Oxid Antioxid Med Sci 2013; 2(3):163-167 *ISSN: 2146-8389*

GESDAV

Oxidants and Antioxidants in Medical Science

available at www.scopemed.org

Original Article

Determination of serum protein desialylation status as an indicator of duration of transfusion therapy in individuals with thalassemia syndrome

Mrinal Pal[1], Indranil Chakraborty[2], Utpal Chowdhuri[3], Jayanta De[4], Kanika Mandi Choudhuri[1]

[1]Department of Biochemistry, Burdwan Medical College, Burdwan;
[2]Department of Biochemistry, Malda Medical College, Malda;
[3]Institute of Hematology and Transfusion Medicine, Medical College, Kolkata;
[4]Department of Biochemistry, Midnapur Medical College, Midnapur; ***West Bengal, India***

Received June 3, 2013
Accepted June 27, 2013

Published Online August 14, 2013

DOI 10.5455/oams.270613.or.046

Corresponding Author
Mrinal Pal
115, Pirpukur, Kalna Road,
PO + Dist: Bardhaman - 713 101, India.
mrinalpal77@rediffmail.com

Key Words
Blood transfusion; Carbonylation; Desialylation; Protein catabolism; Thalassemia

Abstract
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 sialic acid content and oxidative changes in serum proteins of thalassemia subjects on regular blood transfusion. 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 and desialylation of serum protein was estimated by dinitrophenylhydrazine and thiobarbuturic acid method respectively. The effects of *in vitro* treatment of serum protein H_2O_2 on the above mentioned parameters were observed. Carbonylation of serum protein was significantly higher and sialic acid content of serum protein significantly lower in thalassemia subjects on regular blood transfusion. A significant negative correlation exists between carbonylation and sialic acid content of serum protein. Enhanced desialylation and carbonylation of serum protein by *in vitro* H_2O_2 treatment suggest that oxidative stress can cause desialylation of serum proteins.

INTRODUCTION

Sialic acid is a terminal carbohydrate residue of several glycoproteins. It is a negatively charged polar molecule which maintains the conformation of glycoprotein having major role in function and life span of a glycoprotein [1]. Removal of terminal sialic acid known as desialylation and the protein without sialic acid termed as asialoglycoprotein [2]. Desialylation of protein unmask the subterminal galactose residue and thus are removed from circulation by galactose receptor of liver resulting in immature termination [1, 2].

Thalassemia syndrome is an inherited disorder of alpha and beta globin chain biosynthesis. The reduced supply of globin chain diminishes the production of hemoglobin tetramers, causing hypochromia and microcytosis [3]. In beta thalassemia decreased or impaired biosynthesis of beta globin chain leads to accumulation of unpaired alpha chains within red blood cells (RBC) [4]. Oxidation of excess alpha chains results in accumulation of hemichromes, causing structural and functional alteration of RBC membrane [5]

Blood transfusion is the mainstay of therapy in case of thalassemia [3]. Individuals with beta-thalassemia undergo frequent blood transfusion leading to an iron overload [3, 6]. Which again precipitate further oxidative stress by Fenton's reaction [6]. This uncontrolled oxidative stress may alter the overall redox status of beta-thalassemia patients. Several authors have shown higher levels of oxidative status

markers and depletion of antioxidant reserves in thalassemia patients [5, 6]. Though being life saving for the patients, blood transfusion for a long duration can lead to lethal iron overload. Each unit of blood contains approx. 200 mg of iron. A patient, who receives 25-30 units of blood per year in absence of chelation, accumulates more than 70 g of iron by 3rd decade [7]. Iron accumulates in chronically transfused patients because no mechanism exists for increased 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) [6, 7]. This NTBI-induced peroxidative injury to phospholipids of lysosomes and mitochondria produced by free hydroxyl radicals is the most important factor for morbidity in thalassemia [8]. Under physiological condition, iron bound to transferrin is not available to catalyze the conversion of molecular oxygen to highly reactive free ions by Fenton reaction. NTBI, however, causes oxidative damage to cell and organelle membrane by generating free radicals [6, 8, 9].

Free radicals can impart important changes in secondary structure of proteins like cross-linking or carbonylation, *etc*. Carbonylation of proteins is an established marker of oxidative stress [10]. Due to carbonylation the proteins become more susceptible for proteolysis [11, 12]. So, increased oxidative stress causes carbonylation of serum proteins as well as removal of terminal sialic acid residue of the protein molecule [13-15].

Very little study concerning protein oxidative damage in thalassemia has been carried out. The aim of this present study is to find out whether serum protein desialylation can act as an indicator of iron overloading due to long duration of transfusion therapy and oxidative stress in thalassemic individuals.

MATERIALS AND METHODS

Consent and ethical clearance

Present study was done in Department of Biochemistry Medical College, Kolkata, West Bengal, India. During blood collection, informed consent was taken from all subjects/guardians. Ethical clearance for this work was given by the institutinal ethics committee.

Control subjects

Group A: 20 newly diagnosed cases of thalassemia syndrome who never received blood transfusion

Group B: 40 age and sex matched thalassemia carriers or traits who never received blood transfusion

Selection of cases

Group C: 30 Patients of thalassemia syndrome receiving blood transfusion for a long time; > 2 years, receiving ≥ 20 units blood transfusion per year without any chelation therapy.

Collection of blood

Ten milliliters of venous blood was collected from cases and controls with proper aseptic technique without any anticoagulant. The clotted blood was centrifuged at 1500-2000 rpm speed for 3-5 min for separation of serum. For quality control pooled serum free from any hemolysis was used and kept in deep freeze (–20°C) after dividing it in several aliquots.

Assay of carbonylation of serum protein (Levine's method [16])

Serum was first separated and treated with 10% trichloroacetic acid (TCA) to precipitate the protein content. Now this precipitated protein was washed thoroughly with ethanol/ethylacetate mixture to remove impurities. Then 2,4-dinitrophenylhydrazine (DNPH) was added to this precipitated protein. DNPH reacts with carbonylated protein and was converted into 2,4-dinitrophenylhydrazone. 2,4-dinitrophenylhydrazone has a specific color which was measured at 370 nm using a dual beam spectrophotometer (UV5704SS; Electronics Corporation of India, Hyderabad, Andra Pradesh, India). Intensity of color of the measured solution was proportional to the concentration of carbonylated protein which can be calculated by using the molar extinction co-efficient of carbonylated serum protein. HCl was used as blank solution. Intraassay CV% was 3.4 and interassay CV% was 5.16.

Estimation of serum sialic acid (Modified Aminoff's method [17])

Colorimetric assay for sialic acid is based on periodate oxidation followed by the reaction with thiobarbituric acid (TBA) and depends on formation of a hexose, 5-uluronic acid product (which is a pre-chromogen) by the periodate cleavage of $C_{6\text{-}7,}$ $C_{7\text{-}8,}$ $C_{8\text{-}9}$ bonds in free sialic acid. There is eventual formation of β-formyl pyruvic acid from free sialic acid. This in turn reacts with TBA to yield a chromosphere with absorption maximum at 549 nm. Measured using dual beam spectrophotometer (UV5704SS); intraassay CV% 2.9 and interassay CV% 4.4.

Estimation of serum glycoprotein [18]

After removing heat coagulable proteins by means of perchloric acid, glycoproteins, which remain in solution, are precipitated by phosphotungstic acid. The amount of glycoprotein precipitated has been estimated by determining its carbohydrate component by the reaction with an orcinol-sulphuric acid reagent; its protein content was measured by Lowry's method [19] and its tyrosine content by using Folin-Ciocalteu reagent.

 DOI 10.5455/oams.270613.or.046

Estimation of serum ferritin concentration [20]

Ferritin was measured by a solid phase enzyme immunoassay based on the 'sandwich' principle. Two separate antibodies directed against distinct antigenic determinants of the ferritin molecule were utilized in the assay; intraassay CV% 2.8, interassay CV% 4.8.

Estimation of serum iron (ferrozine method [21])

Iron bound to transferrin is released in an acidic medium and the ferric ion is reduced to ferrous state. The ferrous ion reacts with ferrozine to form a violet colored complex. Intensity of the complex is directly proportional to the amount of iron present sample; measure absorbance at 578 nm.

Estimation of serum protein (biuret method [22])

Total protein was measured by biuret method using Autoanalyser XL-600 (Transasia, Mumbai, India).

In vitro treatment of serum protein with hydrogen peroxide and water

Four milliliters of serum from 30 control subjects, *i.e.* the cases of thalassemia carrier or trait never receiving blood transfusion, was collected and divided into two parts. One part was treated with 0.47 mol/l H_2O_2 for 4 h in order to study the effect of oxidative stress on serum protein. Both serum carbonylation and 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 and serum carbonylation and serum sialic acid status is measured by above mentioned methods.

Statistical analysis

Data obtained were first analyzed for significance of differences of means between the case and control groups by independent t test. For assessing intragroup and intergroup variation one way Analysis of Variance (ANOVA) test is done. Finally to find out correlation between the parameters of both case and control groups bivariate correlation analysis is done. For all tests, 'P' value was considered to be significant if it was less than 0.05 at a confidence level of 95%. All statistical analyses were performed with the help of the SPSS software (version 17).

RESULTS AND DISCUSSION

In this study the level of serum protein carbonylation, a marker for oxidative stress, is significantly higher in the patients with thalassemia syndrome with regular blood transfusion than both control groups (Table 1). So it can be concluded that oxidative stress is significantly increased with increased duration of blood transfusion. Apart from albumin most of the serum proteins are glycoprotein in nature [23]. Several recent studies showed that oxidative stress is the main cause of desialylation of glycoprotein of platelets and LDL particle [7, 24]. Though the exact mechanism is not known clearly.

In present study there is a clear relationship between the duration of blood transfusion and desialylation of serum protein: more the duration of transfusion more the level of desialylation. Sialic acid content of serum protein is significantly lower in the case population than both control groups (Table 1). This indicates that desialylation process of glycoprotein is enhanced with blood transfusion of long duration. Serum glycoprotein is also significantly higher in case population than both control groups ($P < 0.001$; Table 1). So, there is hypercatabolism of serum glycoprotein due to long duration of blood transfusion [2].

In this case oxidative stress generation is aggravated by blood transfusion-induced iron overload. Serum iron and ferritin content is significantly higher in the group with repeated blood transfusion which proves that iron load increases the oxidative stress in this population (Table 1). Serum ferritin concentration tends to increase linearly with cumulative transfusion volume [6, 25]. There was strong intra-patient correlation between serum ferritin concentrations and transfused blood volume [25].

Table 1. Overall results of the three groups; results are displayed in the form of mean ± standard deviation and P values (level of significance at 95% CI) were the result of the one way ANOVA test

Parameters (unit)	Subjects without disease and transfusion	Newly diagnosed cases without transfusion	Cases of thalassemia who received blood transfusion for several times	P value
Serum protein carbonylation (nmol/mg of serum protein)	0.81 ± 0.13	1.35 ± 0.12	2.28 ± 0.19	**< 0.001**
Sialic acid content of serum protein (µg/mg of serum protein)	2.57 ± 0.29	2.28 ± 0.33	1.53 ± 0.21	**0.001**
Serum glycoprotein (mg/ml of serum)	74.07 ± 10.11	67.38 ± 10.2	87.39 ± 9.3	**< 0.001**
Serum ferritin (ng/ml)	50.55 ± 7.69	38.29 ± 13.67	163.55 ± 43.84	**< 0.001**
Serum iron (µg/ml)	36.47 ± 13.02	32.5 ± 8.69	157.89 ± 43.5	**< 0.001**
Serum protein (g/dl)	7.92 ± 0.23	7.52 ± 0.44	7.45 ± 0.56	**< 0.001**

Table 2. Results of unpaired t-test among study groups (P values)

Parameters	Group A *vs* B	Group A *vs* C	Group B *vs* C
Serum protein carbonylation	< 0.001	< 0.001	< 0.001
Sialic acid content of serum protein	0.001	< 0.001	< 0.001
Serum glycoprotein	0.015	< 0.001	< 0.001
Serum ferritin	0.001	< 0.001	< 0.001
Serum iron	0.117	< 0.001	< 0.001

Table 3. Carbonylation of serum protein and serum sialic acid content after *in vitro* treatment of serum protein with hydrogen peroxide and water (mean ± SD)

Cases (n = 30)	Carbonylation of serum protein (mmol/mg of protein)	Sialic acid content of serum protein (µg/mg of protein)
Treatment with hydrogen peroxide	2.03 ± 0.43	1.42 ± 0.94
Treatment with water	1.56 ± 0.46	3.01 ±0.23

In vitro treatment of serum protein with H_2O_2 for 4 h caused increased level of carbonylation of serum protein and decreased sialic acid content of serum protein. This indicates that oxidative stress causes desialylation of serum protein (Table-3). Serum protein carbonylation and sialic acid content of serum protein represented a significant negative correlation in both case and control groups: case, r = -0.448 and P = 0.009; control, r = -0.337 and P = 0.003. It can be concluded that these two parameters may be interrelated and dependent on each other. Their mechanism of generation may be same, *i.e.* transfusion induced oxidative stress.

The patients suffering from thalassemia who get regular blood or erythrocyte transfusion suffer from iron overload [5]. Toxic 'free iron' in thalassemia may produce oxidative stress in different tissues of the body [26, 27].This oxidative stress may decrease life span of erythrocytes by altering the secondary structure of membrane proteins of RBC [25, 28]. Free extracellular (labile plasma iron) and intracellular (labile iron pool) iron species that have been identified in thalassemic blood cells are responsible for generation of oxidative stress by catalyzing formation of oxygen radicals over the antioxidant capacity of the cell [29]. On the other side, desialylation of RBC membrane protein unmask the subterminal galactose residue and thus desialylated RBCs are removed from circulation by galactose receptor of liver [30, 31]. So, half life of transfused RBC is decreased resulting in requirement of repeated blood transfusion which ultimately leads to more and more iron overload and thus oxidative stress. So, more the duration of blood transfusion more the iron overload-induced oxidative damage, and more the degree of desialylation of serum protein.

Desialylation can be estimated easily in laboratory by above mentioned simple method and the test is cheap also. So it would be advantageous to use serum protein desialylation as a marker of duration of transfusion therapy.

COMPETING INTERESTS

None to report

 DOI 10.5455/oams.270613.or.046

REFERENCES

1. Stryer L. Carbohydrate. In: Stryer L (ed) Biochemistry, 4th edition, W.H. Freeman, New York, NY, USA, p 477, 1995.

2. Schengrund CL, Lausch RN, Rosenberg A. Sialidase activity in transformed cells. J Biol Chem 1973; 248:4424-8.

3. Jeffery S, Dzieczkowski, Anderson KC. Hematological disorders. In: Kasper DL, Faucci AS, Longo DL, Hauser SL, Loscalzo J, Jameson JL (eds) Harrison's Internal Medicine, 18th edition, McGraw Hill, USA, pp 951-952, 2012.

4. Fessas P, Loukopoulos D, Kaltsoya A. Peptide analysis of the inclusions of erythroid cells in beta-thalassemia. Biochim Biophys Acta 1966; 124:430-2.

5. Schrier SL, Rachmilewitz EA, Mohandas N. Cellular and membrane properties of alpha and beta thalassemic erythrocytes are different: implications for differences in clinical manifestations. Blood 1989; 74:2194-202.

6. Sazama K, DeChristopher PJ, Dodd R, Harrison CR, Shulman IA, Cooper ES, Labotka RJ, Oberman HA, Zahn CM, Greenburg AG, Stehling L, Lauenstein KJ, Price TH, Williams LK. Practice parameters for the recognition, management and prevention of adverse consequences of blood transfusion. College of American Pathologists. Arch Pathol Lab Med 2004; 124:61-70.

7. Rossi EC, Simon TL, Moss GS, Gold SA. Principles of Transfusion Medicines, 2nd edition, Williams and Wilkins, Baltimore, MD, USA, pp 445-456, 1996.

8. Livrea MA, Tesoriere L, Pintaudi AM, Calabrese A, Maggio A, Freisleben HJ, D'Arpa D, D'Anna R, Bongiorno A. Oxidative stress and antioxidant status in beta thalassemia major: iron overload and depletion of of lipid-soluble antioxidants. Blood 1996; 88:3608-14.

9. Meral A, Tuncel P, Sürmen-Gür E, Ozbek R, Oztürk E, Günay U. Lipid peroxidation and antioxidant status in beta-thalassemia. Pediatr Hematol Oncol 2001; 17:687-93.

10. Sripradha R, Sridhar MG, Agrawal A. Can protein carbonyl/glutathione ratio be used as a potential biomarker to assess oxidative stress in alcoholic hepatitis? Indian J Med Sci 2010; 64:476-83.

11. England K, O'Driscoll C, Cotter TG. Carbonylation of glycolytic proteins is a key response to drug-induced oxidative stress and apoptosis. Cell Death Differ 2004; 11**:**252-60.

12. Thomas N. Role of oxidative carbonylation in protein quality control and senescence. EMBO J 2005; 24:1311-7.

13. Goswami K, Nandakumar DN, Koner BC, Bobby Z, Sen SK. Oxidative changes and desialylation of serum protein in hyperthyroidism. Clin Chim Acta 2003; 337:162-8.

14. Gyves PW, Gesundheit N, Thotakura NR, Stannard BS, DeCherney GS, Weintraub BD. Changes in the sialylation and sulfonation of secreted thyrotropin in congenital hypothyroidism. Proc Natl Sci 1990; 87:3792-6.

15. Goswami K, Koner BC. Level of sialic acid residues in platelet proteins in diabetes, aging, and Hodgkin's lymphoma: a potential role of free radicals in desialylation. Biochem Biophys Res Commun 2002; 297:502-5.

16. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990; 186:464-78.

17. Aminoff D. Methods for quantitative estimation of N-acetyl neuraminic acid and their application on hydrolysate of sialomucoprotein. Biochem J 1961; 81:384-92.

18. Winzler RJ .Methods in Biochemical Analysis, 2nd edition, Interscience, New York, NY, USA p 270, 1960.

19. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-75.

20. Fairbanks VF, Klee GG. Biochemical aspect of Hematology. In: Burtis CA, Ashwood ER (eds) Tietz Textbook of Clinical Chemistry, 2nd edition, WB Saunders, Philadelphia, PA, USA, p 2065, 1994.

21. Fairbanks VF, Klee GG. Biochemical aspect of hematology. In: Burtis CA, Ashwood ER (eds) Tietz Textbook of Clinical Chemistry, 2nd edition, WB Saunders, Philadelphia, PA, USA pp 2066-2067, 1994.

22. Kingsley GR. The direct biuret method for determination of serum protein as applied to photoelectric and visual colorimetry. J Lab Clin Med 1942; 27:840-5.

23. Chrostek L, Cylwik B, Krawiec A, Korcz W, Szmitkowski M. Relationship between serum sialic acid and sialylated glycoproteins in alcoholics. Alcohol Alcohol 2007; 42:588-92.

24. Goswami K, Nandeesha H, Koner BC, Nandakumar DN. A comparative study of serum protein-bound sialic acid in benign and malignant prostatic growth: possible role of oxidative stress in sialic acid homeostasis. Prostate Cancer Prostatic Dis 2007; 10**:**356-9.

25. Harrison-Findik DD. Gender-related variations in iron metabolism and liver diseases. World J Hepatol 2010; 2:302-10.

26. Amer J, Goldfarb A, Rachmilewitz EA, Fibach E. Fermented papaya preparation as redox regulator in blood cells of beta-thalassemic mice and patients. Phytother Res 2008; 22:820-1.

27. Marotta F, Pavasuthipaisit K, Yoshida C, Albergati F, Marandola P. Relationship between aging and susceptibility of erythrocytes to oxidative damage: in view of nutraceutical interventions. Rejuvenation Res 2006; 9:227-30.

28. Trombetta D, Gangemi S, Saija A, Minciullo PL, Cimino F, Cristani M, Briuglia S, Piraino B, Isola S, Salpietro CD. Increased protein carbonyl groups in the serum of patients affected by thalassemia major. Ann Hematol 2006; 85:520-2.

29. Fibach E, Rachmilewitz EA. The role of antioxidants and iron chelators in the treatment of oxidative stress in thalassemia. Ann NY Acad Sci 2010; 1202:10-6.

30. Camilla R, Suzuki H, Dapra V, Loiacono E, Peruzzi L, Amore A, Ghiggeri GM, Mazzucco G, Scolari F, Gharavi AG, Appel GB, Troyanov S, Novak J, Julian BA, Coppo R. Oxidative stress and galactose-deficient IgA1 as markers of progression in IgA nephropathy. Clin J Am Soc Nephrol 2011; 6:1903-11.

31. Samanta S, Dutta D, Ghoshal A, Mukhopadhyay S, Saha B. Sundar S, Jarmalavicius S, Forgber M, Mandal C, Walden P, Mandal C. Glycosylation of erythrocyte spectrin and its modification in visceral leishmaniasis. PLoS ONE 2011; 6(12):e28169.