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LEVEL OF NITRIC OXIDE AND ANTIOXIDANT VITAMINS IN SICKLE CELL ANAEMIA PATIENTS

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Abstract : Sickle cell anaemia (SCA) is characterized with sever anaemia and vasoocclussive episodes. Nitric Oxide (NO) a potential vasodilator, synthesized from various cells including endothelial cell. However SCA is associated with endothelial dysfunction, a measure cognitive factor for pulmonary hypertension (PH) and vasoocclussive crisis. The present study was attempted to evaluate level of serum NO and plasma antioxidant vitamins A, E and C in homozygous (n=30) and heterozygous (n=30) sickle cell patients and compared with age and sex matched healthy controls (n=30). We found, significantly (P<0.0001) elevated level of serum NO and significantly (P<0.0001) depleted antioxidant vitamins in homozygous and heterozygous sickle cell patients compared to healthy controls. Our study reveals that oxidative stress may be a responsible factor for the reduced bioavailability of NO which can impair the vasodilation in sickle cell patients.

Key words : sickle cell anaemia, vasoocclussive crisis, nitric oxide, oxidative stress, antioxidant vitamins

INTRODUCTION

Sickle cell anaemia (SCA) was the first disease to be characterized at the molecular level, but some of mechanisms underlying pathophysiology remain unexplained. Similar to the vascular disease like atherosclerosis, SCA is associated with chronic inflammation and ischemia-reperfusion injury due to the occlusion of rigid sickle erythrocytes in capillary beds (1, 2). Vasoocclusive consequences lead to acute episodic pain, infection, cerebral infarction, acute chest syndrome, splenic sequestration, end organ damage and early death (3).

Nitric Oxide (NO) synthesized in an oxygen dependant reaction catalyzed by nitric

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oxide synthase (NOS), converting L-arginine to citrulline. Nitric oxide synthase has 3 major isoforms; neuronal (nNOS) and endothelial (eNOS) being constitutive, and inducible (iNOS). iNOS is expressed after the induction by lipopolysaccharide (LPS), interleukins (IL-1, IL-11), and tumor necrosis factor (TNF-α) the inflammatory mediators in various inflammatory disorders such as inflammatory bowel disease, left ventricular failure, metastatic melanoma and neutropenic sepsis (4, 5).

Elevated level of malondialdehyde (MDA), a lipid peroxidation product and superoxide dismutase (SOD) activity has been also observed in previous studies suggesting excessive formation of reactive oxygen species (ROS) by sickle cells. Lowered level of antioxidant vitamins and enzyme activities were also reported in SCA patients (6, 7). These ROS can react with NO converting into more potent reactive NO species (RNOS) which may damage the cell membrane. This may exaggerate the sickling and hemolytic consequences in sickle cell anaemia. Further, hemolysis may derange the NO metabolism that reduces the bioavailability of NO leading to poor vasodilation and vasoocclussive process.

Therefore, measurement of NO along with antioxidant vitamins can provide a simple and potential inflammatory as well as oxidative stress marker in sickle cell anaemia.

MATERIAL AND METHODS

The present study was carried out in the Department of Biochemistry, Annasaheb Chudaman Patil Memorial Medical College and sickle cell centre, Shri Bhausaheb Hire Government Medical College, Dhule, Maharashtra. Prior to start the study, local ethical clearance was obtained. A total population of 90 subjects were enrolled in the study, including age and sex matched 30 (15 male and 15 female) healthy controls (HbAA), 30 (15 male and 15 female) homozygous (HbSS) and 30 (15 male and 15 female) heterozygous (HbAS) sickle cell patients on the basis of solubility test and HPLC analysis of blood. Subjects were excluded from the study using criteria of age <15 years, other than HbAS and HbSS pattern, past three month history of crisis, blood transfusion, treatment with hydroxyurea, use of vitamins and trace elements supplementation and pregnancy.

After obtaining the written consent from all the subjects included in the study a total of 7 ml of blood withdrawn aseptically from the antecubital vein. From this approximately 3 ml blood in EDTA (0.47 mol/L K3-EDTA) container and 4 ml blood in plain container drawn to obtain plasma and serum respectively. Samples were centrifuged at 3000 rpm for 10 min to separate the plasma and serum. For the estimation of nitric oxide, serum was deproteinised first and nitrate was reduced to nitrite by cadmium granule reduction method which then coupling with N-napthyethylenediamine to give pink coloured complex as per the method of Cortas and Wakid (8). Plasma Vitamin A measured by Carr-Price reaction in which blue colour complex was formed (9). Plasma Vitamin E determined by Baker and Frank method which is based on reduction of ferrous ions which forms a red coloured complex with α-α^1 dipyridyl (10). Plasma Vitamin C measured by Caraway method based on the reaction

with dinitrophenyl hydrazine (11).

Data were analyzed using SPSS program version 16.0. Values were expressed as means±SD, significance of the mean difference between SCA patient and control was assessed by statistical paired student T test. Pearson's correlation coefficient used for the correlation assessment.

RESULTS

Data of Table No. I shows, significantly (P<0.0001) increased level of serum NO and significantly (P<0.0001) decreased antioxidant vitamins (A, E and C) in heterozygous as well as homozygous sickle cell anaemia patients compared to controls. We also seen negative correlation between plasma NO level and Vitamin A (r= –0.62, P<0.01), Vitamin E (r=–0.72, P<0.01) and Vitamin C (r=–0.64, P<0.01) in homozygous sickle cell patients.

DISCUSSION

In the present study, we observed the elevated level of serum NO and depleted antioxidant vitamins in SCA patients compared to the control group. Acute painful vasoocclussive crisis (VOC) is one of the earliest manifestations of sickle cell disease (SCO) may occur in early age of life (3). Endothelial dysfunction is associated with intravascular hemolysis, reduced nitric oxide (NO) bioavailability, oxidative stress and inflammation. This leads to vasomotor instability and ultimately producing a proliferative vasculopathy, leading to the development of the pulmonary hypertension (PH) in adult age. Pulmonary hypertension, a common complication is a major predictor of the mortality rate (12, 13). Reduced bioavailability of NO, act as predisposing factor for the vasoocclusion by promoting RBC adhesion and impairing the regional regulation of blood flow (14).

Among endothelial mediators, nitric oxide (NO) regulates the normal vascular tone, cellular adhesion, platelet aggregation, and thrombosis. Various studies have demonstrated a state of resistance to the vasodilation due to eNOS mediated impaired blood flow (15, 16). Recently, it has been shown that, this state of NO resistance to the endogenous and exogenous NO, correlated with increased plasma hemoglobin levels which coupled to hemolytic rate and

TABLE I: Mean (SD) levels of serum NO and antioxidant vitamins in controls (HbAA), heterozygous (HbAS) and homozygous (HbSS) sickle cell patients.

Parameters	*Controls*	*Heterozygous*	*Homozygous*
N	30	30	30
Age in years	15–60	15–60	15–60
Male	15	15	15
Female	15	15	15
Nitric Oxide µ mol/l	32.11±6.49	63.41±16.75*	82.88±33.18*
Vitamin A µg/dl	42.73±4.88	30.76±2.44*	23.43±2.82*
Vitamin E µg/dl	82.29±3.06	56.97±4.47*	42.86±3.73*
Vitamin C µg/dl	85.05±3.59	70.26±5.21*	46.81±6.62*

*-P<0.0001 – Control Vs Homozygous Sickle cell anaemia.
*-P<0.0001 – Control Vs Heterozygous Sickle cell anaemia.

oxidant stress (13, 17). After, the hemolysis hemoglobin is decompartmentalized and released into plasma, where it rapidly reacts with NO and destroys it (12). This results in the abnormally increased consumption of NO forming NO free radicals and ultimately inhibiting vasodilation. The simultaneous release of erythrocyte arginase during hemolysis may limit the availability of arginine to NOS, contributing to a deficiency of NO in vascular system (18).

Aslan and Freeman have shown that superoxide radical formed in the reaction catalyzed by xanthine oxidase in the endothelium inhibit the action of NO in the vasculature of transgenic sickle cell mice (19, 20). Overproduction of ROS in microvasculature increases the oxidative stress that can disrupt NO homeostasis and produce the highly oxidative peroxynitrite radicals. Our finding of increased level of NO in SCD patients is further supported by a previous study reporting accelerated auto-oxidation, enhanced oxidative stress, increased susceptibility to lipid peroxidation, and increased generation of ROS in sickle cell patients (4, 7).

Essien et al have found significantly low values of plasma vitamins A (retinol), C (ascorbic acid) and E (alpha tocopherol) in patients with sickle cell anemia in steady state compared to controls, which support our results (21). Vitamin E has chain breaking antioxidant, membrane protective and anti-inflammatory actions. While vitamin C synergistically act together with vitamin E by spearing its action. Studies performed with mixed tocopherols supplementation have demonstrated that vitamin E activates endothelial NOS, increases NO release, and decreases platelet aggregation *in vivo* (22). Adelekan and Ray et al, who also noticed significantly low levels of antioxidant vitamins such as β-Carotene, Vitamin E and Vitamin C in sickle cell patients (6, 23).

Regular supplementation of these vitamins may ameliorate some of the sickle cell manifestations such as vasoocclussive crises, acute chest syndrome, recurrent infection and growth retardation (2). Natta et al observed marked reduction in the number of circulating irreversible sickle erythrocytes in their 35 week supplementation of vitamin E in patients with sickle cell anaemia (24). Supplementation of antioxidant vitamins can mitigate the oxidative stress which may improve the bioavailability of NO, vasodilation and prevent vasoocclussive crisis.

Thus, our study shows elevated serum NO and depleted antioxidant vitamins suggests hyphened oxidative stress in SCA patients. We conclude from this study that the regular antioxidant supplementation might be useful in the management of SCA patients.

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REFERENCES

1. Belcher JD, Marker PH, Weber JP, et al. Activated monocytes in sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion. *Blood* 2000; 96: 2451–2459.
2. Kaul DK, Hebbel RP. Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice. *J Clin Invest* 2000; 106: 411–420.
3. Powars D. Natural history of disease: the first two decades. In: Embury SH, Hebbel RP, Mohandas N, Steinberg MH, eds. Sickle Cell Disease: Basic Principles and Clinical Practice. New York, NY: Raven Press; 1994: 395–412.
4. Katherine C, Nail-Granger D. Sickle cell Disease. Role of reactive oxygen and nitrogen metabolites. *Clin and Exp Pharm and Phys* 2007; 34: 926–932.
5. Neilly IJ, Copland M, Haj M, Adey G, Benjamin N, Bennett B. Plasma nitrate concentrations in neutropenic and nonneutropenic patients with suspected septicaemia. *British Journal of Haematology* 1995; 89: 199–202.
6. Adelekan DA, Thurnham DI, Adekile AD. Reduced antioxidant capacity in pediatric patients with homozygous sickle cell disease. *Eur J of Clin Nutr* 1989; 43: 609–614.
7. Das SK, Nair R. Superoxide dismutase, glutathione peroxidase, catalase and lipid peroxidation of normal and sickled erythrocytes. *Br J Haematol* 1980; 44: 87–91.
8. Najwa KC, Nabil WW. Determination of inorganic nitrate in serum and urinary by *Clin Chem* 1990; 36(8): 1440–1443.
9. Kimble-1938, Kaser & Stekol-1943. Varleys Practical Biochemistry, 1980, 5th edition Vol II.
10. Baker and Frank, Varleys. Practical Biochemistry, 1980, 5th edition Vol II.
11. Caraway WT. Carbohydrates N.W. Ed., Fundamentals of clinical chemistry. 2000; 173–176.
12. Reiter CD, Wang X, Tanus-Santos JE, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med* 2002; 8: 1383–1389.
13. Kaul DK, Liu XD, Chang HY, Nagel RL, Fabry ME. Effect of fetal hemoglobin on microvascular regulation in sickle transgenic-knockout mice. *J Clin Invest* 2004; 114: 1136–1145.
14. Moncada S, Higgs A. The L arginine nitric oxide pathway. *N Engl J Med* 1993; 329: 2002–2012.
15. Eberhardt RT, McMahon L, Duffy SJ, et al. Sickle cell anemia is associated with reduced nitric oxide bioactivity in peripheral conduit and resistance vessels. *Am J Hematol* 2003; 74: 104–111.
16. Nath KA, Shah V, Haggard JJ, et al. Mechanisms of vascular instability in a transgenic mouse model of sickle cell disease. *Am J Physiol Regul Integr Comp Physiol* 2000; 279: R1949–R1955.
17. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. *JAMA* 2005; 293: 1653–1662.
18. Morris CR, Kato GJ, Polijokovic M et al. Dysregulated arginine metabolism, hemolysis associated pulmonary hypertension and mortality in sickle cell disease. *JAMA* 2005; 294: 81–90.
19. Aslan M, Ryan TM, Adler B, et al. Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. *Proc Natl Acad Sci* 2001; 98: 15215–15220.
20. Aslan M, Ryan TM, Townes TM, et al. Nitric oxide-dependent generation of reactive species in sickle cell disease. Actin tyrosine induces defective cytoskeletal polymerization. *J Biol Chem* 2003; 278: 4194–4204.
21. Essien EU. Plasma levels of retinol, ascorbic acid and alpha-tocopherol in sickle cell anemia. *Cent Afr J Med* 1995; 41(2): 48–50.
22. Liu M, Wallmon A, Olsson-Mortlock C, et al. Mixed tocopherols inhibit platelet aggregation in humans: Potential mechanisms. *Am J Clin Nutr* 2003; 77: 700–706.
23. Ray D, Deshmukh P, Goswami K, Garg N. Antioxidant vitamin levels in sickle cell disorders. *Natl Med J India* 2007; 20(1): 11–13.
24. Natta CL, Machlin LJ, Brin M. A decrease in reversible sickle erythrocytes in sickle cell anaemia patients given vitamin E. *Am J Clin Nutr* 1980; 33: 968–971.