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Original Article

A comparative evaluation of oxidative status of erythrocytes in normal and sickle cell disease patients

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Abstract

It is well established that G6PD deficient individuals are subjected to high oxidative stress and, hence, stiffening of the cell membrane due to oxidation of important membrane proteins. This effect is pronounced in RBCs where G6PD is the sole producer of NADPH, an essential cofactor in the antioxidant defense mechanism. A different approach is to evaluate oxidative stress as the analysis of antioxidant concentrations. GSH can be oxidized, mainly to glutathione disulfide (GSSG), or can form glutathionylated proteins (PSSG). The measurement of GSG, GSSG give fundamental information on the intracellular redox status. Analytical methods based on spectrophotometry, HPLC, capillary electrophoresis, nuclear magnetic resonance, and mass spectrometry have been reported for the determination of glutathione in biological samples. Our study has concluded that the HPLC method to measure the concentration of reduced (GSH) and oxidized (GSSG) glutathione in the normal (healthy) and Sickle Cell Disease patients. Erythrocyte glutathione depletion has been linked to hemolysis and oxidative stress. Our study revealed that the total antioxidant status in steady-state sickle cell disease (SCD) patients and compared it with some healthy individuals and related it to certain hematological parameters and their recent clinical history. 15 (males & females) adult SCD patients and 15 age-matched controls were studied. And we hypothesized that altered glutathione and glutamine metabolism play a role in this process. Total glutathione (oxidized & reduced) were assayed in erythrocytes of 15 SCD patients and 15 healthy volunteers. Erythrocyte glutathione levels were significantly lower in SCD patients than in healthy volunteers. The ratio of erythrocyte GSH: GSSG correlated inversely to the oxidized levels of the erythrocytes.

1. Introduction

Sickle cell disease is an inherited disorder that affects red blood cells. People with sickle cell disease have red blood cells that contain mostly hemoglobin S, an abnormal type of hemoglobin. The most common form found in North America is homozygous Hb S disease, first described by Herrick in 1910. Hemoglobin – is the main substance of the red blood cells. It helps red blood cells carry oxygen from the air in our lungs to all parts of the body. Normal red blood cells contain hemoglobin A. Hemoglobin S and Hemoglobin C are abnormal type of hemoglobin. Sometimes these red blood cells become sickle cell shaped (crescent shaped) and have difficulty passing through small blood vessels. When sickle-shaped cells block small vessels, less blood can reach that part of the body. Tissue that does not receive a normal blood flow eventually becomes damaged. This is what causes the complications of sickle cell disease. There is currently no universal cure for sickle cell disease. Normal red blood cells are soft and round and can squeeze through tiny blood tubes (vessels). Normally, red blood cells live for about 120 days before new ones replace them. People with sickle cell conditions make a different form of hemoglobin A called hemoglobin S (S stands for sickle). Red blood cells containing mostly hemoglobin S do not live as long as normal red blood cells (normally about 16 days).[1]

Sickle cell disease usually begins in early childhood. Characteristic features of this disorder include a low number of red blood cells (anemia), repeated infections, and periodic episodes of pain. The severity of symptoms varies from person to person. Some people have mild symptoms, while others are frequently hospitalized for more serious complications. (U.S. National Library of Medicine February 2007). Sickle cells condition are inherited from parents in much the same way as blood type, hair color and texture, eye color and other physical traits. The type of hemoglobin a person makes in the red blood cells depends upon what hemoglobin genes the person inherits from his or her parents. Like most genes, hemoglobin genes are inherited in two sets one from each parent.

Cells with a markedly increased Hb S concentration are a prominent feature of sickle cell disease, as a consequence of the loss of K, Cl and water from the erythrocyte. The extreme dependence of polymerization kinetics on Hb S concentration means that these dehydrated erythrocytes rapidly sickle when deoxygenated. Blockade of K loss from the erythrocyte should, therefore, prevent the increase in Hb S concentration and reduce erythrocyte sickling. Detailed knowledge of the mechanisms leading to cell dehydration makes this a viable therapeutic option. Two ion transport pathways, the K-Cl co-transport and the Ca activated K channel play prominent roles in the dehydration of sickle erythrocytes. Possible therapeutic strategies include inhibition of K-Cl co-transport by increasing red cell Mg content and inhibition of the Ca activated K channel by oral administration of clotrimazole.[1]

The blood sample from total number of 30 patients was collected. RBC will be separated on the basis of gravity by Percoll gradient method. Glutathione contents (reduced/oxidized) will be determined by HPLC method by lysis of RBC.

- To fractionate erythrocyte from normal and sickle cell disease patients on the basis of their density by Percoll gradient.
- To estimate levels reduced glutathione and oxidized glutathione in fractionated erythrocytes by HPLC.

2. Materials and methods

2.1 Sample collection:

Blood was collected from 30 different Sickle Cell Disease Patients from Seesha Hospital Coimbatore by venipuncture to the antecubial vein with a 23- gauge butterfly needle attached to a 5ml

syringe. Approximately 2ml blood was drawn into the syringe, the butterfly needle is removed (while the syringe remains attached to the needle), and the venipuncture site is covered with gauze. The subject was asked to hold the gauze while the collector removes and disposal of the butterfly needle and drips 2ml of blood into an EDTA vial. Collected sample were stored at -80^{0}C as soon as possible for storage.

2.2 Sample processing

2ml of blood from the collected sample was then spun in a centrifuge at 1200 RPM for 5 mins in a 15ml centrifugation tube. The supernatant obtained (plasma) was then removed by the help of pipette. The pellet obtained was the whole blood cells. The pellet was collected in a different tube and was mixed with an equal amount of PBS solution. The tube was gently inverted twice for mixing and stored at -40^{0}C.

2.3 Percoll gradient separation:

2.3.1 Stock solution of Percoll:

To prepare a gradient the osmolarity of Percoll was adjusted with saline medium to make Percoll isotonic with physiological salt solutions by adding 9 parts (v/v) of Percoll to 1 Part (v/v) of 1.5 M NaCl and a Stock Isotonic Percoll (SIP) solution was made.

2.3.2 Gradient separation:

RBCs were separated into fraction based on cell density centrifugation. In a 15 ml tube, 2 ml of a solution of Percoll having a density of 1.115 g ml- 1 were added to the bottom of the tube. Next, 2 ml of a similar solution of Percoll having a density of 1.105 g ml- 1 were slowly added on to the top of the first 2 ml of the higher density solution. Finally, 1 ml of RBCs was added to the top of the Percoll layers. The tubes' containing the Percoll and RBCs was then centrifuged at 7500 RPM for 30 mins. After centrifugation, two layers of RBCs are present; those having a density of less than the 1.105 g ml Percoll solution will appear at the top of the tube and represent those RBCs having the lowest density; these RBCs are considered the younger of the RBCs in the original sample. Those below the higher density Percoll solution are the denser, or aged, RBCs. These separates RBC fractions were removed by pipette.

2.4 Determination of GSH and GSSG to reveal oxidative stress:

Standard preparation of Glutathione (oxidized and reduced) 1.6 gm of standard Glutathione oxidized (GSH) and Glutathione reduced (GSSG) was weighed separately in two different tubes. To this 2 ml of 0.1% trifluoro acetic acid (TFA) was added. 0.1% of TFA was prepared by adding 100µl of TFA in 100 ml of HPLC grade filtered water. From these standard solutions 6 different dilutions was made of various concentrations to obtain a standard graph for both GSH and GSSG.

Table 1: Serial dilutions of GSH and GSSG:

Sl. No	GSH mg/ml	GSSG mg/ml	TFA (0.1%) ml	Concentrations µg/ml
1.	1.6	1.6	2	800
2.	1 ml of **1**.	1 ml of **1**.	1	400
3.	1 ml of **2**.	1 ml of **2**.	1	200
4.	1 ml of **3**.	1 ml of **3**.	1	100
5.	1 ml of **4**.	1 ml of **4**.	1	50
6.	1 ml of **5**.	1 ml of **5**.	1	25

2.5 HPLC analysis:

2.5.1 Standard graph for GSH and GSSG

Separation was achieved by C_{18} column. The mobile phase used was 100% Methanol (HPLC grade) and 0.1% Trifluoro acetic acid (TFA). Initial solvent conditions was maintained at 100% TFA, 0% Methanol at 1 ml/min for 10 mins. A linear gradient for 90% TFA, 10% Methanol from 10 to 30 mins was made. From 30 to 120 mins, the conditions are maintained at 90% TFA, 10% methanol. The samples were then loaded; GSH and GSSG eluted from the column are detected at 210 nm. Typical injection volume in HPLC is 20 µl done by the help of Hamilton 20 ml syringe. The retention time for the oxidized and reduced glutathione are recorded. A standard graph was obtained for both GSH and GSSG.

2.5.2 Sample analysis:

Perchloric acid was made by adding 3.5 ml PCA in a tube and making up the volume upto 100 ml with distilled water. 200 µl of blood sample was taken and mixed with 700 µl of PCA in a centrifuge tube and centrifuged in a micro centrifuge for 15 mins at 11,000 RPM at 4^0 C. The pellet was discarded and the supernatant was removed by the help of pipette in a different centrifuge tube. This supernatant solution is then used for injection in HPLC.

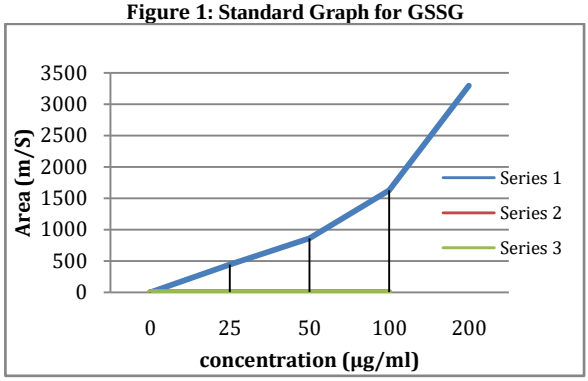
3. Results

Standard graph was obtained by the various dilutions and the concentration of each samples were calculated by the help of standard graph.

Table 2: Peak area obtained for various concentrations of GSSG

Sl. No	Concentration (µg/ml)	Area (mV/sec)
1.	0	0
2.	25	443.23
3.	50	860.28
4.	100	1628.9
5.	200	3282.84
6.	400	6756.11
7.	800	13712.8

Figure 1: Standard Graph for GSSG



The concentrations of the levels of GSSG present in the SC blood sample were calculated by the help of the standard graph.

Table 2.1: Concentration of GSSG in Sickle Cell blood sample

Sl. No	Area (mV/sec)	Concentration (µg/ml)
1.	513.037	30.08485
2.	758.02	44.45083
3.	977.07	57.29608
4.	349.21	20.47792
5.	1297.67	76.09629
6.	350.25	20.53891
7.	678.8	39.80531
8.	778.23	45.63596
9.	967.89	56.75776
10.	911.71	53.46332

Mean concentration of GSSG was found to be 44.42 µg/ml

The concentration of the levels of GSSG present in the normal blood sample was calculated by the help of the standard graph

Table 2.2 Concentration of GSSG in normal blood sample

Sl No.	Area (mV/sec)	Concentration (µg/ml)
1.	378.3	22.18378
2.	158.05	9.268164
3.	163.73	9.601243
4.	30.81	1.80672
5.	32.84	1.925761
6.	162.53	9.530874
7.	94	5.512227
8.	278.09	16.30739
9.	316.36	18.55157
10.	183.99	10.7893

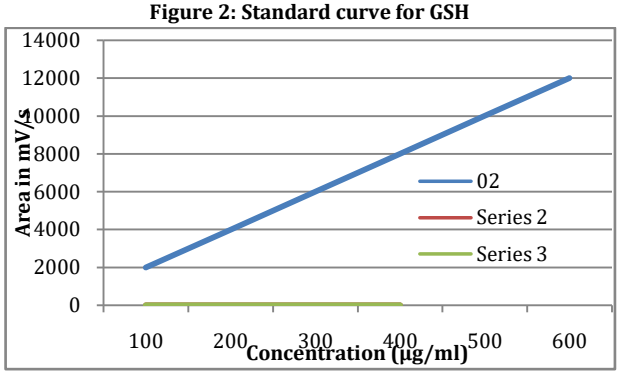
Mean concentration of GSSG was found to be 10.54 µg/ml.

Standard graph was obtained by the various dilutions and the concentration of each samples were calculated by the help of the standard graph

Table 2.3 Peak area obtained for various concentrations of GSH

SI. No	Concentration (µg/ml)	Area (mV/sec)
1.	0	0
2.	25	343.82
3.	50	813.50
4.	100	1460.34
5.	200	3132.81
6.	400	6232.25
7.	800	13240.10

Figure 2: Standard curve for GSH



The concentration of the levels of GSH present in the SC blood sample was calculated by the help of the standard graph.

Table 2.4 Concentration of GSH in sickle cell blood sample

SI No.	Area (mV/sec)	Concentration (µg/ml)
1.	388.97	23.85295
2.	3637.85	223.0852
3.	3008.85	184.4944
4.	3456.69	211.9758
5.	4623.44	283.5249
6.	270.36	16.57938
7.	4835.53	296.5309
8.	7755.34	475.5835
9.	6481.74	397.4821
10.	6668.26	408.9201

Mean concentration of GSH was found to be 252.20 µg/ml.

The concentration of the levels of GSH present in the normal blood sample was calculated by the help of the standard graph

Table 2.5 Concentration of GSH in normal blood sample

SI No.	Area (mV/sec)	Concentration (µg/ml)
1.	7107.63	435.8637
2.	7412.67	454.5698
3.	6389.13	391.8029
4.	4991.2	306.0771
5.	5735.22	351.7029
6.	4810.6	295.0021
7.	3191.6	195.7196
8.	3334.19	204.4637
9.	6120.7	375.3419
10.	4091.9	250.929

Mean concentration of GSH was found to be326.14 µg/ml.

4. Discussion

Sickle cell pathophysiology comprises a complex interplay of episodic vasoocclusive events, ischemia-reperfusion injury, over production of reactive oxygen species (ROS), inflammation, endothelial activation, and hemolysis. Hemolysis and oxidative stress act synergistically to promote vascular dysfunction in sickle cell disease (SCD).

A different approach is to evaluate oxidative stress as the analysis of antioxidant concentration. An ROS attack can lead to a major depletion of antioxidants such as vitamin E, vitamin C, reduced glutathione (GSH), and urate. The measurement of GSH, GSSG may therefore give fundamental information on the intracellular redox status. Analytical methods based on spectrophotometry, HPLC, capillary electrophoresis, nuclear magnetic resonance, and mass spectrometry have been reported for the determination of glutathione in biological samples. [2]

In order study Percoll gradients was used to fractionate erythrocytes on the basis of density in self-generated gradients, and used two different gradients of densities 1.105.g/ml and 1.115 g/ml. There is evidence that the colloidal silica particals of Percoll adhere to the surface of erythrocytes (even after washing) and cause progressive hemolysis; the elongation index of erythrocytes is thus difficult to measure and their deformability is affected. Discontinuous gradient are normally most easily prepared by under layering (i.e. low density first); over layering solutions, particularly those which differ in density by only a small amount, is more difficult. One alternative for over layering is to use a small alone (low-pulsating) peristaltic pump; first to take up the required volume of solution into the attached tubing and second, reverse the flow, to expel it slowly on to a denser layer in the centrifuge tube. [3] In our study RBCs were separated into fractions based on cell density centrifugation. In a 15ml tube, 2 ml of a similar solution of Percoll having density solution. Finally, 1 ml of RBCs was then centrifuged at 7500 RPM for 30 mins. After centrifugation, two layers of RBCs are present; those having a density of less than the 1.105 g ml Percoll solution will appear at the top of the tube and represent those RBCs having the lowest density; these RBCs are considered the "younger" of the RBCs in the original RBC sample. Those below the higher density Percoll solution are the more dense, or aged, RBCs. These separated RBC fractions were removed by pipette and diluted to a hematocrit of 7% using the PBS solution. GSH and GSSG levels were then measured with the help of HPLC. [4]

HPLC method was used to measure the concentration of reduced (GSH) and oxidized (GSSG) glutathione in the normal (healthy) and Sickle Cell Disease patients. We made use of the standard GSH and GSSG for calculating the amount present in the samples. The detection limit was found out to be 4 min for GSH and 6 min for GSSG at an UV range of 210 nm and flow rate at 1ml/min. We made a gradient of 2 different mobile phase i.e. 90% TFA and 10% Methanol and run the sample for 10 min and found out the concentration of GSH and GSSG in 30 different samples (15 Normal Healthy individuals; 15 Sickle Cell Disease patients). The A mean concentration of GSH in blood sample of 15 healthy individuals was found out to be 361.45 µg/ml and in 15 Sickle Cell disease patients was found out as 248.67 µg/ml. Also the mean concentration of GSSG in blood sample of 15 healthy individuals was found out to be 11.03 µg/ml. And in 15 Sickle Cell Disease patients as 39.20 µg/ml. Blood GSSG but not GSH was found significantly higher in Sickle Cell Disease patients as compared to normal healthy individuals. [4]

5. Summary and conclusion

Sickle cell pathophysiology comprises a complex interplay of episodic vasoocclusive events, ischemia- reperfusion injury, and overproduction of reactive oxygen species (ROS), inflammation, endothelial activation and hemolysis, all somehow driven by a single amino acid substitution in the β-globin chain of hemoglobin. Hemolysis and oxidative stress act synergistically to promote vascular dysfunction in sickle cell disease (SCD). It is well established that G6PD deficient

individuals are subjected to high oxidative stress and, hence, stiffening of the cell membrane due to oxidation of important membrane proteins. This effect is pronounced in RBCs where G6PD is the sole producer of NADPH, an essential cofactor in the antioxidant defense mechanism. Oxidative stress may thus play a role in the pathophysiology of the clinical manifestations of the disease. A different approach is to evaluate oxidative stress as the analysis of antioxidant concentrations. An ROS attack can lead to a major depletion of antioxidants such as vitamin E, vitamin C, reduced glutathione (GSH), and urate. GSH can be oxidized, mainly to glutathione disulfide (GSSG), or can form glutathionylated proteins (PSSG). The measurement of GSG, GSSG give fundamental information on the intracellular redox status. Analytical methods based on spectrophotometry, HPLC, capillary electrophoresis, nuclear magnetic resonance, and mass spectrometry have been reported for the determination of glutathione in biological samples.

Our study has concluded that the HPLC method to measure the concentration of reduced (GSH) and oxidized (GSSG) glutathione in the normal (healthy) and Sickle Cell Disease patients. Erythrocyte glutathione depletion has been linked to hemolysis and oxidative stress. Our study revealed that the total antioxidant status in steady-state sickle cell disease (SCD) patients and compared it with some healthy individuals and related it to certain hematological parameters and their recent clinical history. 15 (males & females) adult SCD patients and 15 age-matched controls were studied. And we hypothesized that altered glutathione and glutamine metabolism play a role in this process.

Total glutathione (oxidized & reduced) were assayed in erythrocytes of 15 SCD patients and 15 healthy volunteers. Erythrocyte glutathione levels were significantly lower in SCD patients than in healthy volunteers. The ratio of erythrocyte GSH: GSSG correlated inversely to the oxidized levels of the erythrocytes.

References

[1]. Carroll J, Raththagala M, Subasinghe W, Baguzis S, Oblak T Da, Root P, Spence D. An altered oxidant defence system in red blood cells affects their ability to release nitric oxide-stimulating ATP. *Molecular BioSystem* 2006; 2: 305-311.

[2]. Risso A, Turello M, Biffoni F, Antonutto G. Red blood cell senescence and neocytolysis in humans after high altitude acclimatization. *Blood Cells, Molecules, & Disease* 2007; 38: 83-92.

[3]. Claudia R. Morris, Jung H. Suh, Ward Hagar, Sandra Larkin D. Anton Bland, Martin H. Steinberg, Elliott P. Vichinsky, Mark Shigenaga, Bruce Ames, Frans A. Kuypers and Elizabeth S. Klings. Red Cells Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. *Blood* 2008 January 1; 111(1): 402-410.

[4]. Enika Nagababu, Mary E. Fabry, Ronald L. Nagel and Joseph M. Rifkind. Heme Degradation and Oxidative Stress in Murine Models for Hemoglobinopathies: Thalassemia, Sickle Cell Disease and Hemoglobin C Disease. *Blood Cells Mol Dis*. 2008; 41(1): 60-66.