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TBARS LEVEL IN SOCIOECONOMICAL POOR RURAL SICKLE CELL PATIENTS OF BILASPUR ZONE OF CHHATTISGARH STATE

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ABSTRACT: Sickle cell disease (SCD) is an inherited disorder of hemoglobin synthesis and the oxidative stress has major role in the pathogenesis of SCD. Thiobarbituric acid reactive substances formation by lipid peroxidation is a marker of oxidative damage in membrane.The study was under taken to measure the oxidative stress in sickle cell patients of Chhattisgarh rural population because of low natural antioxidants in their diet. The patients of sickle cell anemia group (homozygous, n=9) and sickle cell trait (heterozygous, n=21) shows TBARS level 2.28±0.66μM/l and 2.23±0.7μM/l respectively as compared to control (without S.C.D) 1.25±0.41μM/l. the study shows TBARS concentration is approximately double in sickle cell disease than control.

Key words: antioxidants, sickle cell disease, TBARS, thiobarbituric acid reactive species.

INTRODUCTION

Sickle cell disease (SCD), an inherited disorder of haemoglobin synthesis has been traced to a single point mutation that substitutes valine for glutamic acid in the β-globin subunit. This induces the polymerization of Sickle haemoglobin (HBS) and a resultant elongation and stiffening of sickle erythrocytes (RBC). However phenotypic expression of the disorder is complicated and is characterized by episodic vaso-occlusive events that elicit ischemia-reperfusion (I/R) related inflammatory responses in multiple organ systems, producing pain crises and end organ damage[1, 2]. The reactive oxygen species (ROS) are also recognized major players in ischemia-reperfusion injury. It is unsurprising that oxidative stress has also been implicated in the pathogenesis of SCD[3].

Under normal physiological conditions, anti-oxidant enzymes and oxygen radical scavengers ensure that basal fluxes of ROS do not injure the host organism. Major ROS defence mechanism include enzymatic (Superoxide dismutase (SOD), Catalase, Glutathione peroxidase) and non-enzymatic systems (reduced glutathione (GSH), Ubiquinols, Uric acid, vitamin C and E, Lipoic acid, Selenium, Riboflavin, Zinc, Carotenoids) as well as metal binding proteins[4].

Oxidative stress is defined as a shift in the normal pro-oxidant/anti-oxidant balance due to formation of reactive oxygen species.[5] The extent of oxidative damage in membranes was assessed by measuring lipid peroxidation i.e. formation of TBARS (Thiobarbutyric acid reactive substances formation).[6]

The exposure of SCD samples to antioxidants such as N-acetylcysteine, vitamin C and vitamin E decreases their oxidative stress. These findings suggest that antioxidant treatment of patients with SCD could reduce oxidative damage to RBC, PMN and platelets.[7] The dietary pattern of Chattisgarh rural population contains restricted amount of natural antioxidants. Hence our aim of the study includes the measurement of oxidant status in sickle cell patients of Chattisgarh rural population.

MATERIAL AND METHODS

Thirty sickle cell patients were taken for the study along with thirteen controls of different age and sex, divided into three groups.
Group A is a Sickle cell patients (Homozygous and Heterozygous), group B is control without Sickle cell disease.
The TBARC assay is done by AMCDD Protocol[8] as follows:
Protocol:

Reagent Preparation:

Thiobarbituric Acid (TBA): 67mg in 1ml DMSO then add 9ml H_2O.

10% Trichloroacetic Acid (w/v): in H_2O.

1,1,3,3-tetramethoxypropane: 4.167µL in 1mL, Ethanol then add 49mL H_2O.(500µM)

Sample Preparation:

Plasma:

- Place 100µL plasma into a labeled 1.5mL micro-centrifuge tube.

Tissue:

- Label 1 set of 1.5mL micro-centrifuge tubes, 1 set screw top tubes and 1 set of 0.5mL tubes.
- Weighed out 20mg and sonicate in 200µL RIPA buffer + inhibitors.
- Sonicate.
- Centrifuged @ 3000 for 10 min @ 4^0.
    1. Remove 10µL aliquot into the 0.5mL tubes for protein analysis.
    2. Place 100µL lysate into a labeled 1.5mL micro-centrifuge tube.
    3. Add 200µL ice cooled 10% Tricholoacetic acid to precipitate protein.
    4. Incubate for 15 minutes on ice.
    5. Prepare standards as follows:
    6. Centrifuge samples @ 2200 × g for 15 min. at @ 4^0C.
    7. Place 200µL supernatant and standards into new screw top 1.5mL tube.
    8. Add and equal volume of 0.67% (w/v) TBA.
    9. Incubate in a boiling water bath for 10 min.
    10. Cool. Sample is ready for assay.

Table – I Standard Preparation

S.No.	CONCENTRATION(µM)	H_2O	TETRAMETHOXYPROPANE
1.	0	500	-----------
2.	0.625	500	500 from tube 3
3.	1.25	500	500 from tube 4
4.	2.5	500	500 from tube 5
5.	5	500	500 from tube 6
6.	10	800	200 from tube 7
7.	50	500	500 from tube 8
8.	100	800	200 OF 500µm stock

Performing Assay:

1. While samples are cooling, layout on computer and save as TBxxxxxx.sed where xxxxxx is the date in yyddmm format.
2. Load 150µL into each standard well in duplicate.
3. Load 150µL into each sample well in duplicate.
4. Put in plate reader and press start.
5. Record absorbance at 532nm.

RESULTS AND DISCUSSION

Reactive Oxygen Species in general and hydroxyl radicals in particular are highly reactive and can react with any molecule in their vicinity e.g. causing damage to proteins, initiating or enhancing the process of lipid peroxidation (Halliwell and Gutteridge, 1985)[9]. Oxidation of membrane proteins and lipids is a self perpetuation process that can damage the cellular membrane integrity and ultimately result in cell dysfunctions (Mead, 1991)[10].
The oxidative stress of SCD-RBC could be attributable to the inherent instability of Hbs (Kings and Farber, 2001)[11]. It was shown that SCD-RBC produces greater quantities of O_2, H_2O_2 and OH free radicals than normal RBC, deoxygenation of hemoglobin is followed by the generation of O_2 free radicals is generated in SCD-RBC. Once Hb undergoes oxidative damage, it denatures, precipitates and forms hemochromes and ROS.
The patients of S.S. anemia group A of 30 with 9 homozygous and 21 heterozygous shows TBARS level 2.28±0.66 µm/l and 2.23±0.7 respectively. Group B of control having estimated value of 1.25±0.41µm/l (without SS anemia's) (Table II, III, IV and fig-1).

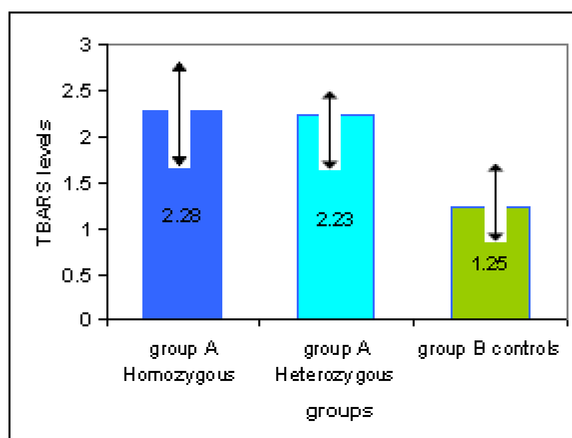

Fig-1: Comparative TBARS levels (µM/L) of group A (Homozygous 'SS', Heterozygous 'AS') and group B (control)

In TBARS assay the reaction between all components (specific and non specific) and thiobarbutaric acid is been measured. Therefore it is not the most specific approach for assessing the extent of lipid peroxidation. However, due to its popular use and ease of the assay it was chosen for this study.

Table II: Group A Homozygous Sickle Cell Patients with TBARS levels.

Patient No.	Age/Sex	TBARS (µM/L)	Medication
1.	24/F	2.25	F.A, H.U.
2.	20/M	1.95	F.A, H.U.
3.	26/M	2.6	F.A.
4.	18/F	3.65	F.A, H.U.
5.	7/F	2.6	F.A.
6.	26/M	2.2	F.A.
7.	13/F	1.8	F.A.
8.	13/F	2.3	F.A.
9.	9/M	1.25	F.A.

F.A: Folic Acid, H.U: Hydroxy Urea Drug.

The result shows that TBARS concentration is approximately double in sickle cell anemia patients. This may be due to generation of TBARS by SSD-RBC, PMN, platelets and also may be due to low content of antioxidant such as vitamin E, vitamin C, N-Acetyl cysteine. The sickle cell disease is more common in socioeconomic poor population and their dietary pattern might be having low antioxidant content.

The vaso-occlusive processes characteristic of SCD, might also be affected by oxidative stress, which results from abnormal adherence of sickle RBC, WBC and platelets to the vascular endothelium in which areas of to ischemia/reperfusion develop. (Dhalla et.al., 2000; Klings and Farber, 2001)[11, 12].

The previous study suggests that the rationale of using antioxidants in the treatments of SCD. Hence sufficient amount of antioxidant supplementation might be helpful to control oxidative stress of sickle cell anemia patients of Chhattisgarh State population.

Table III: Group A, Heterozygous Sickle Cell Patients with TBARS levels.

Patient No.	Age/Sex	TBARS (µM/L)	Medication
1.	6/M	2.25	F.A.
2.	17/F	2.2	F.A.
3.	20/F	2.3	F.A.
4.	23/F	1.9	---
5.	14/F	2.2	---
6.	10/M	2.1	---
7.	48/F	1.8	---
8.	17/F	2.5	---
9.	15/M	2.7	F.A, H.U.
10.	20/M	2.8	---
11.	22/M	2.1	---
12.	6/F	1.95	---
13.	11/M	2.5	F.A.
14.	25/F	2.3	F.A.
15.	37/M	1.12	---
16.	40/F	0.37	---
17.	33/F	1.8	F.A.
18.	7/M	2.5	F.A.
19.	26/M	4.8	F.A, H.U.
20.	27/M	2.5	F.A.
21.	6/F	2.2	---

F.A: Folic Acid, H.U: Hydroxy Urea Drug.

Table IV: TBARS levels of Group B, controls without Sickle Cell Disease.

Patient No.	Age/Sex	TBARS level (µM/L)
1.	60/M	1.2
2.	46/M	1.4
3.	27/M	0.7
4.	21/M	1.4
5.	24/M	1
6.	21/M	0.35
7.	19/M	1.71
8.	20/F	1.41
9.	19/F	1.42
10.	19/M	0.90
11.	21/M	1.52
12.	19/f	1.5
13.	19/F	1.25

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