

Estimation of the Level of Total Carbonyl and Malondialdehyde in Thalassemic Patients and Study their Correlation with Iron Status Parameters

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Abstract

In the present study, an attempt is carried out to estimate the degree of iron overload in thalassemia major patients in addition to the measurement of lipid peroxidation end product, malondialdehyde (MDA), and protein end product, total carbonyl. The level of these compounds will indicate the risk of tissue damage caused by oxidative stress and iron overload in thalassemia patients.

One hundred and eighteen Arabic Iraqi patients with major thalassemia were participated in the present study. Their age range was 4-12 years old. Thirty apparently healthy children were selected as control group. Serum levels of iron and total Iron Binding Capacity (TIBC) were measured spectrophotometrically while unsaturated iron-binding capacity (UIBC), estimated total iron body stores (ETIBS), transferrin saturation percentage (TS%) and transferrin concentration were calculated mathematically. Serum ferritin, MDA, and total carbonyl were measured using ELISA technique.

The results showed significant increase ($p < 0.05$) in all iron indices of thalassemic patients in comparing with healthy control group except TIBC, UIBC, and transferrin concentrations, which decreased significantly ($p < 0.05$) in those patients in comparing with control group. Serum total carbonyl and MDA are increased significantly in thalassemia patients as compared with control group.

It can be concluded that Iraqi thalassemic patients are at high risk for iron overload and iron-induced toxicities. Protein carbonyls and MDA are elevated in those patients. These patients are prone to tissue injury caused by oxidative stress. There is no statistically significant correlation noticed between each serum MDA and total carbonyl with every component of iron status. The mechanism of action of iron in the body is differ from that of the oxidation products of proteins and lipids (total carbonyl and MDA, respectively).

Key words: thalassemia, iron, malondialdehyde, total carbonyl

Introduction:

Thalassemia in Iraq is a real problem due mainly to the deficiency in the equipments and drugs during different periods of wars and lack of security. Out of 1064 couples recruited from the Public Health Laboratory in Basra, southern Iraq, about 5% had beta-thalassemia trait and the carriers of major beta-globin disorders comprised 11.48% ⁽¹⁾. In Najaf Governorate (about 1.2 million people in 2010), till July 2010 there are (503) patients' files who are still treating in the "Thalassemia Unit" at AL-Zahra'a Teaching Hospital. Thalassemia major can result in severe complications and even death due to absence of hemoglobin A synthesis and the patients are more dependent on transfusion of blood ⁽²⁾. Iron metabolism disorders are common in the human including both iron deficiency anemia and excessive iron storage. Iron is essential for oxidation-reduction catalysis and bioenergetics, but unless appropriately

shielded, iron plays a key role in the formation of toxic oxygen radicals that can attack all biological molecules. Hence, specialized molecules for the acquisition, transport (transferrin), and storage (ferritin) of iron in a soluble nontoxic form have evolved ⁽³⁾Reactive oxygen species (ROS) degrade polyunsaturated lipids, forming malondialdehyde that mainly exists in the enol form ⁽⁴⁾. This compound is one of the many *reactive electrophile species* that cause toxic stress in cells and form covalent protein adducts which are referred to as advanced lipoxidation end products ⁽⁵⁾. Protein oxidation is defined as the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary byproducts of oxidative stress. Reactive carbonyl compound such as aldehydes and dicarbonyls, including hydroxyalkenals, glyoxal and methylglyoxal, exhibit a large panel of biological properties. These aldehydes react on cellular and tissular proteins to form adducts advanced lipid peroxidation end product that induce protein dysfunctions and alter cellular responses ⁽⁶⁾. In the present study, the iron status was estimated in thalassemic patients and compared with control group. The second aim is to study the possible dependent of MDA and total carbonyl levels on the concentration of iron status parameters in blood transfusion dependent thalassemic patients were examined.

Subjects and Methods

A-Patients: One hundred and eighteen Arabic Iraqi patients with β -thalassemia major were participated in the present study. Their age range was 4-12 years old. These patients were registered as β -thalassemic major patients in "Thallasemia Unit" at "AL-Zahra'a Teaching Hospital" in Najaf city, Iraq. The diagnosis was established by clinical symptoms, hematological, and hemoglobin high-pressure liquid chromatography (HPLC) analysis. Hemoglobin HPLC were done using (VARIANTTM β -Thalassemia Short Program) HPLC instrument. All these patients were on blood transfusion as a part of their treatment regimen. Serum C-reactive protein (CRP) is negative in all samples (CRP<6mg/L). A normal [C-reactive protein](#) can be used to exclude elevated ferritin caused by acute phase reactions ⁽⁷⁾. The present study excluded the patients with apparent diabetes mellitus, infection and inflammation, heart diseases, and patients from non-Arabic ethnic group.

B-Controls: Thirty apparently healthy children were selected as a control group. Their age ranges were comparable to that of patients. None of these subjects was anemic or has an obvious systemic disease.

Measurements: Blood samples were collected from individuals in the morning in plain tubes and the serum separated by centrifugation after clotting. Serum levels of iron were estimated using Ferrozine colorimetric method ⁽⁸⁾, total Iron Binding Capacity (TIBC) were estimated colorimetrically by the following procedure ⁽⁹⁾: An excess of iron is added to the serum to saturate the transferrin. The unbound iron is precipitated with basic magnesium carbonate. After centrifugation, the iron in the supernatant was determined. Unsaturated iron-binding capacity (UIBC), the amount of protein (apotransferrin) still available to bind iron, can be estimated from the formula: $UIBC = TIBC - \text{Serum iron}$.

The ferritin quantitative kit based on a solid phase enzyme-linked immunosorbent assay (ELISA) was supplied by Monobind[®] Inc. USA. The assay system utilizes one rabbit anti-ferritin antibody for solid phase (microtitre wells) immobilization and a mouse monoclonal anti-ferritin antibody in the antibody-enzyme horseradish peroxidase (HRP) conjugate solution.

Estimated Total Iron Body Stores (ETIBS) were calculated using the following formula ⁽¹⁰⁾:

$$ETIBS \text{ (in } \mu\text{mol)} = (\text{serum ferritin in } \mu\text{g/L}) * 143$$

Transferrin saturation percentage (TS%) was calculated from the following equation ⁽¹¹⁾:

$$TS\% = (\text{Serum Iron/TIBC}) * 100\%$$

Transferrin concentration was calculated using the following formula ⁽¹²⁾:

$$\text{Transferrin Conc. (g/L)} = \text{S.Iron (}\mu\text{mol/L)} / (TS\% * 3.98)$$

The formula is based on the maximal binding of 2 mol Fe³⁺/mol of transferrin and a molecular weight of 79,570gm/mol for transferrin ⁽¹²⁾.

Total carbonyl levels were measured using CellBiolabs[®] Protein Carbonyl ELISA Kit. Briefly, bovine serum albumin (BSA) standards or protein samples are adsorbed onto a 96-well plate for two hrs at 37°C. The protein carbonyls present in the sample or standard are derivatized with dinitrophenylhydrazine (DNPH) to DNP-hydrazon and probed with anti-DNP antibody, followed by an HRP conjugated secondary antibody. The protein carbonyl content in unknown sample is determined by comparing with a standard curve that is prepared from predetermined reduced and oxidized BSA standard ⁽¹³⁾.

CellBiolabs[®] MDA Adduct ELISA Kit was used to measure MDA level. It is an enzyme immunoassay for detection and quantization of MDA-protein adducts. The quantity of MDA adduct in protein samples is determined by comparing its absorbance with that of a known MDA-BSA standard curve. BSA standard or protein samples are adsorbed onto a 96-well plate for 2 hours at 37°C. The MDA-protein adducts present in the sample or standard are probed with an anti-MDA antibody, followed by an HRP conjugated secondary antibody. The MDA protein adducts in an unknown sample is determined by comparing with a standard curve that is prepared from predetermined MDA-BSA standard ⁽¹⁴⁾.

Biostatistical analysis:- The results were expressed as (mean±standard deviation). Pooled t-test was used for the comparison between the patients and control groups in the measured parameters. Correlation coefficient (r) values were calculated using Microsoft Excell[®] 2007 program.

Results and Discussion:

The results of iron indices in thalassemic patients and control group are presented in Table (1). There is a significant increase ($p < 0.05$) in all iron indices of thalassemic patients in comparing with healthy control group except TIBC, UIBC, and transferrin concentrations, which decrease in those patients in comparing with control group. The results also showed that total carbonyl and MDA level were significantly increased ($p < 0.05$) in thalassemic patients in comparing with healthy control group.

The results of iron indices in thalassemic patients indicated a state of iron overload. In iron overload state, the iron which is initially stored as ferritin, is deposited in organs as haemosiderin and this is toxic to tissue, probably at least partially by inducing oxidative stress ⁽¹⁵⁾. Most humans prevent iron overload solely by regulating iron absorption. Those who cannot regulate absorption well enough get disorders of iron overload. In these diseases, the toxicity of iron starts overwhelming the body's ability to bind and store it. Patients with thalassemia major accumulate body iron over time as a consequence of continuous red blood cell transfusions which cause hepatic, endocrine, and cardiac complications ^(16, 17). The hemolytic anemia in

thalassemia, caused by unstable hemoglobin variants and the heme iron released from the hemolysis, has been more consistently associated with increased risk of coronary heart diseases and cardiovascular mortality^(18, 19). Furthermore, the increase in ferritin in thalassemic patients may be due to acute [malnourishment](#) that accompanied with anemia of thalassemic patients⁽⁷⁾. The other potential cause for hyperferritinemia in thalassemic patients includes the fact that these patients suffer from anoxia due to low hemoglobin level in their blood. Ferritin concentration has been shown to increase in response to stresses such as anoxia⁽²⁰⁾. Others have found increased production of ferritin mediated by several cytokines, mainly interleukin IL-1, IL-6 and TNF α ⁽²¹⁾.

The results of MDA in thalassemia patients showed a significant increase ($p < 0.05$) by 1.3-fold relative to control group (Table (1)). In comparing with Walter *et al* (2006) study⁽²¹⁾, MDA was significantly increased 1.8-fold in thalassemic patients relative to controls indicating an increase in the superoxidation of lipids that due to increase oxidative stress in thalassemia. Meerang *et al* (2009)⁽²²⁾ found that iron overload in thalassemia patients can stimulate lipid peroxidation. Free extracellular iron and intracellular iron species that have been identified in thalassemic blood cells are responsible for generation of oxidative stress through reaction with hydrogen peroxide to form the deleterious hydroxyl radical that damages cellular macromolecules,⁽²³⁾ by catalyzing formation of oxygen radicals over the antioxidant capacity of the cell. Consequently, there is a rationale for iron chelation to eliminate the free-iron species, which in this respect, act like antioxidants⁽²⁴⁾.

The correlation coefficient values (r) for iron indices parameters with each MDA and total carbonyl level were presented in Table (2). The results showed slight dependence of MDA and total carbonyl on iron indices parameters in thalassemic patients extracted from low values for r -value.

Although increase serum iron is well known to affect the oxidative stress compounds in the body, there is no direct association in the literature between serum iron parameters with MDA and total carbonyl. In one research, it's found that, the strongest predictors of elevated MDA in thalassemia patients were liver iron concentration⁽²¹⁾ which required a special type of MRI techniques. Hence, the estimation of liver iron is more important than serum iron in estimation of the effect of iron status increase on MDA level. Biomarkers of oxidative damage are increased in thalassaemia⁽²⁵⁾. In spite of the iron overload in thalassemia disease, oxidants originate from sources other than the iron loaded tissues. For example, in β -thalassaemia the excess unpaired α -haemoglobin chains denature and autoxidise, contributing to increased oxidants, ineffective erythropoiesis, haemolysis and shortened erythrocyte survival⁽²⁶⁾. Malondialdehyde, a product of lipid peroxidation and protein carbonyls, representing oxidation of the circulating proteins, are elevated in thalassaemia^(27, 28).

Concerning duration of chronic transfusion, elevated MDA is probably a real-time marker of oxidative injury⁽²⁹⁾ and indicative of current liver iron concentration. Duration of chronic transfusion correlates better with cumulative tissue injury⁽³⁰⁾. Walter *et al* (2006)⁽²¹⁾ suggested that this may explain why duration of transfusion is not a predictor of MDA in multivariate analysis. Furthermore, plasma MDA may be increased in thalassaemia because of peroxidation of tissues that leak MDA into the plasma. Parallel to alanine transferase enzyme, plasma MDA may also rise partly as a result of possible liver lipid peroxidation and leakage into the plasma. It is an attractive suggestion that MDA may leak from the liver because of its strong correlation to liver iron concentration in multivariate analysis⁽²¹⁾.

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تقدير الكربونيل الكلي والمالوندايديهايد عند مرضى الثلاسيميا وعلاقتهما بمتغيرات الحديد

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الخلاصة

في هذه الدراسة أجريت محاولة لتحديد درجة زيادة الحديد في أجسام مرضى الثلاسيميا الشديدة بالإضافة إلى قياس تركيز النواتج النهائية لأكسدة الدهون (مالون ثنائي الديهايد (MDA) وأكسدة البروتينات (الكربونيل الكلي)

في مصل الدم حيث أن مستوى هذه المواد يؤشر إلى احتمالية التعرض لتلف الأنسجة الذي ينتج عن ارتفاع تركيز الحديد أو من الشدة التأكسدية.

اشترك في هذه الدراسة مئة وثمانية من مرضى الثلاسيميا الشديدة العراقيين العرب تتراوح أعمارهم بين 4-12 سنة بالإضافة ما اشترك ثلاثون شخصا سليما كمجموعة سيطرة.

تم قياس تركيز الحديد وسعة الارتباط بالحديد الكلية (TIBC) باستخدام الطرق الطيفية اللونية بينما تم حساب كل من الدوال الآتية رياضيا: سعة الارتباط بالحديد غير المشبع (UIBC)، مخزون الحديد الكلي المخمن (ETIBS)، نسبة تشبع الترانسفيرين (TS%)، وتركيز الترانسفيرين. تم قياس تراكيز الفيريتين و MDA و الكربونيل الكلي في المصل بتقنية الـ HPLC. أظهرت النتائج ارتفاعا معنويا ($p < 0.05$) في مؤشرات الحديد لدى المرضى مقارنة بمجموعة السيطرة عدا تراكيز الترانسفيرين و TIBC و UIBC والتي أظهرت انخفاضا معنويا ($p < 0.05$). كما أن تراكيز MDA والكربونيل الكلي كانت أعلى عند مرضى الثلاسيميا مقارنة بمجموعة السيطرة ($p < 0.05$). يمكن الاستنتاج من هذه النتائج أن مرضى الثلاسيميا العراقيين لديهما احتمال عالي للتعرض إلى عمليات التسمم المختلفة الناتجة من فرط الحديد في أجسامهم. كما أن لديهم تراكيز عالية من MDA و الكربونيل الكلي. إن هؤلاء المرضى معرضين لتلف الأنسجة الناتجة من الشدة التأكسدية. كما لا توجد علاقة احصائية تربط بين تراكيز متغيرات الحديد مع دلائل الشدة التأكسدية المقاسة. ويفسر ذلك باختلاف ميكانيكيات تكون وعمل هذه المركبات داخل الجسم.

منااتج الكلمات: مالون ثنائي الديهايد-الكربونيل الكلي-الثلاسيميا