ease in statistical analysis.

The body mass index (BMI) of all the patients was calculated by the following formula.

$$\text{BMI} = \text{Weight (kg)} / [\text{Height (m)} \times \text{Height (m)}]$$

Initially, 5ml of venous blood as specimen were obtained and sera were separated in small aliquots and stored at -4°C till analysis. The samples were analysed in the laboratories of the Department of Physiology and Pharmacology, University of Agriculture, Faisalabad (UAF).

Parameters examined included a total oxidant status (TOS) of the body which was measured according to the method described by Erel.<sup>9</sup> Absorbance was taken at (Bichromatic) wavelength of 500nm and 800nm (Biosystem, BTS-330, Biosystems, S.A. Costa Brava, Barcelona, Spain). Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) was used to calibrate the assay and results were expressed in terms of  $\mu\text{M}$  hydrogen peroxide equivalent per litre ( $\mu\text{mol H}_2\text{O}_2$  equiv./L). Sensitivity of this assay was 1.13  $\mu\text{mol/L H}_2\text{O}_2$  equiv./L.

Total antioxidant capacity TAC was determined according to the method of Erel.<sup>10</sup> Absorbance was taken at 660nm wavelength (Biosystem, BTS-330, Biosystems, S.A. Costa Brava, Barcelona, Spain). The reaction rate was calibrated with Trolox, which is widely used as a traditional standard for TAC measurement assays. The results of assay were expressed in mmol Trolox Equiv./L. The sensitivity of this assay was 0.04 mmol/L Trolox Equiv./L (Figure).

Serum malondialdehyde (MDA) reacts with thiobarbituric acid (TBA) and concentration of thiobarbituric acid (TBA) was measured by the method described in literature.<sup>11</sup> Absorbance was measured at 532nm using spectrophotometer Biosystem, BTS 330, (Biosystem, S.A. Costa Brava, Barcelona, Spain). Results were expressed in nmol/L.

Enzymatic activity of catalase was determined by method described in an earlier study.<sup>12</sup>  $\text{H}_2\text{O}_2$  and molybdate ions formed a yellowish complex. Its absorption was measured against a blank yielding a high peak between 352nm and 360nm depending on the concentration of ammonium molybdate upto 48.5mmol/L. Absorbance was measured at 405nm wavelength with the help of

**Table-1:** Mean values of different serum enzymes in normal and thalassaemic children.

Parameters	Normal Children (n = 90)		Thalassaemic Children (n = 90)		P-value
	Mean $\pm$ SD	Confidence Interval (CI)	Mean $\pm$ SD	Confidence Interval (CI)	
Total oxidant status ( $\mu$ mol/L)	0.66 $\pm$ 0.27	0.61-0.69	1.09 $\pm$ 0.42	1.03-1.12	0.114*
Total antioxidant capacity (mmol/L)	1.37 $\pm$ 0.19	1.32-1.41	1.60 $\pm$ 0.21	1.24-1.94	0.108*
Serum Malondialdehyde (nmol/L)	4.67 $\pm$ 0.48	4.62-4.71	7.96 $\pm$ 0.64	7.79-8.14	0.141*
Serum catalase (kU/L)	71.31 $\pm$ 1.69	67.8-76.4	78.33 $\pm$ 1.32	73.5-82.4	0.126*
Serum paraoxonase (U/L)	158.85 $\pm$ 4.57	153.2-162.7	89.53 $\pm$ 3.44	86.8-94.2	0.757*
Serum arylesterase (U/L)	17.66 $\pm$ 2.34	15.5-19.4	11.71 $\pm$ 2.16	9.2-14.6	0.229*
Serum glutathione peroxidase (U/L)	7695.18 $\pm$ 42.7	7642.6-7723.9	5090.48 $\pm$ 69.4	4932.2-5103.6	0.687*
Serum ceruloplasmin (U/L)	35.83 $\pm$ 1.07	33.2-39.7	67.01 $\pm$ 2.43	62.4-71.8	0.649*

\*P &gt;0.01

SD: Standard deviation.

spectrophotometer (Biosystem, BTS-330, Biosystems, S.A. Costa Brava, Barcelona, Spain).

The activity of paraoxonase assay was performed with 2 mmol/L paraoxon in the absence of sodium chloride (NaCl), which has a basal activity and in the presence of 1 Mol/L NaCl by measuring the release of p-nitrophenol due to initial hydrolysis of paraoxon at 405nm wavelength.<sup>13</sup> The absorbance was taken against paraoxonase substrate reagent which was taken as blank on spectrophotometer Biosystem, BTS-330 (Biosystems, S.A. Costa Brava, Barcelona, Spain).

The activity of Arylesterase was measured by using phenylacetate as a substrate at 270nm wavelength using Biosystem, BTS-330 (Biosystems, S.A. Costa Brava, Barcelona, Spain). Initial rate of hydrolysis was determined by observing the increase in phenol concentration.<sup>14</sup>

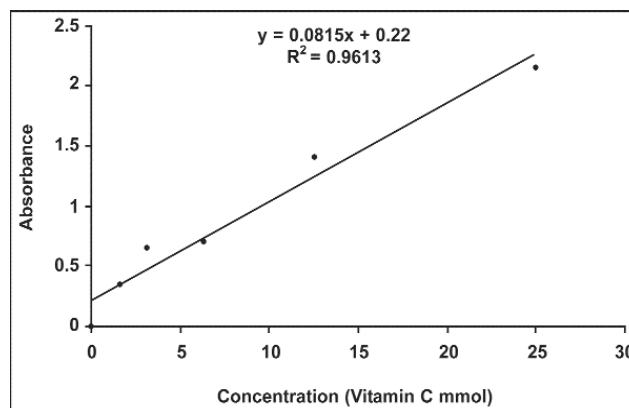
The ceruloplasmin oxidase reacts with O-dianisidinedi hydrochloride and its enzymatic activity was measured by using the colorimetric method.<sup>15</sup> Absorbance was measured at 540nm wavelength against de-ionised water as a blank with the help of spectrophotometer (Biosystem, BTS-330, Biosystems, S.A. Costa Brava, Barcelona, Spain).

Glutathione peroxidase (GPX) absorbance was taken on Ultrospec 100 Pro. Enzymetic activity was expressed as picokatals (Pkat) per 106 sperm cells. Absorbances were read in a spectrophotometer (Shimadzu UV 240; Shimadzu, Kyoto, Japan) at 340nm. A unit of GPX activity was defined as being equivalent to the oxidation of 1 $\mu$ mol of Nicotinamide adenine dinucleotide phosphate (NADPH) per second at 37°C.

Data was analysed by unpaired, two-tailed student's t test using Microsoft Excel 2007. The level of significance was kept at p $\leq$ 0.01.

## Results

There were 180 children in the study; 90(50%) cases and

**Figure:** Standard curve for antioxidant capacity in thalassaemic children.

90(50%) controls. Of the cases, 48(53.3%) were underweight while the weight of 42(46.7%) was in the normal range. Mean values of different serum enzymes in cases and controls were worked out (Table). The values of TOS and TAC were significantly (p<0.01) higher in the cases, suggesting the increased lipid peroxidation during multiple blood transfusions in thalassaemic children. Serum MDA and catalase levels were also considerably elevated (p<0.05), suggestive of increased activity of these enzymes. However, the concentrations of serum paraoxonase, arylesterase, glutathione peroxidase were significantly (p<0.01) lower in cases than the controls, displaying diminished activities during multiple blood transfusions in these patients.

## Discussion

Patients of thalassaemia require frequent blood transfusions to combat the anaemia. These repeated transfusions result in excessive iron accumulation in the body. This iron overload triggers the extreme production of reactive oxygen species (ROS) leading to oxidative stress (OS). In the presence of this stress, a variety of damages may occur to some cells/organelles that could

disturb the normal physiology of the organs. For example, the rapid apoptosis and ineffective erythropoiesis may appear due to the oxidative damage to the red blood cells (RBCs) in thalassaemic patients.<sup>16</sup>

The results of high level of TOS in the present study are concurrent with the results described by<sup>17</sup> that OS was increased in thalassaemic patients. These elevated values of TOS may appear in response to OS, which is attributed to the production of ROS, possibly induced by the multiple blood transfusions in thalassaemic children. In addition, OS may result in the body due to any interruption in the formation of ROS during the metabolic and physiological processes or with the unnecessary production of ROS beyond the enzymatic and non-enzymatic capacity of the body. These high levels of ROS can induce oxidation of biological molecules and may alter the metabolic pathways leading to some abnormalities. In company with ROS, the accumulation of toxic quantities of iron may cause multiple endocrine damages in the body. Moreover, the oxidants can be derived from some of the other sources, excluding iron overload. For example, surplus unpaired  $\alpha$ -haemoglobin chains were disintegrated and autoxidised in  $\beta$ -thalassaemia, resulting in ineffective erythropoiesis, increased oxidant production, haemolysis and reduced RBC survival. Previous studies have documented different morphological, metabolic and biochemical changes in RBCs of thalassaemia patients. These changes were associated with constant OS within the cells caused by precipitation of excess  $\alpha$ -globin chains and release of free iron.<sup>17</sup> There might be a number of additional reasons for the increased level of TOS in thalassaemic patients, but all the processes mentioned above may be responsible for high level of TOS in thalassaemic children.

In general, the formation of ROS during cell metabolism is balanced by the similar rate of antioxidants in the body. Antioxidants are a set of enzymes with low molecular mass components, which have physiological role to maintain cellular redox state and to neutralise and/or suppress oxidative damage induced by ROS. The protective intracellular enzymes and non-enzymatic antioxidants prevent the ROS accumulation in the body. These enzymes show resistance against OS by scavenging the free radicals and inhibiting lipid peroxidation or OS. In the current study, the overall TAC level was significantly ( $p < 0.01$ ) higher in thalassaemic children. The possible reason of high values of TAC might be due to the counteractive effects of antioxidant enzymes in response to the elevated OS in thalassaemic children. Previous studies have some miscellaneous results about TAC levels in  $\beta$ -thalassaemic patients. According to some

researchers,<sup>18</sup> the TAC level was remarkably decreased in thalassaemic patients while some investigators<sup>19</sup> supported the same consequences in thalassaemic patients, receiving chelation therapy with desferrioxamine (DFO). However, some investigators<sup>20</sup> have reported insignificant differences in the level of TAC apart from high level of TOS and OS among thalassaemic and control groups. Our results showed high level of TAC in thalassaemic patients which are not in line with the results of previously described studies.<sup>18-20</sup>

Serum MD Aisa three-carbon dialdehyde, formed as a by-product of prostaglandin synthesis. It is the end product of lipid peroxidation and responsible for harmful effects of free radicals on deoxyribonucleic acid (DNA) or cell membrane. MDA is supposed to act as indicator of tissue injury and OS.<sup>21</sup> In the current study, the higher concentration of MDA in thalassaemic patients are in line with the results described earlier.<sup>21</sup> The non-transferrin bound iron (NTBI) and duration of blood transfusions are indicative as predictors of MDA, because iron stress is reliant upon the duration of blood transfusions and NTBI displays the availability of free iron in the body. The possible reason of high level of MDA in the present study could be due to the increased level of circulating erythroid precursors and existence of high concentration of unpaired  $\alpha$ -globin chains in RBCs. The occurrence of excess  $\alpha$ -chains in thalassaemic erythrocytes renders them unstable and lead to early disintegration and eventual oxidation.

Catalase is an intracellular enzyme formed by four polypeptide chains, hence incorporating four porphyrin heme groups in the structure. The function of catalase is detoxification of hydrogen peroxide molecules present in the cells. In the current study, the high values of catalase in thalassaemic patients are concurrent with the reports documented by other investigators.<sup>22</sup> Contrary to this, some researchers<sup>23</sup> reported insignificant change in the catalase level in thalassaemic patients, which has been attributed to the presence of normal RBCs infused with fresh blood transfusions. For the thalassaemic patients, there are ambiguous results regarding catalase values in literature. Some authors<sup>22</sup> suggest increased activity due to the compensation mechanism of increased OS while the others<sup>23</sup> proposed decreased activity of catalase in thalassaemic patients. Our study found noticeably high values of catalase in thalassaemic patients.

Paraoxonase (PON-1) is an esterase enzyme in the company of high density lipoprotein (HDL) transporting apolipoprotein, bringing the antioxidant properties to

HDL. It is proposed to link with peroxidase-like activity on pre-existing peroxides and modify the proportion of oxidative products. Arylesterase is another aromatic esterase, strongly linked to PON-1. It is suggested that PON-1 and arylesterase are identical and genetically function as one enzyme but their activities are assessed as two separate enzymes. The low level of PON-1 seemed to be related with the degree of OS and anaemia and may result from increased inactivation of the enzyme. One study<sup>24</sup> reported that PON-1 activity was down-regulated by interleukin-1 and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) in thalassaemic patients. Some other investigators<sup>25</sup> also reported decreased activity of PON-1 in thalassaemic children. Hence the results of PON-1 in the current study are similar with the result described previously.

Glutathione peroxidase (GPX) is related to the antioxidant selenoenzymes group that protects the cellular damage by catalysing the reduction of lipid hydroperoxides in the presence of glutathione. Some miscellaneous results are found in literature regarding GPX in thalassaemic patients. A study<sup>26</sup> documented high level of GPX, while another<sup>27</sup> reported decreased level of GPX. We also found low level of GPX which might be due to the enzyme inhibition process or may be attributed to the unwarranted production of hydrogen peroxide in thalassaemic patients.

Ceruloplasmin (CP) has a chain-breaking capacity and is considered an important plasma antioxidant. It protects the body against oxygen free radicals, because of its oxidase activity. The high levels of CP in the current study are in line with results of some investigators.<sup>28</sup> The exact reason of increased activity of CP is still not fully understood. Some researchers<sup>29</sup> assumed that the inflammatory conditions might have some role in the high level of CP in thalassaemic patients.

Most of the reasons for the shifting pattern of serum enzyme levels in thalassaemic patients are hypothetical, but the exact mechanisms behind these are still not clear. Therefore, there is a need to conduct further detailed studies to explore the accurate mechanisms of these enzymes.

## Conclusion

Multiple blood transfusions disconcert the oxidant and antioxidant status in thalassaemic children. OS is seen because of excessive iron overload, which in turn leads to oxidative damage. The increase in MDA levels is the best marker of this damage. Various serum enzymes levels are also disturbed due to multiple blood transfusions. These findings suggest regular monitoring of oxidant and antioxidant status during the initial few years of life and

proper treatment along with replacement therapy should be employed when necessary.

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