

Role of Antioxidants by comparative evaluation of oxidative stress in Heterozygous v/s Homozygous Sickle cell disease patients: Research Protocol

ABSTRACT:

Background: Red blood cells are sources for oxidants in Sickle cell anaemia, a genetic disorder of itself. Heme iron and oxygen in oxygenated hemoglobin have bonding interaction and association with electron transfer. In response, antioxidant has modus operandi to reduce oxidative stress and damage to RBC and tissue.

The studies on biochemical indicators for oxidative stress in sickle cell would further enhance the understanding and present knowledge of effects of antioxidants on the status of iron metabolism and consequent relief to the sickle cell anaemia patients.

Objectives: To evaluate oxidative stress and Antioxidant levels and iron indices factors in heterozygous and homozygous sickle cell disease patients and compare between them along with normal healthy control and Iron Deficiency anaemia

Methodology: Comparative Cross-Sectional Study is designed to explore specific antioxidant levels and oxidative stress along with Iron Indices among sickle cell anaemia diagnosed cases attending/admitted in the department of Medicine, Paediatrics and Community Medicine at Jawaharlal Nehru Medical College and its Hospital, Sawangi, Meghe, along with age and sex matched healthy individuals from general population.

Results: Oxidative stress as evaluated by stress markers may be higher among the Homozygous sickle cell disease than among the Heterozygous.

Conclusion: Oxidative stress may be compounded in sickle cell disease patients in conjunction with iron deficiency.

Keywords: Sickle Cell Disease, Oxidative stress, Iron deficiency, Antioxidants, Ferritin, Transferrin

INTRODUCTION:

Red blood cells are the sources of free radicals in Sickle cell anaemia. RBC's compact with redox-active haemoglobin having rich oxygen supply. The bonding intercommunication within heme iron and oxygen in oxygenated haemoglobin is connected with electron transfer. With these antioxidants help to reduce oxidative stress and Injury to RBC and tissue. Iron extricated by haemolysis is obtained for recirculation and therefore iron deficiency is less common, in turn iron stores increases owing to blood transfusion¹, but still some studies shows iron deficiency in untransmuted patients.² Iron deficiency anaemia patients reported increased oxidant activity³. Compounded iron deficiency in Sickle cell may lead to increase in fold of oxidant activity and decrease in antioxidant activity. Moreover, Iron indices like Ferritin and Transferrin plays an important role as metabolic antioxidant.

In view of the above observations, it is felt that the studies on biochemical markers of oxidative stress in sickle cell anaemia would further enhance the understanding and present knowledge of effects of antioxidants on the status of iron metabolism and consequent relief to

the sickle cell anaemia patients. Hence it is proposed to study the relationship and bearing of levels of antioxidants in sickle cell heterozygous (AS) and sickle cell homozygous (SS) disease patients along with non - sickle cell iron deficiency anaemia cases in the region of Wardha and adjoining districts in Maharashtra.

OBJECTIVES OF THE STUDY:

- 1) To measure and compare the oxidative stress among Homozygous and Heterozygous cases Sickle Cell anaemia among themselves and among the healthy controls with or without iron deficiency anaemia.
- 2) To determine the factors associated with the levels of antioxidants, indices of oxidative stress among SCD cases and healthy controls.
- 3) To study the effects of antioxidants levels on the bearing of oxidative stress in Sickle Cell Disease patients.

HYPOTHESIS:

Oxidative stress as evaluated by stress markers is more among the Homozygous (SS) Sickle Cell Disease (SCD) than among Heterozygous (AS). The association between oxidative stress among patients of Anaemia due to SCD and iron deficiency states without SCD. Whether there exists the higher level of oxidative stress among the individuals of homozygous SCD as compared with their heterozygous counterpart and among the normal healthy individuals.

MATERIAL AND METHODS:

The current study is designed to explore specific antioxidant levels and oxidative stress among Sickle Cell Anaemia (SCA) cases. The study will explore the factors associated with antioxidant levels and oxidative stress among SCA cases, also the specific associated factors among homozygous and heterozygous SCA cases will be determined.

Study design:

The proposed hospital-based case control study will be conducted among the cases of homozygous SCD & heterozygous SCD, which will be compared among themselves and among the healthy controls with or without iron deficiency anaemia.

Study settings:

The current study will be conducted at *Jawaharlal Nehru Medical College (JNMC)* and *Acharya Vinoba Bhave Rural hospital (AVBRH)*, Sawangi (Meghe) Wardha in Central India.

Study participants:

The JNMC and AVBR Hospital is a tertiary care super speciality health care centre, located approximately 80 km southwest to the city of Nagpur.

Selection of cases:

The hospital conducts weekly specialized clinic for patients of SCD. The clinic collects and maintains an appropriate database of registered cases. This database is evaluated and updated with regular interval. The information of each case of SCD consists of baseline biochemical characteristics including conformation by haemoglobin electrophoresis⁵ and solubility test⁶, done in Department of

Biochemistry. Accordingly, the potential participants that is, homozygous (SS) SCD and heterozygous (AS) SCD will be identified from the database.

The stratified random sampling will be applied for selection of cases. The strata will be based on the type of SCD either homozygous (SS) or heterozygous (AS). Among the SCD cases

based on their status of haemoglobin 50% will be selected from homozygous (SS) and remaining 50% will be selected from heterozygous (AS) SCD.

Selection of controls

The participants in the control group will be selected among the apparently healthy individuals accompanying with patients other than SCD Clinic, such persons will be matched for the age and sex of the case group individuals (as above). A baseline screening of potential controls will be conducted with routine physical examination and primary investigations consisted of hemogram, urine routine, liver function test and kidney function tests. The stratified random sampling for selection of control will be based on the haemoglobin status. Among the controls 50% individuals will be selected with iron deficiency anaemia and remaining 50% with normal haemoglobin concentration.

Study Period : 2016-2019

Sample size estimation

The study size is calculated on the formula $n=4pq/E^2$

Where p is prevalence of oxidative stress among SCD (22.5%)⁴, q is 100-p, E is anticipated relative precision (20% of p, that is $20*22.5/100=4.5$)

Therefore, the sample of $n= 4*22.5*77.5/4.5^2=344$. As there are three groups, each group will be consisting of 115 participants.

Accordingly, the case group will be consisting of 240 participants with 120 having homozygous SCD and remaining 120 with heterozygous SCD.

The control group will consist of 240 participants with 120 having iron deficiency anaemia and remaining 120 without IDA.

Inclusion criteria

Following is the inclusion and exclusion criteria that will be applied for while selecting the potential participants in each of the Case and Control groups.

Inclusion criteria for cases:

- 1) A diagnosed case of Sickle Cell Disease currently on the register of SCD Clinic with documented haemoglobin electrophoresis report.

Exclusion criteria for cases

- 1) Case of SCD admitted for ailments other than routine blood transfusion. (As they will be biased to have higher oxidative stress)
- 2) Case of SCD with its complications such as acute crisis
- 3) History of recent blood transfusion 3 months preceding the enrolment.
- 4) Case of SCD presented as apparently healthy but detected illness during the baseline screening, infectious diseases presented with acute febrile illness or concomitant chronic non communicable diseases such as Ischemic Heart Diseases, diabetes mellitus, hypertension etc.

Inclusion criteria for controls

- 1) Apparently healthy individuals without SCD or any concurrent symptoms.
- 2) No known SCD or any other haemoglobinopathies among first degree relative.
- 3) No known case of acute or chronic concurrent disease.

Exclusion criteria for controls

- 1) Unreliable or unknown history of current past illness.
- 2) Persons initially selected as apparently healthy but detected illness during the baseline screening, infectious diseases presented with acute febrile illness or concomitant chronic non communicable diseases such as Ischemic Heart Diseases, diabetes mellitus, hypertension etc.

Method of Collection of Data

After due consent from the study subject, 4ml venous blood will be collected in plain bulb and allowed to stand for 30 min on the table. After 30 minutes the blood sample in plain bulb shall be centrifuged at 3000rpm for 15 min. Separated serum will be stored at -20⁰c and analysed within two weeks of storage. Collected serum shall be used to estimate following parameters:

1. Serum iron level
2. TIBC
3. Serum ferritin level
4. Vitamin E
5. Ceruloplasmin
6. Uric acid
7. Zinc

Under aseptic conditions another 4ml of venous blood shall be taken in EDTA container and used for the analysis of the following parameters.

1. Complete blood cell count.
2. Malondialdehyde. (MDA)
3. Catalase
4. Glutathione peroxidase (GPx)
5. Superoxide dismutase (SOD)
6. Glutathione reductase
7. Vitamin C

BIOCHEMICAL ANALYSIS:

I) Complete blood count: Will be done by either by ABX MICROS 60 / SYSMEX KX-21/ BECKMAN COUNTER blood cell counter.

II) Measurement of Oxidative stress markers :

1. Malondialdehyde (MDA):MDA expressed in nmol by result of Thiobarbituric acid estimation in serum using spectrophotometer⁷.
2. Glutathione peroxidase (GPx): This enzyme will be measured by using Fortress Diagnostic U.K. kit using ELICO SL 244 UV-VIS/BL 198 BIO spectrophotometer/ ROBONikprieteste XP Biochemistry Analyzer⁸.
3. Catalase: This enzyme will be measured by using Merck Diagnostic kit using ELICO SL 244 UV-VIS/BL 198 BIO spectrophotometer/ ROBONikprieteste XP Biochemistry Analyzer^{9,10}
4. Superoxide dismutase (SOD): This will be measured by using Fortress Diagnostic kit using ELICO SL 244 UV-VIS/BL 198 BIO spectrophotometer/ ROBONikprieteste XP Biochemistry Analyzer¹¹.

III) Measurement of Antioxidants and Iron Indices for metabolic antioxidants

1. The concentrations of iron in serum will be measured by Ferrozine method, using Human diagnostics Worldwide – Wiesbaden-Germany Kit using /ELICO SL 244 UV-VIS/ BL 198 BIO spectrophotometer /ROBONikprietesteXP Biochemistry Analyzer¹².
2. The concentrations of TIBC in serum will be measured by Ferrozine method, using (Human diagnostics Worldwide – Wiesbaden-Germany) using ELICO SL 244 UV-VIS/ BL 198 BIO spectrophotometer/ ROBONikprietesteXP Biochemistry Analyzer¹².
3. Serum Ferritin will be estimated by ELISA method, using DiaMetra Milano–Italy Diagnostics UK, kits using ROBONiK / B4B Diagnostic divisions ELISA Reader¹³.

4. Transferrin will be calculated by formula : $0.70 \times \text{TIBC}$ and Transferrin saturation (%) will be calculated by formula: $\text{Iron}/\text{TIBC} \times 100$ ¹⁴.
5. Ceruloplasmin will be measured by using Audit Diagnostic kit using ELICO SL 244 UV-VIS/ BL 198 BIO spectrophotometer/ ROBONikprieteste XP Biochemistry Analyzer¹⁵.
6. Uric acid will be measured by by using Randox. kit using ELICO SL 244 UV-VIS / BL 198 BIO spectrophotometer/ ROBONikprieteste XP Biochemistry Analyzer¹⁶.
7. Glutathione reductase will be measured by using Randox kit using ELICO SL 244 UV-VIS/ BL 198 BIO spectrophotometer/ ROBONikprieteste XP Biochemistry Analyzer¹⁷.
8. Zinc will be measured by colorimetric method using Centronicdiagnostic Warternberg Germany kit using ELICO SL 244 UV-VIS/BL 198 BIO spectrophotometer/ ROBONikprieteste XP Biochemistry Analyzer¹⁸.
9. Ascorbic acid (vitamin C) level will be measured by the method of Ayekyaw (1978) by colorimetric method using ELICO SL 244 UV-VIS/ BL 198 BIO spectrophotometer¹⁹.
10. Vitamin E will be assayed by colorimetric method using ELICO SL 244 UV-VIS / BL 198 BIO spectrophotometer²⁰.

Statistical Analysis:

The information on the source documents will be digitized in MS excel and further analysed using SPSS version 17.0. The quantitative variables will be analysed by percentages, mean & SD etc. The qualitative variables will be analysed by proportions. The comparative analysis will be performed by individual values with degree of dispersion with 95% confidence interval and the appropriate test of significance with 5% level of significance. The factors associate with oxidative stress will be analysed in the individual groups and compared with bivariate analysis using appropriate test of significance. The quantification of association will be determined by intergroup odds ratio. The logistic regression analysis will be performed to determine the role of independent association of the key factors as determined in the bivariate analysis.

Expected outcomes:

Oxidative stress as evaluated by stress markers is more among Homozygous (SS) sickle cell disease than in sickle cell Heterozygous (AS) disease patients. Association of oxidative stress in Sickle cell anaemia and iron deficiency states without sickle cell disease.

Chart 1. LOG FRAME MATRIX FOR ACHIEVEMENT OF EXPECTED OUTCOME

Objective	Key variables (Type)	Measurement (cut off levels)	Source of information	Expected outcome	Conclusion
Baseline assessment of study participants	Mean age & SD (Nominal)	Years	Source document (Case report format)	No significant statistical difference among the groups	Comparability of the groups
	Sex (Dichotomous)	M/F			
	Locality (Dichotomous)	Urban/Rural			
	SE Status (Ordinal)	Prasad's SE scale			
	Obesity Yes / No (Nominal)	BMI			
	Smoking Yes / No (Nominal)	History			
	Alcohol consumption Yes /No (Nominal)	History			
	Type of SCD (Dichotomous)	Hb electrophoresis	SCD clinic database		
	Anaemia Yes / No (Dichotomous) Severity (ordinal)	Blood Hb(g%)	Lab report format		
To measure and compare the antioxidants, oxidative stress among SCD cases and healthy controls.	1. Serum iron level (Nominal)	µg/dl	Source document (Lab report format)	High	Presence and degree of oxidative stress
	2. TIBC (Nominal)	µg/dl		High	
	3. Serum ferritin level (Nominal)	ng/ml		High	
	4. Vitamin E (Nominal)	µg/ml		Low	
	5. Ceruloplasmin (Nominal)	mg/dl		High/Low	
	6. Uric acid (Nominal)	mg/dl		High/Low	
	7. Zinc (Nominal)	µg/dl		Low	
	8.Malondialdehyde (MDA) (Nominal)	mmol/dl		Low	
	9.Catalase (Nominal)	nmol/min/ml		High	
	10.Glutathione peroxidase (GPx) (Nominal)	U/ml		Low	
	11.Superoxide dismutase (SOD) (Nominal)	U/ml		High/low	
	12.Glutathione reductase(Nominal)	U/L		High /Low	
	13.Vitamin C (Nominal)	mg%		Low	

DISCUSSION:

Polymerization of deoxygenated red blood cells results in a cascade of ischemic injury, inflammation, and vaso-occlusion crises. Cumulative effects of these increases reactive oxygen species (ROS), in turn reflecting increased intensity towards symptoms of SCD with generation of vicious circle²¹.

Oxidative stress has a role to play in the pathophysiology of SCA and intervention aimed at increasing the antioxidant capacity of these patients may be beneficial²². Striking increased levels of superoxide dismutase and lipid peroxidation are found in both sickle cell carrier and diseases²³.

Normally iron stores is reflected by Ferritin, a metabolic antioxidant produced during iron metabolism. When need arises, they are released by apoferritin shell and binds with transferrin. Transferrin is another metabolic antioxidant, transports iron towards erythropoietic cells. Iron is generally 25 to 30 % saturated with transferrin. Additionally, iron status is constituted by serum iron and total iron binding capacity²⁴.

In India suggestion towards iron deficiency needs an attention with its caution supplementation in sickle cell anemia, in light of iron status of the body already under the stress due to haemolysis²⁵. Also, iron deficiency anaemia seems to be more in heterozygous than homozygous Sickle cell disease²⁶. Owing to higher iron content in the reticuloendothelial, sickle cell children show comparatively higher ferritin considering healthy children²⁷.

Conclusion: Oxidative stress may be compounded in sickle cell diseases patients in conjugation with iron deficiency.

Competing interests: None declare

REFERENCES:

1. Cohen A, Schwartz E. Iron chelation therapy in sickle cell anemia. *Am J Hematol* 1979; 7:69-76.
2. Vichinsky E, Kleman K, Embury S, Lubin B. The diagnosis of iron deficiency anemia in sickle cell disease. *Blood* 1981; 58:963-8.
3. Yoo JH, Maeng HY, Sun YK et al., Oxidative status in Iron deficiency anemia. *J Chin Lab Anal*.2009;23:319-23.
4. Deshmukh P , Garg BS , Garg N, Prajapati NC, Bharambe MS. Prevalence of sickle cell disorders in rural Wardha. *Indian J Community Med*.2006;31: 26-7.
5. Marengo-Rowe AJ. Rapid electrophoresis and quantitation of hemoglobin on cellulose acetate. *J Clinical Path*.1965;18:790-92.
6. Nalbandian RM, Nichols BM, Camp FR Jr, Lusher JM, Conte NF, Henry RL et al., Dithionite tube test-- a rapid, inexpensive technique for the detection of haemoglobin S and non-S sickling haemoglobin. *Clinical Chemistry* .1971; 17(10) :1028-32.
7. Devasagayam T.P.A, Pushpendran CK, Eapen J. Differences in lipid peroxidation of rat liver rough and smooth microsomes. *Biochem Biophys Acta* 750.1983; 91-7.
8. Thomas JP, Maiorino M, Ursini F, Girotti AW. Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. *J Biol Chem*. 1990; 265:454-61.
9. Johansson, LH, Borg, LAH. A Spectrophotometric method for determination of catalase activity in small tissue samples. *Anal. Biochem*.1988;174,331-336.
10. Wheeler CR., Salzman JA, Elsayed NM, Omaye ST, Korte DW. Automated assays for superoxide dismutase activity, catalase, glutathione peroxidase and glutathione reductase activity. *Anal. Biochem*.1990;184,193-9.

11. Arthur JR., Boyne R. Superoxide dismutase and Glutathione peroxidase activities in neutrophils from selenium deficient and copper deficient cattle. *Life sciences*.1985; 36:1569–1575. doi10.1016/0024-3205(85)90381-9
12. Garcic A.A high-sensitive simple determination of serum iron using chromazurol. *B. Clin. chim. Acta*94. 1979 ;115.
13. Van Oost BA, Willekens. FL, Neerbos BRV and Beld BVD. Implication of using different tissue ferritins as antigen for ferritin in serum four radioimmunoassay kits compared.*Clin.Chem*.1982;28/12,2429-33.
14. HigginsTE, Beutler, Doumas BT, “Hemoglobin, Iron and Bilirubin,” *Textbook of Clinical Chemistry and Molecular Diagnostics*, Burtis CA,Ashwood ER and Bruns DE, Eds.pp. 1186–1191, Elsevier, St. Louis, Mo, USA.
15. Ceron JJ,Subiela SM. Anautomated spectrophotometric method for measuring canine ceruloplasmin in serum.*Vet.Res*.35. 2004; 671-9.
16. Buchanan MJ, Isdale IC and Rose BS.Serum uric acid estimation Chemical and enzymatic methods compared.*Ann.rheum.Dis*.1965; 25,285-8.
17. Bompert GJ. Prevot DS, and Bascands JL. Rapid automated analysis of glutathione reductase,peroxidase and s-transferase activity.*Clin Biochem* 1990; 23,501-504.
18. Johnsen and R. Eliasson. Evaluation of a commercially available kit for the colorimetric determination of zinc. *International J Andrology*. 1987; April 10 (2): 435-40.
19. Kwaw A. A simple colorimetric method for ascorbic acid determination in blood plasma. *ClinicaChimica Acta* .1978; 86 (2): 153-157.
20. JargarJG ,Hattiwale SH, Das S, Dhundasi SA , Das KK. A modified simple method for Determination of serum α -tocopherol (Vitamin E). *J Basic Clin PhysiolPharmacol* .2012; 23(1): 45-8.
21. Chirico EN, Pialoux V. Role of Oxidative Stress in the Pathogenesis of Sickle Cell Disease. *IUBMB Life*. 2012; 64(1): 72–80.
22. Fasola F, Adedapo K, Anetor J. Total Antioxidants Status and Some Hematological Values in Sickle Cell Disease Patients in Steady State. *J Natl Med Assoc*. 2007; 99:891-894
23. Titus J, Chari S, Gupta M and Parekh N. Pro-oxidant and anti-oxidant status in patients of sickle cell anemia. *Indian Journal of Clinical Biochemistry*. 2004; 19 (2) 168-172.
24. Addison G.M, Beamish M.R, Hales C.N, Hodgkins. M, Jacobs A, Llewelin P, “An immunoradiometric assay for ferritin in the serum of normal subjects and patients with iron deficiency and iron overload,” *Journal of Clinical Pathology*,1972; 25 (4) 326–329.
25. Mohanty D, Mukherjee MB, Colah RB, Wadia M. Iron deficiency anemia in sickle cell disorders in India. *Indian J Med Res*. 2008;127, 366-369.
26. Debkumar R, Ramkrishna M, Ujjai KC, Assessment of iron status in patients of sickle cell disease and trait and its relationship with frequency of blood transfusion. *International journal of scientific study*. 2014; 2(4).
27. Akodu SO, Diaku-Akinwumi IN, Kehinde OA, Njokanma.OF. Serum Iron Status of Under-Five Children with Sickle Cell Anemia. *Hindawi Publishing Corporation Anemia* .2013; Vol (2013). Article ID 254765.

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