CARBONYLATION OF SERUM PROTEIN CAN ACT AS AN INDICATOR OF DURATION OF TRANSFUSION THERAPY IN INDIVIDUALS WITH THALASSEMIA SYNDROME

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Abstract:
Background: In thalassemia patients blood transfusion is the mainstay of therapy. Blood transfusion is associated with oxidative stress. Oxidative stress has its influence on plasma proteins. The present study was carried out to explore the level of carbonylation of serum protein content and oxidative changes in serum proteins of thalassemia subjects on regular blood transfusion.

Method: Blood was collected from thalassemia patients receiving long duration of blood transfusion and from newly diagnosed cases of thalassemia who never receive blood transfusion and from carriers or traits of thalassemia as control. The extent of carbonylation of serum protein was estimated by thiobarbuturic acid method. The effects of in vitro treatment of serum protein with H2O2 on the above mentioned parameters were observed.

Observation: Carbonylation of serum protein was significantly higher in thalassemia subjects on regular blood transfusion. Enhanced carbonylation of serum protein by in vitro H2O2 treatment suggests that oxidative stress can cause carbonylation of serum proteins.

Conclusion: Carbonylation of serum protein can be used as an indicator of duration of transfusion therapy.

Key Words: Carbonylation, Oxidative stress, Serum Protein, Thalassemia, Transfusion

INTRODUCTION

Thalassemia Syndrome is an inherited disorder of alpha & beta chain biosynthesis. The reduced supply of globin chain diminishes the production of hemoglobin tetramers, causing hypochromia & microcytosis. In beta Thalassemia decreased or impaired biosynthesis of beta globin chain leads to accumulation of unpaired alpha chains within RBCs. In 1966 Fessas et al. described the presence of inclusion bodies in RBCs of thalassemia patients, suggesting that they were precipitated alpha chain. Oxidation of excess alpha chains results in accumulation of hemichromes, causing structural & functional alteration of RBC cell membrane.

There is a strict correlation between oxidative stress and anemia in thalassemia. In fact, reactive oxygen species (ROS) are generated in increased amounts in thalassemic erythrocytes, and the oxidative insult leads to hemolysis and erythrocyte immune recognition and removal from the circulation. Furthermore, individuals with beta-thalassemia undergo frequent blood transfusion leading to an iron overload which again precipitate further oxidative stress by Fenton’s reaction. This uncontrolled oxidative stress may alter the overall redox status of beta-thalassemic patients and cause other health problems. For example, thalassemic patients have high circulating levels of mediators of inflammation, which are responsible for a continuous proinflammatory status. One has to point out that oxidative
stress plays an important role in the evolution and perpetuation of various kinds of inflammation through modulation of the intracellular redox control machinery [4,7]. Several authors have shown higher levels of oxidative status markers and depletion of antioxidant reserves [2,5] in thalassemic patients. However very few study concerning protein oxidative damage in thalassemia has been carried out. Carbonyl stress may be due to toxic effects of ROS and of various mono-dicarbonyls and α-dicarbonyls on proteins. The level of these modified molecules can be quantified by measurement of the protein carbonyl content, which has been shown to increase in a variety of diseases and processes, such as Alzheimer’s disease, inflammatory bowel disease, arthritis, chronic lung disease, chronic renal failure, diabetes, and sepsis [8]. Because the circulating proteins destroyed by oxidation have a quite long half-life period, the evaluation of carbonyl group content in blood proteins is considered a useful biomarker of oxidation induced by ROS [9,10].

On the other hand, chronic blood transfusion is indicated in thalassemia patients for survival. In fact for many years, after the description of thalassemia as a clinical entity, therapy was limited to blood transfusion [3]. Though life saving for the patients but chronic blood transfusion can lead to several adverse effects like blood borne infections, alloimmunisation, febrile reactions & lethal iron overload. Each unit of blood contains approx. 200 mg of iron. A patient who receives 25-30 units of blood/year in absence of chelation, accumulates more than 70g of iron by 3rd decade [4,5]. Iron accumulates in chronically transfused patients because no mechanism exists for increasing iron excretion. Patients who have fully saturated transferrin, a significant factor of total iron in plasma circulates in the form of low molecular weight complex, not bound to transferrin or Non-Transferrin Bound Iron (NTBI) [11,12]. This NTBI induced peroxidative injury to phospholipids of lysosomes & mitochondria, produced by free hydroxyl radicals is the most important factor for morbidity in thalassemia. Under physiological condition, iron that are bound to transferrin, not available to catalyze the conversion of molecular oxygen to highly reactive free ions by Fenton reaction [8,9,10,11]. NTBI causes oxidative damage to cell & organelle membrane by generating free radicals [13,14]. Iron overload mainly affects the cells of liver, heart, pancreas & pituitary [15].

In 2003, Goswami K, Nandakumar N, Koner BC, et al. [15] showed that in hyperthyroidism there was increase oxidative stress & which increased carbonylation of serum protein. There was increase in carbonylation of serum protein which was a specific marker for peroxidation of serum protein. There was significant negative correlation between level of carbonylation & sialic acid content of serum protein in hyperthyroid cases (r= -0.77, p<0.05).

The present study was undertaken to find out whether carbonylation of serum protein can act as a indicator of iron overloading vis a vis oxidative stress in thalassemic individuals.

**AIM OF THE STUDY**

Determination of carbonylation of serum protein status as an indicator of duration of transfusion therapy among individuals with thalassemia syndrome.

**OBJECTIVES OF THE STUDY**

1. To measure Carbonylation of serum protein as an indicator of oxidative stress in individuals with Thalassemia Syndrome receiving transfusion therapy
2. To estimate the serum ferritin for assessment of iron status among individuals with Thalassemia Syndrome
3. To estimate the serum iron for assessment of serum iron status
MATERIAL & METHODS:

The present study was a hospital based, non interventional, cross sectional study. This study was undertaken in the Dept of Biochemistry of Burdwan Medical College & Hospital, Burdwan in collaboration with the Institute of Hematology & Transfusion Medicine (IHTM). The cases of Thalassemia syndrome were selected from the patients attending out patient department of Institute of Hematology and Transfusion Medicine, Medical College and Hospital, Kolkata. The age and sex matched controls were selected from carrier or trait of thalassemia syndrome subjects preferably parent or sibling of the patients. Both the cases and controls were informed about the risks and benefits of the study and written consent was taken before drawing blood from them. The study was approved by the Institutional Ethics committee, Medical College and Hospital, Kolkata

A. Case: 30 Patients of thalassemia syndrome receiving blood transfusion for a long time (> 2 yrs).

Control:

A. 40 Age & sex matched thalassemia carrier or trait who never received blood transfusion (1st control group)

B. 20 Untransfused newly diagnosed cases of thalassemia syndrome (2nd control group)

Sample Collection: 10 ml venous blood was collected from cases and controls with proper aseptic technique in plain vial. The collected clotted blood was then centrifuged at 1500 rpm speed for 3-5 minutes for separation of serum. All the tests were done immediately with serum obtained from clotted blood.

Estimation Of Serum Protein Carbonylation (Levine’s Method) [16]

Principle of The Test

Serum was first separated and treated with 10% Tri-chloro Acetic Acid (TCA) to precipitate the protein present in the serum. Now this precipitated protein was washed thoroughly with ethanol / ethylacetate mixture to remove impurities. Then 2,4-dinitrophenyl hydrazine (DNPH) was added to this precipitated protein. DNPH reacted with carbonylated protein and converted into 2,4-dinitro phenyl hydrazone. 2,4-dinitro phenyl hydrazone has specific color which was measured spectrophotometrically at 370 nm wavelength. Intensity of color of the measured solution was proportional to the concentration of carbonylated protein which can be calculated by using Molar Extinction Coefficient of carbonylated serum protein-21 x 10^3 L / mol cm.2 mol /L HCl was used as blank solution. Concentration of carbonylated protein is expressed in terms of n mol / mg of serum protein. Serum protein is estimated at first by Biuret Method.

Estimation Of Serum Ferritin Concentration [17]

The Ferritin Enzyme Immuno Assay was a solid phase Enzyme Immuno Assay based on the ‘Sandwitch’ principle.

Estimation Of Serum Iron (Ferrozine Method) [18]

Principle Of The Test

Iron bound to transferrin, is released in an acidic medium & the ferric ion is reduced to ferrous state. The ferrous ion react with Ferrozine to form a violet colored complex. Intensity of the complex is directly proportional to the amount of iron present sample.

In vitro treatment of serum protein with hydrogen peroxide & water

4ml of serum from 12 control subjects i.e, the cases of thalassemia carrier or trait, never
receiving blood transfusion was collected & divided into two parts. One part treated with 0.47 mol/l \( \text{H}_2\text{O}_2 \) for 4hrs to study the effect of oxidative stress on serum protein. Both serum carbonylation & sialic acid content is measured.

Other part of the samples is treated as control by adjusting the volume with water.

Now both the samples are washed with 95% ethanol & serum carbonylation & serum sialic acid status is measured by above mentioned methods.

Results of the tests are tabulated in table No-8

RESULT & ANALYSIS

In this study we got three groups of population

A. Subjects Without Disease And Blood Transfusion, Diagnosed As Trait Or Carrier Of Thalassemia Syndrome (1st Control Population)

B. Newly Diagnosed Without Any Blood Transfusion (1st Control Group)

C. Diagnosed Cases Of Thalassemia Who Received Several Blood Transfusion

Gr.C population again divided into two groups

1) Those who received BT more than 5 years (Gr.C1)

2) Those who received BT less than or equal 5 years (Gr.C2)

All data are collected & using SPSS software (version:17) Mean & SD is calculated of all parameters. Now unpaired t – test is done for equality of means and t - value, degree of freedom ( df ) , 2-tailed significance are calculated in between the three groups mentioned above to determine the level of significance.

<table>
<thead>
<tr>
<th>Parameters (unit)</th>
<th>Subjects without disease and blood transfusion (gr. A) No of cases (n)=55</th>
<th>Newly diagnosed cases without any blood transfusion (gr.b) no of cases (n)=21</th>
<th>Cases of thalassemia who received several blood transfusion (gr. C) No of cases (n)=33</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum protein carbonylation (nmol / mg of serum protein)</td>
<td>0.81 ± 0.13 (0.017)</td>
<td>1.35 ± 0.12 (0.026)</td>
<td>2.28 ± 0.19 (0.034)</td>
</tr>
<tr>
<td>Mean ± SD (SE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum ferritin (ng / ml)</td>
<td>50.55 ± 7.69 (1.36)</td>
<td>38.29 ± 13.67 (2.98)</td>
<td>163.55 ± 43.84 (7.63)</td>
</tr>
<tr>
<td>Mean ± SD (SE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum iron (µg / ml)</td>
<td>36.47 ± 13.02 (1.10) *</td>
<td>32.5 ± 8.69 (2.17) **</td>
<td>157.89 ± 43.5 (8.37) ***</td>
</tr>
<tr>
<td>Mean ± SD (SE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum protein (g / dl)</td>
<td>7.92 ± 0.23 (0.31)</td>
<td>7.52 ± 0.44 (0.092)</td>
<td>7.45 ± 0.56 (0.56)</td>
</tr>
<tr>
<td>Mean ± SD (SE)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NB: * here n is 49 instead of 55; ** here n is 16 instead of 21; *** here n is 27 instead of 33.
Table-2:
TEST OF SIGNIFICANCE BETWEEN SUBJECTS WITHOUT DISEASE AND BLOOD TRANSFUSION (Gr. A) AND NEWLY DIAGNOSED CASES WITHOUT ANY BLOOD TRANSFUSION (Gr. B)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>t-score</th>
<th>Significance (2-tailed)</th>
<th>95% confidence interval of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>upper</td>
</tr>
<tr>
<td>Serum protein carbonylation</td>
<td>-17.27</td>
<td>0.000</td>
<td>-0.60</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>3.54</td>
<td>0.001</td>
<td>5.24</td>
</tr>
<tr>
<td>Serum iron</td>
<td>1.62</td>
<td>0.117</td>
<td>-1.08</td>
</tr>
</tbody>
</table>

[significance (2-tailed) is at 0.05 level]

It is seen from Table 2, that serum protein carbonylation is significantly higher in newly diagnosed cases than control groups. Serum ferritin level significantly increased in newly diagnosed cases but serum iron status showed no significant difference. Serum glycol protein is significantly higher in newly diagnosed cases.

Table-3:
TEST OF SIGNIFICANCE BETWEEN SUBJECTS WITHOUT DISEASE AND BLOOD TRANSFUSION (Gr. A) AND DIAGNOSED CASES OF THALASSEMIA WHO RECEIVED SEVERAL BLOOD TRANSFUSION (Gr. C)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>t-score</th>
<th>Significance (2-tailed)</th>
<th>95% confidence interval of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>upper</td>
</tr>
<tr>
<td>Serum protein carbonylation</td>
<td>-38.13</td>
<td>0.000</td>
<td>-1.544</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>-17.9</td>
<td>0.000</td>
<td>-125.3</td>
</tr>
<tr>
<td>Serum iron</td>
<td>-19.2</td>
<td>0.000</td>
<td>-134</td>
</tr>
</tbody>
</table>

[significance (2-tailed) is at 0.05 level]

It is seen that serum protein carbonylation is significantly higher in diagnosed cases with multiple blood transfusions than control groups. Serum ferritin level & serum iron significantly increased in diagnosed cases with chronic blood transfusion. Serum glycol protein is significantly higher in diagnosed cases.
Table 4

TEST OF SIGNIFICANCE BETWEEN NEWLY DIAGNOSED WITHOUT ANY BLOOD TRANSFUSION & DIAGNOSED CASES OF THALASSEMIA WHO RECEIVED CHRONIC BLOOD TRANSFUSION

<table>
<thead>
<tr>
<th>Parameters</th>
<th>t-score</th>
<th>Significance (2-tailed)</th>
<th>95% confidence interval of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>upper</td>
</tr>
<tr>
<td>Serum protein carbonylation</td>
<td>-21.53</td>
<td><strong>0.000</strong></td>
<td>-1.01</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>-12.66</td>
<td><strong>0.000</strong></td>
<td>-145.1</td>
</tr>
<tr>
<td>Serum iron</td>
<td>-11.34</td>
<td><strong>0.000</strong></td>
<td>-147.71</td>
</tr>
</tbody>
</table>

[significance (2-tailed) is at 0.05 level]

Table 4 displays the results of unpaired t-test for equality of means of the Diagnosed Cases Without Any Blood Transfusion and Diagnosed Cases Of Thalassemia Who Received Chronic Blood Transfusion. Results shows that serum protein carbonylation, serum protein desialylation, serum glycoprotein, serum iron & ferritin all are significantly increased in cases of diagnosed cases of thalassemia with multiple blood transfusion than newly diagnosed cases without receiving blood transfusion.

Table 5
RESULT OF MEASUREMENT OF SERUM CARBONYLATION & SIALIC ACID AFTER IN VITRO TREATMENT OF SERUM PROTEIN WITH HYDROGEN PEROXIDE AND WATER

<table>
<thead>
<tr>
<th>Control (n=12)</th>
<th>Carbonylation of serum protein [Mean ±SD] (mmol / mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment with hydrogen peroxide</td>
<td>2.03 ± 0.43</td>
</tr>
<tr>
<td>Treatment with water</td>
<td>1.56 ± 0.46</td>
</tr>
</tbody>
</table>

Results of the table-5 shows that there is increase in serum carbonylation status & also serum desialylation status in samples treated with hydrogen peroxide. This is comparable to our present study. Hydrogen peroxide generates oxidative stress which causes increase carbonylation & desialylation of serum protein.

DISCUSSION

The objective of our present study was based on the assumption that the patients suffering from thalassemia who get regular blood or erythrocyte transfusion, may suffer from iron overload. Toxic ‘free iron’ may produce oxidative stress in different tissues of the body as per Fenton Chemistry and may produce decreased life span of erythrocytes. Under physiological condition, iron are not available to catalyze the conversion of molecular oxygen to highly reactive radical species by Fenton chemistry. This is because ferric iron is bound to proteins, preventing it from participating in reactions that could lead to cellular injury. Elevation in low molecular weight iron-binding...
complex in serum and intracellular transit pool are associated in pathological condition of iron overload \(^{[5,16]}\). This promotes peroxidative damage to cell and organelle membrane. Files B et al (2002) showed serum ferritin concentration to increase linearly with cumulative transfusion volume \(^{[18]}\). There was strong intra-patient correlation between serum ferritin concentrations and transfused blood volume. Increased oxidative stress causes increased fragility of RBC membrane which leads to easy destruction \(^{[5,7,19]}\).

Many studies showed previously that oxidative stress was increased in Thalassemia syndrome \(^{[5,11,15]}\). Chronic blood transfusion also related to increased oxidative damage to the RBCs \(^{[16,17]}\). Due to chronic blood transfusion there is accumulation of iron which further aggravates the damage. Oxidative stress induced damage cause increased fragility of RBC membrane which ultimately results in lysis of red cell \(^{[5,6]}\).

We know that blood transfusion is the mainstay of therapy in Thalassemia. As oxidative stress induced destruction of RBCs decreased the life span of the cells, so more frequent blood transfusion is required among these patients.

Carbonylation of serum protein is a well known marker for oxidative stress \(^{[5,9]}\). In this study the level of serum protein carbonylation is significantly higher among the patients with Thalassemia syndrome with regular blood transfusion than both the control groups eg. Newly diagnosed cases of Thalassemia syndrome without any blood transfusion & control population who are carrier or trait of thalassemia syndrome without any history of blood transfusion \(^{[2,3,11]}\). So it can be deduced that serum protein carbonylation is significantly increased with chronicity of blood transfusion.

In vitro treatment of serum protein with H\(_2\)O\(_2\) for 3 hours caused increased level of carbonylation of serum protein. This indicates that oxidative stress causes carbonylation of serum protein (Table-5).

In the present study the carbonylation of serum protein is significantly higher among the patients with Thalassemia syndrome with regular blood transfusion than the other two groups e.g. newly diagnosed cases of Thalassemia syndrome without any blood transfusion & control population who are carrier or trait of thalassemia syndrome without any history of blood transfusion.

So it shows a clear relationship between the chronicity of blood transfusion and carbonylation of serum protein. More the duration of transfusion more the level of carbonylation of serum protein \(^{[5,11,12,17]}\).

Carbonylation of serum protein is highest with the cases of thalassemia who have undergone chronic transfusion. In this case oxidative stress generation is aggravated by chronic blood transfusion induced iron load associated with pathological process. Serum iron & ferritin content is significantly higher in this group with repeated blood transfusion which proves that iron load increases the oxidative stress in this population. Due to increased oxidative stress osmotic fragility of erythrocytes is increased \(^{[5,7,8]}\). Thus increase in morbidity & complications. So more the duration of blood transfusion more the iron over-load induced oxidative damage.

It is also noted that carbonylation of serum protein status also increased with the duration of blood transfusion. Patients receiving blood transfusion more than five years showed significantly more carbonylation of serum protein than patients receiving blood transfusion less than five years.

**CONCLUSION:** From our study it can be concluded that carbonylation of serum protein
can be used as an indicator of both oxidative stress and also duration of transfusion

REFERENCES


[16]. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S,


