Lack of correlation between non-labile iron parameters, total carbonyl and malondialdehyde in major thalassemia

Hussein Kadhem Al-Hakeim,* Furquan Muein Auda and Basim Muhammed Ali

Department of Chemistry, College of Science, Kufa University, Najaf 221, Iraq

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Thalassemia patients are at high risk of iron-induced toxicity and oxidative stress consequences. The present cross-sectional study is conducted to determine whether or not lipid peroxidation or protein oxidation is correlated with iron parameters in patients with thalassemia major. To prove this hypothesis, malondialdehyde and total carbonyl were correlated with the degree of excess iron concentration in the patients. A total of 118 Arabic Iraqi patients and 30 healthy children were participated in the present study. Results showed a significant increase (p<0.05) in serum total carbonyl, malondialdehyde and the iron indices of patients as compared with the control group. Total iron binding capacity and transferrin concentrations decreased significantly (p<0.05) in patients with thalassemia compared with the control group. The results also showed a lack of a significant correlation between each serum malondialdehyde and total carbonyl with each component of iron status. In conclusion, total carbonyls and malondialdehyde were increased in thalassemia patients indicating the vulnerability of these patients to tissue injury caused by oxidative stress. The formation of total carbonyl and malondialdehyde are independent of excess non-labile iron concentration, indicating that different mechanisms are involved in injury caused by the labile iron and in the formation of oxidation end products.

Key Words: Thalassemia, non-labile iron, malondialdehyde, total carbonyl

Thalassemia major can result in severe complications and death because of deficiency in or lack of synthesized hemoglobin A; patients with this disease are dependent on blood transfusion. β-Thalassemia is an important health problem in different Iraqi governorates because this disorder displays a high genetic carrier rate and frequency of consanguineous marriages. However, genetic carriers of β-thalassemia account for a higher percentage than patients manifesting β-thalassemia and comprise a significant percentage of the total population. Iron metabolism disorders, including iron deficiency anemia and excessive iron storage, are common in humans. Iron is essential for oxidation-reduction catalysis and bioenergetics; however, this element may pose health risks because of the formation of toxic oxygen radicals that can attack biological molecules if such radicals are not eliminated properly. This condition is possible in the presence of excess iron concentrations as in transfusion-dependent patients with thalassemia. Hence, specialized molecules for the acquisition, transport (transferrin), and storage (ferritin) of iron in a soluble, non-toxic form have evolved.

Oxidative stress is important in the pathophysiology of thalassemia and other congenital and acquired hemolytic anemia cases. Reactive oxygen species (ROS) degrade polyunsaturated lipids and form malondialdehyde (MDA), which is mainly present in enol form. MDA is one of many reactive electrophilic species that cause toxic stress in cells and form stable covalent protein adducts that are referred to as advanced lipoxidation end products. These modifications by MDA can cause both structural and functional changes in oxidized proteins. This naturally occurring ROS is a marker of oxidative stress and used as a biomarker to determine oxidative stress level in organisms.

Organisms are constantly exposed to various ROS that induce protein oxidation. Protein carbonyls are efficient biomarkers of oxidative stress because of the relatively early formation and relative stability of carbonylated proteins. However, the nature of relationships among high levels of protein carbonyls, oxidative stress, and diseases remains uncertain. Reactive carbonyl compounds, such as aldehydes and dicarbonyls, exhibit many biological properties. Aldehydes react with proteins to form adducts that induce protein dysfunctions and alter cellular responses.

The present study was conducted to examine the possible dependence of MDA and total carbonyl formation on non-labile iron status parameters in blood transfusion-dependent patients with thalassemia.

Subjects and Methods

Patients. A total of 118 Arabic Iraqi male patients (aged 4 years to 12 years) with β-thalassemia major participated in the present study. These patients were registered as patients with β-thalassemia major in Thalassemia Unit at Al-Zahra’a Teaching Hospital in Najaf City, Iraq. This condition was diagnosed by observing clinical symptoms and conducting hematological and hemoglobin HPLC analysis. Hemoglobin HPLC analysis was conducted using an HPLC instrument (VARIANT™ β-Thalassemia Short Program).

The patients received approximately 15 ml of packed red blood cells/kg of body weight at each transfusion (2–6 week intervals) to maintain hemoglobin levels above 9.5 g/dl. Patients were under chelation therapy with desferrioxamine B (Desferal) at least four times a week, as a subcutaneous infusion. The range of dose was 30 to 60 mg/kg body weight/day. The median duration of thalassemia was 6.2 years with a range of 1.8 to 9.3 years. The duration of the treatment was 3.1 ± 8.7 years. All participated patients had not undergone splenectomy. Endocrinologic, hematologic and cardiac evaluations were performed regularly by the physicians. Blood samples from patients were collected after 7–10 days after the last transfusion and just before the next transfusion.

Serum C-reactive protein (CRP) is negative in all of the samples...
Transferrin saturation percentage (TS%) was calculated from the standard curve prepared from predetermined MDA–BSA standard in an unknown sample was determined by comparing with a standard curve. Excess iron concentration was added to the serum to saturate transferrin. The unbound iron was then precipitated with basic magnesium carbonate. Afterward, iron in the supernatant was determined. Unsaturated iron-binding capacity (UIBC) and the amount of protein (apotransferrin) still available to bind iron can be estimated from the formula, UIBC = TIBC – Serum iron.

A ferritin quantitative kit based on a solid phase enzyme-linked immunosorbent assay (ELISA) was supplied by Monobind® Inc. (Lake forest, CA). The assay system utilized one rabbit anti-ferritin antibody in solid phase (microtiter 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 equation:

\[
\text{ETIBS (in μmol) = (Serum ferritin in μg/L)} \times 143
\]

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

\[
\text{TS\%} = \left(\frac{\text{Serum Iron}}{\text{TIBC}}\right) \times 100\%
\]

Transferrin concentration was calculated using the following equation:

\[
\text{Transferrin Conc. (g/L) = S. Iron (μmol/L)/(TS\% \times 3.98)}
\]

This equation is based on the maximal binding of 2 mol Fe\(^{3+}\)/mol of transferrin and a molecular weight of 79,570 g/mol of transferrin.

Total carbonyl concentrations were determined using CellBiolabs® Protein Carbonyl ELISA kit. Briefly, bovine serum albumin (BSA) standards or protein samples were adsorbed on a 96-well plate for 2 h at 37°C. The protein carbonyls present in the sample or standard were derivatized with dinitrophenylhydrazine to dinitrophosphonyl (DNPH)-hydrazone and then probed with anti-DNPH antibody and HRP-conjugated secondary antibody. The protein carbonyl content in an unknown sample was determined by comparing with a standard curve prepared from a predetermined reduced and oxidized BSA standard.

CellBiolabs® MDA Adduct ELISA kit, an enzyme immunoassay used to detect and quantify MDA-protein adducts was used to determine MDA concentration. The quantity of MDA adduct in protein samples is determined by comparing the absorbance of MDA with that of a known MDA–BSA standard curve. BSA standard or protein samples were adsorbed on a 96-well plate for 2 h at 37°C. The MDA-protein adducts present in the sample or standard were probed with an anti-MDA antibody and then with an HRP-conjugated secondary antibody. The MDA protein adducts in an unknown sample was determined by comparing with a standard curve prepared from predetermined MDA–BSA standard.

Statistical Analysis. The distribution types of the variables results were examined using Kolmogorov-Smirnov test. The results of the analysis divided the variables into two types according to the statistical distribution; the normally distributed variables and nonparametric variables. For the variables that are normally distributed, the results were expressed as (mean ± SD). Pooled t test has been used for the comparison between the patients and control groups. Spearman’s correlation coefficients (\(\rho\), \(\rho\)) were calculated to estimate the correlation between parameters. For the nonparametric variables, that are not normally distributed, the results have been expressed as medians, in addition to (mean ± SD). Mann-Whitney U test was used for the comparison between the patients and control groups. Spearman’s correlation coefficients (\(\rho\), \(\rho\)) were calculated to estimate the correlation between parameters. All statistical analysis was performed using SPSS Statistics ver. 19.0.1 multilingual program (2010), IBM, Armonk, New York. Forecasting study was performed using “Regression Forecasting Model” software purchased from Business Spreadsheets, USA.

Results and Discussion

The iron indices in patients with thalassemia and the control group are presented in Table 1. A significant increase (\(p<0.05\)) in all iron indices was observed in patients with thalassemia compared with the healthy control group except TIBC, UIBC, and transferrin concentrations. These concentrations decreased in patients the compared with the control group. Total carbonyl and MDA concentrations significantly increased (\(p<0.05\)) in patients with thalassemia compared with the healthy control group. The list of \(\rho\) of the iron indices of each MDA and total carbonyl level is presented in Table 2.

The results showed that MDA and total carbonyl were slightly dependent on iron index parameters in patients with thalassemia as indicated in the low \(r\), however, this dependence was not significant. These results are different from the results of Livrea et al.\(^{(15)}\) and Naithani et al.\(^{(16)}\) They reported serum iron level showed correlation with oxidative stress markers in the beta thalassemia major patients. Both studies did not exclude the patients with positive CRP test as they excluded in the present work. Therefore, the correlations and huge changes in the measured parameters may be due to inflammation rather than thalassemia disorder. Furthermore, in Livrea et al.\(^{(15)}\) research, the patients mostly adult (age mean 21 ± 10 year), patients were male and females, more than half of patients had positive hepatitis C virus and many of them had another diseases. Therefore the correlations and other results may be due to the differences in the patient’s criteria. Another important factor is the low number of patients involved in the studies of Livrea et al.\(^{(15)}\), Naithani et al.\(^{(16)}\) and Dasgupta et al.\(^{(17)}\) in comparing with the higher number of patients in the present research in addition to the involvement of patients with positive CRP test and race difference lead to different results and correlations.

The iron indices in patients with thalassemia indicated excess iron concentrations. In this condition, iron initially stored as ferritin is deposited in organs as hemosiderin, a toxic substance affecting tissues at least partially by inducing oxidative stress. Iron induces toxicity when the body fails to deal safely with this excess iron concentration. The outcome of multiple blood transfusions and an inappropriate transferrin concentrations. These concentrations decreased in patients the compared with the control group. Total carbonyl and MDA concentrations significantly increased (\(p<0.05\)) in patients with thalassemia compared with the healthy control group. The list of \(\rho\) of the iron indices of each MDA and total carbonyl level is presented in Table 2.

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been identified in thalassemic blood cells are responsible for oxidative stress by reacting with hydrogen peroxide to form deleterious hydroxyl radicals that damage cellular macromolecules by catalyzing oxygen radical formation; this process then induces stress on the antioxidant capacity of cells.\(^{(25)}\) As a result, iron chelation can eliminate free-iron species, which function as antioxidants. In addition, antioxidants such as vitamin E and polyphenols are also capable of ameliorating increased oxidative stress parameters, along with iron chelators, may substantially improve the pathophysiological characteristics of hemolytic anemia, particularly thalassemia.\(^{(26)}\) Nevertheless, these findings require further investigation and discussion on the basis of the principles of oxidative stress and the mechanism of iron toxicity in patients with thalassemia.

Correlation coefficients (\(p\)) in Table 2 showed no statistically significant correlation between iron status parameters and MDA or total carbonyl. A similar finding of oxidative stress with excess iron concentration in thalassemia has been described in many studies. However, the direct correlation between iron status parameters and MDA or total carbonyl is not well examined; in the present study, this correlation was statistically analyzed. However, some researches mentioned that excess iron concentration can stimulate lipid peroxidation\(^{(27)}\) which is a well-defined mechanism of cellular damage in animals. Increased levels of erythrocyte free reactive iron and lipid peroxidation end product are associated with low erythrocyte glutathione level. This result indicates non-heme iron-mediated cellular damage in \(\beta\)-thalassemia.\(^{(28)}\)

Many possible factors may explain the lack of correlation between iron parameters with MDA and total carbonyl. Continuous blood transfusion in patients with thalassemia affects the concentrations of blood components, thereby leading to variable findings in MDA and total carbonyl contents. Considering the duration of chronic transfusion, Kadiiska et al.\(^{(29)}\) showed that increased MDA is probably a real-time marker of oxidative injury and correlated more significantly with cumulative tissue injury.\(^{(30)}\) Increased plasma MDA levels in thalassemia may result from several mechanisms. First, plasma MDA can be enhanced in patients with thalassemia because this factor may be dependent on the amount of circulating erythrocyte precursors and peripheral blood erythrocytes that contain a high density of unpaired \(\alpha\)-hemoglobin chains.\(^{(31)}\) Furthermore, plasma MDA may be increased in thalassemia because of peroxidation in tissues; as a result, MDA leaks into the plasma. Similar to alaninetransferase, plasma MDA may increase partly as a result of possible liver lipid peroxidation and leakage into the plasma. MDA may also leak from the liver as indicated by the strong correlation between MDA and liver iron concentration in multivariate analysis.\(^{(32)}\) Second, many oxidative stress reactions occur intracellularly, and some of the molecular products of oxidation may enter the

\[ \text{Table 2. Correlation coefficient (}\rho\text{) of iron indices with MDA and total carbonyl in thalassemic patients} \]

| Iron indices | MDA | Total carbonyl |
|--------------|-----|----------------|
| Hb           | 0.01 | -0.07          |
|              | 0.74 | 0.68           |
| PCV          | 0.02 | -0.04          |
|              | 0.25 | 0.64           |
| Ferritin     | 0.11 | 0.29           |
|              | 0.6  | 0.06           |
| ETIBS        | 0.11 | 0.24           |
|              | 0.42 | 0.09           |
| S.Iron       | -0.15| 0.14           |
|              | 0.62 | 0.52           |
| TIBC         | -0.2 | 0.21           |
|              | 0.12 | 0.18           |
| TS %         | 0.07 | -0.15          |
|              | 0.63 | 0.46           |
| Transferrin  | -0.18| 0.33           |
|              | 0.25 | 0.36           |
| UIBC         | 0.23 | 0.27           |
|              | 0.1  | 0.08           |

Hb, hemoglobin; PCV, packed cell volume; ETIBS, estimated total iron body stores; TIBC, total iron-binding capacity; TS %, transferrin saturation percentage; UIBC, unsaturated iron-binding capacity; MDA, malondialdehyde.

Table 1. Parameters of thalassemic patients in comparison with control group

| Parameters    | Control group | Thalassemia group | \(p\) value |
|---------------|---------------|-------------------|-------------|
| Hb (g/dl)     | 12.84 ± 1.05  | 7.01 ± 1.70       | \(<0.001\)  |
| PCV %         | 41.51 ± 3.15  | 24.41 ± 4.13      | \(= 0.008\) |
| Ferritin (pM) | 164.34 ± 115.49 | 1133.06 ± 613.94 | \(<0.001\)  |
| ETIBS (mmol)  | 10.46 ± 7.35  | 75.79 ± 39.35     | \(<0.001\)  |
| S.Iron (μM)   | 18.04 ± 6.36  | 33.91 ± 14.07     | \(<0.001\)  |
| TIBC (μM)     | 56.17 ± 9.96  | 45.55 ± 13.12     | \(= 0.017\) |
| TS %          | 32.72 ± 11.52 | 75.84 ± 20.99     | \(<0.001\)  |
| Transferrin (g/L) | 0.14 ± 0.03 | 0.12 ± 0.03       | \(= 0.021\) |
| UIBC (μM)     | 38.13 ± 10.68 | 12.07 ± 11.79     | \(<0.001\)  |
| MDA (μM)      | 0.81 ± 0.18   | 1.04 ± 0.30       | \(= 0.014\) |
| T. Carbonyl (μM) | 3.56 ± 0.99 | 4.34 ± 1.54       | \(<0.009\)  |

Hb, hemoglobin; PCV, packed cell volume; ETIBS, estimated total iron body stores; TIBC, total iron-binding capacity; TS %, transferrin saturation percentage; UIBC, unsaturated iron-binding capacity; MDA, malondialdehyde.

Consistently associated with an increased risk of coronary heart diseases and cardiovascular mortality.\(^{(25)}\) Other potential causes of hyperferritinemia are anoxia as a consequence of low hemoglobin level in the blood of patients with thalassemia. Ferritin concentration increases in response to stresses, such as anoxia.\(^{(22)}\)

MDA content in patients with thalassemia showed a significant 1.3-fold increase \((p<0.05)\) compared with that of the control group (Table 1). In agreement with the results of Patrick et al.,\(^{(23)}\) MDA content significantly increased by 1.8-fold in patients with thalassemia relative to the control group. This result indicated an increase in lipid superoxidation because of an increase in oxidative stress in patients with thalassemia. MDA, a product of lipid peroxidation and protein carbonyls, represents the oxidation of circulating proteins and is increased in patients with thalassemia.\(^{(24)}\)

Free extracellular iron and intracellular iron species that have been identified in thalassemic blood cells are responsible for
blood and be transported into other tissues where these products participate in more harmful events or become degraded. Hence, the quantity of oxidative stress-inducing compounds in tissues is more accurate than that in the blood.

Increased serum iron content affects the concentration of oxidative stress-inducing compounds in the body. However, studies have indicated that no direct association between serum iron parameters and MDA and total carbonyl concentrations is indicated. In one study, the strongest predictor of increased MDA in patients with thalassemia is liver iron concentration. Hence, estimated liver iron is more important than serum iron in the investigation of the effect of an increase in iron status on MDA level. Iron can potentially promote cellular damage by causing the formation of highly reactive hydroxyl radicals and inducing unsaturated lipid peroxidation. Iron imbalance/accumulation has been implicated in oxidative injury associated with many diseases, including β-thalassemia, via multiple mechanisms.

Oxidative stress is associated with an increase in or the production of oxidizing species; oxidative stress is also associated with a significant decrease in the antioxidant-defense molecules, such as glutathione. The effects of oxidative stress depend on the magnitude of these changes, considering that a cell can overcome small perturbations and regain normal status.

Conclusions

This study found a lack of correlation between oxidative stress represented by increased serum total carbonyl and MDA with non-labile iron status parameters in patients with thalassemia major. This result indicated that different mechanisms are involved in injuries caused by the labile iron and in the formation of oxidation end products.

Conflict of Interest

No potential conflicts of interest were disclosed.

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