Lipid Peroxidation and Antioxidant Status in β-Thalassemic Patients: Effect of Iron Overload

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Abstract

To study the effect of iron overload due to continuous blood transfusions on peroxidation products, such as malondialdehyde (MDA) and peroxynitrite, with evaluation of some antioxidants like, glutathione (GSH), superoxide dismutase (SOD), vitamin A, vitamin C, vitamine E, Ceruloplasmin, uric acid and albumin in thalassemia patients. Forty patients with thalassemia major, aged 5 to 15 years, were carried out in Abn-Alatheer Teaching Hospital in Mosul city, during the period from October 2007 to April 2008. They were on Chelation therapy with desferrioxamine. They were divided into two groups, the first one without iron overload (90,97±12.92), and the second one with iron overload (157.75±7.57). All the patients were received whole blood. Blood samples were collected before and after blood transfusion. The results showed that there were significant increase in MDA and peroxynitrite in patients with iron overload five days before and after blood transfusion in compared with groups having normal iron level. On the other hand, glutathione, superoxide dismutase activity, Vitamin A, vitamin C, vitamin E, albumin and ceruloplasmin were significantly decreased whereas, uric acid was increased significantly. It is concluded that, Iron over load due to continuous blood transfusion in thalassemia causes increase in oxidative tissue damage with a changes in antioxidants status.

Key Words: Beta-thalassemia, lipid peroxidation, antioxidants, Malondialdehyde, Iron

الخلاصة

لدراسة تأثير فرط تحميل الحديد نتيجة إعطاء الدم المستمر في نواتج البيروكسدة المتمثلة بالمالونديالديهايد ونترات البيروكسيد, مع قياس بعض من مضادات الأكسدة كالكلوتاثايون والسوبر اوكسايد دسميوتيز وكل من فيتامين A وفيتامين C وفيتامين E والسيروبلازمين وحامض اليوريك والألبومين عند مرضى الثلاسيميا. أختير ٤٠ مريضا من المصابين بالنوع الرئيسي لمرض الثلاسيميا, واللذين هم تحت علاج عقار الدسفيروكسامين. حيث تراوحت أعمارهم من ٥–١٥ سنة ومن الراقدين في مستشفى أبن الأثيرالتعليمي بالموصل, خلال الفترة من تشرين الأول ٢٠٠٧ولغاية نيسان ٢٠٠٨. تم تقسيمهم إلى مجموعتين, الأولى اتصفت بعدم وجود فرط تحميل للحديد (٩٠,٩٧±١٢,٩٢) والثانية بوجود فرط تحميل للحديد (١٥٧,٧٥±٧,٥٧). بعدها تم إعطاء الدم لكل أفراد العينة المدروسة, وأخذت عينات الدم قبل وبعد عملية نقل الدم. أظهرت نتائج هذه الدراسة وجود زيادة معنوية في مستويات المالونداياالديهايد ونترات البيروكسيد في المرضى الذين يعانون من فرط تحميل الحديد مقارنة بالآخرين الذين يكون مستوى الحديد عندهم طبيعي. من ناجية أخرى, فقد انخفضت معنويا مستويات الكلوتاثايون وفعالية أنزيم السوبر اوكسايد دسميوتيز وكل من فيتامين A وفيتامين C وفيتامين E والألبومين والسيريوبلازمين, بينما ارتفع معنويا مستوى حامض اليوريك. وقد أستنتج من الدراسة بأن فرط تحميل الحديد الناتج من نقل الدم المتواصل لمرضى الثلاسيميا قد سبب زيادة شدة الكرب التاكسدي مع اختلاف في مستويات مضادات الأكسدة .

Introduction

Increased level of lipid peroxidation and decreased level of antioxidants play important roles in the pathogenesis of anemias[1]. It is well documented that disturbances of oxidant - antioxidant balance occur in hemoglobinopathies, especially in thalassemia[2]. In beta-thalassemia, decreased or impaired biosynthesis of beta-globin leads to accumulation of unpaired alpha globin chains[3]. Excess presence of the alpha-globin chains primarily[3] and also iron overload, as a result of multiple transfusions, are the main reasons for the cellular oxidative damage in thalassemias[4].

Iron overload is still a major concern in homozygous β-thalassemia. Under physiological conditions, iron ions are not available to catalyze the conversion of molecular oxygen to highly reactive radical species by Fenton reaction, because ferric iron is bound to proteins, preventing it from participating in reactions that could lead to cell injury[5]. Under various pathological conditions associated with iron overload, including thalassemia, due to blood transfusion used for treatment of thalassemia. There is evidence of an increase in iron in both serum and cells[6].

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Received : 26 / 11 / 2008
Accepted : 23 / 6 / 2009

This increases generation of free radicals[7], and promotes peroxidative damage to cell and organelle membranes in organs that accumulate excess iron, including liver, pituitary gland, pancreas, and heart[8]. This study evaluates the total antioxidant potential and several individual antioxidants, as well as parameters of peroxidative stress, including malondialdehyde (MDA) (the breakdown product of lipid peroxidation), in serum of patients with β-thalassemia major, transfusion-dependent, and under regular iron chelation therapy with or without signs of iron overload, before and after transfusion.

Subjects and Methods

Experimental Design

This study was conducted at Abn-Alatheer Teaching Hospital in Mosul city, from October 2007 to April 2008. Forty patients with β-thalassemia major, aged 5 to 15 years (mean, 7.3±3.7), were divided into two groups. The first group included twenty patients without signs of iron overload as revealed from the mean value of serum iron (90,97±12.92), referred as control, while the second group included twenty patients with clinical and biochemical signs of iron overload (157.75±7.57). Both groups were under continuous and regular blood transfusion program (one transfusion process/ month). Chelation therapy with desferrioxamine (DFO) was administered to each of the patients (by pump, five days a week, 40-60mg/kg/day, 12 hours infusion)[9]. All of the patients were examined regularly once a month in Pediatric Hematology Department of this hospital.

Sample Collection and Clinical Chemistry Analysis

Ten milliliters of blood samples were obtained from each patient five days before and after blood transfusion process. After clotting, serum was separated by centrifugation and divided in several aliquots. The analytical determinations described below were either performed immediately, or serum was stored at -20°C and used within 72 hours. The readings of the measured parameters were done at clinical biochemical laboratory in the College of Science. Clinical laboratory examinations on serum including, MDA and peroxynitrite as an oxidant indicator and the total antioxidant capacity like glutathione, SOD, vitamin A, vitamin C, vitamin E, ceruloplasmin, uric acid, and albumin levels were evaluated. The level of serum MDA was determined by a modified procedure using the thiobarbituric acid reaction substance (TBARS) methods[10], and the activity of SOD levels in blood serum was determined using photochemical method described by Brown and Goldstein[11]. This methods depends on an indirect approach to determine the SOD activity through the change in formazene absorbance formed from the reduction of $O_2^{\cdot -}$, which is produced by radiating the sample of serum with light) for nitroblue tetrazolum (NBT) dye. Decreased difference in formazene absorbance means increased SOD activity. Serum glutathion is determined by a modified procedure utilizing Ellman`s reagent[12].Serum vitamin A[13], vitamin C[14] and vitamin E[15] were measured spec-trophotometrically. Ceruloplasmin, Peroxy nitrite activity were measured by modified method described by Menden *et al.*[16] and Vanuffelen *et al.*[17] respectively. The level of uric acid[18] and serum albumin[19] were measured.

Statistical Analysis

All data were compared by t-test between patient groups in SPSS 10.0 program. The values within the tables were given as mean ± standard deviation. Statistical significance was considered at $p<0.05$[20].

Results

The effects of blood transfusion in thalassemic patients, without iron overload, represented with normal serum iron (90,97±12.92), on lipid peroxidation and antioxidant status are presented in table (1). Glutathione, vitamin A, vitamin C vitamin E, and albumin increased significantly, while MDA, peroxynitrite, SOD activity, ceruloplasmin, and uric acid did not changed significantly after blood transfusion in comparison with the control group (before transfusion). On the other hand, with the exception of peroxynitrite, SOD activity, albumin and ceruloplasmin other parameters like Glutathione, vitamin A, vitamin C, and vitamin E were increased significantly, while MDA and uric acid were decreased significantly in thalassemic patients, with iron overload, after receiving blood transfusion as shown in table (2). The effects of iron overload in thalassemic patients, represented with increased serum iron (157.75±7.57), before blood transfusion, on lipid peroxidation and antioxidant status are presented in table (3). MDA, peroxynitrite, and uric acid were found to be higher in thalassemic patients with iron overload when compared with non-iron overload patients. On the other hand, glutathione, SOD activity, vitamin A, vitamin C, vitamin E, ceruloplasmin, and albumin were significantly decreased in the same group. Moreover, after blood transfusion, all parameters were altered significantly in iron overload group in comparison with thalassemic

patient without iron overload as shown in table (4). Glutathione, SOD activity, vitamin A, vitamin C, vitamin E, albumin and ceruloplasmin were decreased significantly. In contrast, MDA, peroxynitrite, and uric acid were shown to be increased significantly.

Table 1: Effects of blood transfusion in thalassemic patients, without iron overload, on lipid peroxidation and antioxidant status.

Parameters / Groups	Malondialdehyde (MDA)	Peroxy nitrite	Superoxide Dismutase (SOD)	Vitamin A	Vitamin C	Vitamin E	Ceruloplasmin	Glutathione (GSH)	Uric Acid	Albumin
	µmol/L	µmol/L		µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	g/L
Pre-Blood Transfusion Without Iron Overload	4.58 ±0.579	78.8 ±8.573	0.130 ±0.010	1.52 ±0.346	40.59 ± 7.034	20.6 ±4.502	289.19 ± 22.518	10.33 ±1.310	204.83 ± 54.661	43.16 ±6.634
Post-Blood Transfusion Without Iron Overload	(NS) 4.79 ±0.417	(NS) 75.8 ±5.462	(NS) 0.127 ±0.008	* 1.69 ±0.249	** 46.45 ±5.735	** 24.34 ±3.165	(NS) 298.7 ± 24.372	*** 11.92 ±1.349	(NS) 177.75 ± 18.496	** 48.13 ±3.265

- Values are expressed as means ± SD from 20 subjects per group.
- (NS): Not significant

*Significantly different from the control ($p<0.05$)
** Significantly different from the control ($p<0.01$)
*** Significantly different from the control ($p<0.001$)

Table 2: Effects of blood transfusion in thalassemic patients, with iron overload, on lipid peroxidation and antioxidant status.

Parameters / Groups	Malondialdehyde (MDA)	Peroxy nitrite	Superoxide Dismutase (SOD)	Vitamin A	Vitamin C	Vitamin E	Ceruloplasmin	Glutathione (GSH)	Uric Acid	Albumin
	µmol/L	µmol/L		µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	g/L
Pre-Blood Transfusion With Iron Overload	6.69 ±0.900	85.1 ±6.337	0.145 ±0.011	1.21 ±0.236	29.05 ±2.344	15.22 ±0.610	259.44 ± 22.866	7.79 ±1.073	316.55 ± 43.848	35.11 ±3.080
Post-Blood Transfusion With Iron Overload	*** 5.54 ±1.169	(NS) 83.3 ± 7.513	(NS) 0.143 ±0.013	** 1.44 ±0.267	* 34.42 ±9.449	* 16.73 ±3.048	(NS) 266.44 ± 23.249	* 8.97 ±2.440	** 265.00 ± 74.337	(NS) 37.76 ±7.335

- Values are expressed as means ± SD from 20 subjects per group.
- (NS): Not significant

*Significantly different from the control ($p<0.05$)
** Significantly different from the control ($p<0.01$)
*** Significantly different from the control ($p<0.001$)

Table 3: Effects of iron overload in thalassemic patients, before blood transfusion, on lipid peroxidation and antioxidant status.

Parameters / Groups	Malondialdehyde (MDA)	Peroxy nitrite	Superoxide Dismutase (SOD)	Vitamin A	Vitamin C	Vitamin E	Ceruloplasmin	Glutathione (GSH)	Uric Acid	Albumin
	µmol/L	µmol/L		µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	g/L
Pre-Blood Transfusion Without Iron Overload	4.58 ±0.579	78.8 ±8.573	0.130 ±0.010	1.52 ±0.346	40.59 ± 7.034	20.6 ±4.502	289.19 ± 22.518	10.33 ±1.310	204.83 ± 54.661	43.16 ±6.634
Pre-Blood Transfusion With Iron Overload	*** 6.69 ±0.900	* 85.1 ±6.337	*** 0.145 ±0.011	** 1.21 ±0.236	*** 29.05 ±2.344	*** 15.22 ±0.610	*** 259.44 ± 22.866	*** 7.79 ±1.073	*** 316.55 ± 43.848	*** 35.11 ±3.080

- Values are expressed as means ± SD from 20 subjects per group.
- (NS): Not significant

*Significantly different from the control ($p<0.05$)
** Significantly different from the control ($p<0.01$)
*** Significantly different from the control ($p<0.001$)

Table 4: Effects of iron overload in thalassemic patients, after blood transfusion, on lipid peroxidation and antioxidant status.

Parameters / Groups	Malondialdehyde (MDA)	Peroxy nitrite	Superoxide Dismutase (SOD)	Vitamin A	Vitamin C	Vitamin E	Ceruloplasmin	Glutathione (GSH)	Uric Acid	Albumin
	µmol/L	µmol/L		µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	g/L
Post-Blood Transfusion Without Iron Overload	4.79 ± 0.417	75.8 ± 5.462	0.127 ± 0.008	1.69 ± 0.249	46.45 ± 5.735	24.34 ± 3.165	298.7 ± 24.372	11.92 ± 1.349	177.75 ± 18.496	48.13 ± 3.265
	*	**	***	**	***	***	***	***	***	***
Post-Blood Transfusion With Iron Overload	5.54 ± 1.169	83.3 ± 7.513	0.143 ± 0.013	1.44 ± 0.267	34.42 ± 9.449	16.73 ± 3.048	266.44 ± 23.249	8.97 ± 2.440	265.00 ± 74.337	37.76 ± 7.335

- Values are expressed as means ± SD from 20 subjects per group.
- (NS): Not significant

*Significantly different from the control ($p<0.05$)
** Significantly different from the control ($p<0.01$)
*** Significantly different from the control ($p<0.001$)

Discussion

It has been postulated that the biochemical and metabolic changes of thalassemic patients are associated with a constant oxidative stress within the red cell caused by precipitation of excess alpha-globin chains, and release of free iron[21]. The measurement of the peroxidation products, together with the evaluation of the antioxidants may be the simple measurement of iron overload due to blood transfusion in thalassemia[9]. Increased plasma MDA level, which is measured by the thiobarbituric acid reaction substance (TBARS) methods, was found in beta-thalassemia patients[22], where MDA is a good indicator of oxidative damage. The increased in serum MDA levels in patient with thalassemia in our study, as shown in tables (3 and 4) can be compared with those obtained by other investigators[9] . In addition, Peroxynitrite, was measured in the present study. Serum levels of this pro-oxidants was increased significantly in thalassemic patient with iron overload compared with other without iron overload.. Nitric oxide ($NO^{\cdot}$) contains unpaired numbers of electrons and are therefore free radicals. It was first recognized as a distinct gas in 1772 by Joseph Priestley[23]. It can be produced by vascular endothelium. It can react with another endogenous free radical, superoxide, to produce a reactive intermediate, peroxynitrite ($ONOO^{-}$), which is a powerful oxidant, able to damage many biological molecules, and can decompose at acid pH to release small amounts of hydroxyl radicals.[24]

$$ONOO^{-} + H^{+} \longrightarrow {}^{\cdot}OH + NO_2$$

As a result of continuous blood transfusions, our patients might be subjected to peroxidative tissue injury by the secondary iron overload[4]. These finding might support the idea of iron overload in beta-thalassemia leads to an enhanced generation of reactive oxygen species and oxidative stress. Iron is also an important nutritional metal for many physiological functions[25]. However, persons receiving multiple transfusions as part of the treatment for thalassemia, are faced with problem of iron overload and consequent metabolic dearrangements. Under normal circumstances there is virtually no free iron. All irons are protein bound. In patients with thalassemia, the irons liberated from the haemoglobin saturate the transferrins, and the transferrins transfer the iron on to a storage iron protein called apoferritin. When the ferritin is saturated, another storage iron protein called hemosiderin is formed.[7] On this bases, iron overload, in the present study, was not created immediately until multiple blood transfusion were performed. For this reason, MDA level was not altered significantly in patients after blood transfusion was achieved immediately.During the 1984, the Cambridge chemist H.J.H. Fenton described a reaction between iron salts and H_20_2 that caused oxidative damage to organic molecules such as tartaric acid[26]. The Fenton reaction is widely represented as in the following Eqs..

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{-} + {}^{\cdot}OH$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HO_2^{\cdot} + H^{+}$$

overall reaction

$$\text{Iron salt} + 2\ H_2O_2 \longrightarrow 2H_2O + O_2$$

In the process of changing from the ferrous to the ferric state, an electron is transferred from iron to oxygen to make superoxide as shown in in the following Eq. 5.

$$Fe^{2+} + O_2 \rightleftharpoons Fe^{2+}O_2 \leftrightarrow Fe^{3+}\ O_2^{-} \rightleftharpoons Fe^{3+} + O_2^{\cdot -}$$

Therefore, the presence of iron complexes stimulate peroxidation by peroxide decomposition of unsaturated fatty acids generating alkoxyl ($LO^{\cdot}$) and peroxyl ($LO_2^{\cdot}$) radicals[27]. Oxidative stress is a prominent contributor to the premature destruction of RBC as well as anemia in thalassemia. The oxidative status within red blood cells is maintained by the balance between oxidative systems, such as Reactive Oxygen Species (ROS), and antioxidative systems, like reduced glutathione (GSH)[28]. Glutathione is a low-molecular-mass, thiol-containing tripeptide, It is synthesized endogenously in the human cell, It acts to protect the body against the production of free radicals[29]. As a result, increased production of H_2O_2 in thalassemia major induces glutathione peroxidase, an enzyme that lead to decreased glutathione concentration[30]. On this basis, a significant decrease in serum glutathione concentration was noticed in the present work in patients with iron overload, serving as oxidative stress, versus those from non-iron overload group. During the course of metabolism, a superoxide anion is produced. Normally the superoxide anion is converted by the enzyme SOD to produce H_2O_2[31], which in turn is converted to innocuous compounds by the action of catalase and peroxidase. But if free ferrous iron is available it reacts with H_2O_2 to produce hydroxyl radical which is an extremely reactive species leading to depolymerisation of polysaccharide[32]. The production of free radicals due to thalassemia was associated with a significant decrease in enzymatic antioxidants activity like SOD as shown in the present study in tables (3 and 4). It can be compared with other research of Şimşek, *et al.*,[9] in which erythrocyte SOD (a preventive antioxidant) levels was found to be higher in beta-thalassemic patients than healthy children.

Moreover, marked changes in the other antioxidant pattern were also observed in all patients. Evidence is presented of a net drop in the concentration of vitamin A, vitamin C, and vitamin E in all patients with iron overload when compared with those without iron overload, as shown in tables (3 and 4). On the other hand, a significant increase of nutrient antioxidants was observed in all patients received blood transfusion in comparison with the same patient before receiving blood transfusion as shown in tables (1 and 2). As vitamin C is essential to maintain vitamin E status and function, depletion of vitamin C, in turn, contributes to further exacerbate the depletion of vitamin E[33]. Although efficient antioxidants such as uric acid and bilirubin are high, they cannot compensate for lipid-soluble antioxidants, so that tissue lipid compartments are not suitably preserved. The observed depletion of serum levels of vitamin E and vitamin A can be explained by impairment of liver function and peroxidative processes causing a substantial reduction of serum lipids, producing a concurrent reduction of serum vitamin E and vitamin A[34]. A significant drop in nutrient antioxidants obtained in the present work can be compared with another research made by Livrea et al.,[4] who observed a significant decline in the concentration of vitamin A, vitamin C, and vitamin E in all patients affected with thalassemia due to continuous blood transfusions. Another antioxidant parameter involves uric acid and albumin was included in the present study. Uric acid was increased significantly in opposite to albumin which decreased significantly in patients with iron overload. Similarly, Livrea et al.,[4] showed an increase of uric acid whereas serum albumin was in the normal range in all thalassemic patients.Uric acid provides an excellent example of the adaptation of the organism to oxidative stress. It is a cellular waste product originating from the oxidation of hypoxanthine and xanthine by xanthine oxidase and dehydrogenase. High uric acid levels may provide efficient antioxidant activity for the organism. Urate, the physiological state of uric acid, reacts with hydroxyl radicals producing a stable urate radical that can be regenerated by ascorbate. This compound can act with peroxyl radicals, superoxide dismutase, ozone, nitrous oxide$^{\cdot}$, and other nitrogen-oxygen radicals. Urate also protects protein from nitration; it can chelate metal ions, such as copper and iron, and prevent them from participating in redox cycling[35]. Ceruloplasmin, is a chain breaking antioxidant, it can protect the body against the deleterious effects of oxygen free radical (OFR)[36]. The antioxidant property of ceruloplasmin is through its oxidase activity, which is directed towards ferrous ions (ferroxidase activity). It also inhibits ferrous ion stimulated lipid peroxidation and is known to be involved in the decomposition of lipid peroxides[37]. Serum Ceruloplasmin was significantly lower in the iron-overload group compared to the non iron-overload patients, as shown in tables (3 and 4). The inverse relationship between serum ceruloplasmin and the level of iron is indicating the anti-thalassemic nature of this antioxidant.

Conclusion

These results point out that the iron-induced oxidative stress in thalassemia may play a major role in the depletion of most

antioxidants, including lipid-soluble antioxidants. Our results suggest that the measurement of peroxidation products, matched with evaluation of antioxidants, may be a simple measure of oxidative stress in thalessemia.

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