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Research Article

L-arginine and no levels are diminished in Children of African Descent with Acute Vaso-Occlusive Sickle Cell Crisis in Sokoto, Nigeria

Erhabor Osaro[1*], Ibrahim Bagudo Aliyu[1], Abdulrahaman Yakubu[1], Safiya Mohammed[1], Imoru Momodu[1], Udomah Francis[1],Ahmed Marafa[1], Ibrahin Kwaifa[1], Buhari H[1], Zama Isaac[1], Okwesili Augustine[1], Onuigwe Okechukwu[1], Jiya N[2], Knox Van Dyke[3], Egenti Nonye[4]

[1]*Department of Haematology and Blood Transfusion Science, Usmanu Danfodiyo University Sokoto, Nigeria*

[2]*Department of Paediatrics, Usmanu Danfodiyo University Sokoto, Nigeria*

[3]*Department of Biochemistry, West Virginia University Morgantown, USA*

[4]*Department of Community Medicine University of Abuja, Nigeria*

**Corresponding author: Prof. Osaro Erhabor, Haematology Department, Faculty of Medical Laboratory Science, Usmanu Danfodiyo University, Sokoto, Nigeria, E-mail: n_osaro@yahoo.com*

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Abstract

Sickle cell disease is an autosomal recessive disorder and the most common genetic disease affecting Africans. Alterations in nitric oxide production may have an important role in the pathophysiology of SCD. The aim of this study was to evaluate L-arginine and nitric oxide levels among children of African descent presenting to the Children Emergency Unit of Usmanu Danfodiyo University Sokoto with sickle cell disease (SCD). Plasma levels of L- arginine and Nitric Oxide (NO) were measured among 90 children aged 1-6 years and mean age 4.01 ± 0.87 years with sickle cell disease and 50 apparently healthy age-matched controls. The NO and L-arginine levels were significantly higher among normal control children compared to the SCD children (p=0.05). The study subjects were also classified based on the presence or absence of vaso-occlusive crisis (VOC) at the time of sample collection. Plasma L-arginine and serum NO levels were examined among 43 children with SCD with episodes of VOC and 47 age -matched children with SCD at steady-state. L-arginine levels was lower among SCD children with VOC (40.7 ± 3.77) compared to those in steady state (48.92± 6.10) (p=0.05). Similarly, the NO levels were significantly decreased among SCD children with VOC (94.80 ± 14.50) compared to those in the steady state (425.70 ± 15.05) (p=0.01). The findings from this study suggest that there may be a relationship between the L-arginine-nitric oxide pathway and vaso-occlusive crisis in SCD. Low arginine levels during VOC may be a reflection of acute L-arginine substrate depletion that may have resulted in decreased nitric oxide production. It may be necessary to routinely monitor the L-arginine and NO particularly among children presenting with vaso-occlusive crisis. Finding from this study may be a justification for L-arginine supplementation of children of African descent with SCD.

Keywords: L-arginine; Nitric oxide; Sickle cell disease; Vaso-occlusive crisis; Children; African Descent

Introduction

Sickle cell disease (SCD) is the most common and clinically haemoglobinopathy particularly among children of African descent. It is a collective name for a group of conditions causing clinical symptoms that result from the formation of sickle red cells. Normal red blood cells are biconcave discs. Sickle shaped red cells have a characteristic crescent shape. It results from a substitution of a single amino acid called glutamic acid by valine at position 6 of the beta globin polypeptide chain. It is inherited as an autosomal recessive trait. Homozygotes

only produce abnormal haemoglobin chains that make haemoglobin S (HbS, termed SS), and this results in the clinical syndrome of sickle cell disease. Heterozygotes produce a mixture of normal and abnormal beta chains that make normal HbA and HbS (termed AS), and this results in the clinically asymptomatic sickle-cell trait [1]. Sickle cell anaemia (SCA) is one type of anaemia which occurs if the red blood cells don't contain enough haemoglobin. Haemoglobin is the oxygen carrying pigment of red blood cells. SCA results both from haemolysis and reduced oxygen affinity of haemoglobin S. When haemoglobin S is deoxygenated, the molecules of haemoglobin polymerizes to form pseudocrystalline structures known as "tactoids". These distort the red cell membrane and produce characteristic sickle –shaped cells [1]. In addition, intracellular polymers lead to red cell membrane changes, generation of oxidant substances and abnormal adherence of red cells to vascular endothelium [2]. Although there is no cure for this ailment, novel or promising therapies have been tested [3-5]. One of the novel and promising therapies is L- Arginine supplementation. Sickle cell subjects have a dysregulated arginine metabolism, leading to increased oxidative stress and possible depleted arginine levels. L- arginine is considered to be a nutritionally dispensable amino acid in humans. Preterm infants are unable to synthesize or create arginine internally, making the amino acid nutritionally essential for them [6]. Most healthy people do not need to supplement with arginine because their body produces sufficient amounts [7]. L-arginine is the nitrogen donor for the synthesis of Nitric Oxide, a potent vasodilator that is deficient during times of sickle cell crisis. One of arginine's key functions in the body is to convert into Nitric oxide. It is the substrate for the endothelial nitric oxide (NO) synthase (eNOS), which metabolizes this amino acid to L-citrulline and NO which is a powerful vasodilator with antiplatelet properties [8]. NO is inactivated by haemoglobin and other heme proteins, which bind it tightly. NO is of major importance in the maintenance of blood pressure invivo. L- arginine is deficient in sickle cell disease. Increased NO consumption by cell-free plasma haemoglobin and reactive oxygen species leads to decreased NO bioavailability. L- arginine is low in HbSS adults in the steady state and appears to decrease to even lower levels during acute pain episodes [9,10].

Vasoocclusive "crises" comprise a variety of syndromes that are typically recurrent and potentially catastrophic. Clinical manifestations are sudden in onset and are directly attributable to obstruction of the microcirculation by intravascular sickling. They are the most frequent type of crisis in sickle cell anaemia. Possible risk factors for the development of sickle cell vasoocclusion include Hb S polymerization, sickle cell deformability, sickle blood viscosity, the fraction of dense cells, sickle cell–endothelial cell adherence, endothelial cell activation, haemostatic activation, vascular tone, contributions from white blood cells and platelets, local and regional environmental factors, and psychosocial factors [11]. They are precipitated by such factors as infection, acidosis, dehydration or deoxygenation (altitude, operations, obstetric delivery, stasis of the circulation, exposure to cold and violent exercise.

NO is the major endothelium-derived relaxing factor in normal physiology. It plays a central role in vascular homeostasis by maintaining vasomotor tone, limiting ischemia-reperfusion injury, and modulating endothelial proliferation [12]. Plasma levels of the NO metabolites nitrite and nitrate and plasma arginine levels are depressed in sickle cell patients during vaso-occlusive crisis and the acute chest syndrome (ACS) [13]. L-arginine is an orally available dietary supplement that produces NO-dependent increased blood flow and reduced pulmonary artery pressure in patients with pulmonary hypertension.

Clinicians in developing countries including Nigeria face significant challenges in managing patients with sickle cell disease. A better understanding the disease, its progression and appropriate prophylactic treatment can have a positive impact of the quality of life of the patients and by extension, the overall management. There is paucity of studies on L-arginine and nitric oxide levels among patients with SCD. It is not known what the effect of vaso-occlusive crisis is on the L-arginine and nitric oxide levels of Nigerian children with SCD. The aim of this study was to compare the levels of L-arginine and NO among three groups of children of African descent in Sokoto, North Western Nigeria: healthy, steady state with SCD and SCD presenting with vaso occlusive crisis (VOC).

Materials and Methods

Study Design, Informed Consent and Ethical Considerations

This research was a case- control study and included children with SCD (Subjects). Age and gender- matched children who were homozygous for haemoglobin A was monitored as controls. The subjects were recruited from among the children presenting to the Paediatric Department of Usmanu Danfodiyo University Teaching Hospital Sokoto. Control children were recruited from among healthy children presenting to the paediatric department for routine health check. Socio- demographic data of the patients was obtained by using an interviewer- administered questionnaire which included the age, gender and other socio-demographic data. Quantitative data was gotten by estimating the serum l-arginine and NO on presenting to the paediatric department of Usmanu Danfodiyo University Teaching Hospital Sokoto. Ethical approval was obtained from the ethical committee of Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto. Verbal informed consent was obtained from the parents or guardians of the subjects prior to the commencement of the study.

Study Site and Participating Hospital

The study was carried out in Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto, Nigeria. Sokoto State

is located in the extreme North Western corner of Nigeria, it occupies 25,973 square kilometres and is situated along latitude $13^0$3'39 N and longitude 5^0 14' 20 E. As of 2005, it had an estimated population of more than 4.2 million (NPC, 2007). It shares its borders with Niger Republic to the North, Zamfara State to the East, Kebbi State to the South-East and Benin Republic to the West. With an annual average temperature of 28.3°C (82.9°F). Sokoto is in the dry Sahel, surrounded by Sandy Savannah and isolated Hills. Sokoto is on the whole, a very hot area. Study was conducted in the Paediatric Department of Usmanu Danfodiyo University Teaching Hospital Sokoto in collaboration with the Haematology Department of the Faculty of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria.

Study Participants

The study included children with a confirmed HbSS electrophoretic pattern presenting to the paediatric unit of Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto. Verbal informed consent was obtained from the parents/guardian of all subjects. Ethical approval was obtained from the Usmanu Danfodiyo University Teaching Hospital Ethical and Research Committee before commencement of the study. The study included subjects with SCA aged 1-6 years and age and gender-matched controls.

Inclusion Criteria

Inclusion criteria included; African descent, confirmed homozygozity for haemoglobin-S, aged (1-6 years) and willingness of parents/ guardians to offer verbal informed consent for their ward to participate as subject in this study.

Exclusion Criteria

The following were excluded from the study; subjects not homozygous for HbS, children >6 years and < 1 year old, children who had a recent transfusion in the last 4 months and children whose parents or guardian refused to provide verbal informed consent for their ward to participate in the study.

Data Collection

Sample Collection

About 2 millilitres of blood sample was collected from each subject and control participants aseptically using the venepuncture technique. The blood was collected into tubes containing dipotassium ethylenediamine tetra-acetic acid (K2EDTA) anticoagulant from each subject and control participants on presentation to paediatric department of Usmanu Danfodiyo University Teaching Hospital Sokoto. The samples were left undisturbed at room temperature and later centrifuged at 3000rpm for 5 minutes to obtain clear non- haemolysed plasma. The plasma was transferred into sterile labelled test tubes and assayed (in batches) for l-arginine (Immundiagnostik Ltd Co, Germany) and Nitric Oxide (ENZO Blood Sciences, UK).

Statistical Analysis

Statistical analysis was performed using statistical package for social sciences (SPSS) version 20. Frequencies and percentages were calculated. Student t- test (independent t test and paired sample t-test) and ANOVA were used for comparison of data. The results were presented as mean ± standard error of mean. A p- value of ≤ 0.05 was considered as significant in all statistical comparisons.

Determination of Plasma L-arginine level

Plasma L- arginine levels was estimated using the Enzyme Linked Immunosorbent Assay (ELISA) technique (Immundiagnostik, Germany). This assay is based on the method of competitive enzyme linked immunoassays. The sample preparation includes the addition of a derivatization reagent for L-arginine derivatization. The treated samples and the polyclonal L-arginine antiserum are incubated in wells of a microtitre plate coated with L-arginine derivative (tracer). During the incubation period, the target L-arginine in the sample competes with the tracer immobilized on the wall of the microtiter wells for the binding of the polyclonal antibodies. The L-arginine in the sample displaces the antibodies out of the binding to the tracer. Therefore, the concentration of the tracer bound antibody is inversely proportional to the L-arginine concentration in the sample. During the second incubation step, a peroxidase-conjugated antibody is added to each microtiter well to detect the anti- L-arginine antibodies. After washing away the unbound components, tetramethylbenzidine (TMB) is added as a peroxidase substrate. Finally, the enzymatic reaction is terminated by an acidic stop solution. The colour changes from blue to yellow, and the absorbance is measured in a photometer at 450nm. The intensity of the yellow colour is inversely proportional to the L-arginine concentration in the sample; this means, high L-arginine concentration in the sample reduces the concentration of tracer- bound antibodies and lowers the photometric signal. A dose response curve of absorbance unit (optical density, OD at 450nm) versus concentration is generated using the values obtained from the standards. L- arginine present in the patient samples is determined directly from this curve.

Procedure

Determination of Plasma Nitric Oxide Levels

Plasma Nitric Oxide levels were estimated using the Griess Reaction (ENZO Blood Sciences, UK). This assay determines nitric oxide concentrations based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the two step diazotization reaction in which acidified NO2- produces a nitrosating agent, which reacts with sulphanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphth-

yl) ethylenediamene to form the chromophoric azo-derivative which absorbs light at 540-570nm. It is read using an ELISA microtiter plate reader.

Results

In this present study, the plasma levels of L- arginine and Nitric Oxide (NO) were measured among 90 children (at the time of admission) aged 1-6 years and mean age 4.01 ± 0.87 years with SCD and 50 apparently healthy age-matched controls. Table 1 shows the demographic characteristics of the patients and the control. It shows that the majority of the patients (35.6%) and control (24.5%) were aged 5 years old. The distribution of the patients and control group based on ethnicity shows that majority were Hausa, 67.8% and 66.7% respectively. The distribution of the patients and control based on gender showed that majority were males with 67.8% and 66.6% respectively. The mean –arginine and nitric oxide levels were significantly lower sickle cell disease subjects (43.7± 5.77 µmol/l and 363.2 ± 12.5 µmol/l) respectively compared to controls (52.2 ± 4.13 µmol/l and 373.3 ± 17.1 µmol/l) respectively (p=0.05). The study subjects were also classified based on the presence or absence of vaso-occlusive crisis at the time of sample collection. Of the 90 subjects, 43 who presented with crisis on admission were found to have significantly lower levels of L-arginine and nitric oxide levels (40.7 ± 3.77 µmol/l and 94.80 ± 14.50 µmol/l) compared to those in their steady state (48.92± 6.10 µmol/l and 425.70 ± 15.05 µmol/l) respectively (p=0.05 and 0.01 respectively). Table 3 above shows the effect of VOC on the plasma levels of L- arginine and NO in subjects.

Characteristics	Controls		Subjects	
	N= 50	Percentage (%)	N= 90	Percentage (%)
Age (years)				
1	7	13.7	3	3.3
2	6	11.8	9	10.0
3	9	17.6	13	14.4
4	5	9.8	15	16.7
5	12	24.5	32	35.6
6	11	22.6	18	20.0
Ethnicity				
Hausa	33	66.7	61	67.8
Fulani	13	25.5	25	27.8
Yoruba	2	3.9	2	2.2
Igbo	2	3.9	2	2.2
Gender				
Male	34	66.6	61	67.8
Female	16	33.4	29	32.2

N= number of subjects

Table 1. Demographic characteristics of Controls and Patients.

Parameter	Subjects N= 90	Control N= 50	t-value	p- value
L-arginine (µmol/l)	43.7± 5.77	52.2 ± 4.13	19.53	0.05
Nitric Oxide (µmol/l)	363.2 ± 12.5	373.3 ± 17.1	5.60	0.05

Level of significance is considered when p<0.05

Table 2. Comparison of the Plasma levels of L-Arginine and Nitric Oxide among both the study and control groups.

Parameters	VOC			p-value
	Yes (N= 43)	No (N= 47)	t-test	
L- arginine (µmol/l)	40.7 ± 3.77	48.92± 6.10	8.84	0.05
Nitric Oxide (µmol/l)	94.80 ± 14.50	425.70 ± 15.05	144.2	0.01

Level of significance is considered when p<0.05
VOC = Vaso-occlusive crisis

Table 3. The Effect of Vaso-Occlusive Crisis on the Plasma Levels of L- arginine and Nitric oxide levels in Children with Sickle Cell Anaemia.

Discussion

Information about the levels of L- arginine and Nitric Oxide is useful for providing better services and effective management of SCD. This research work was carried out to determine the levels of plasma L- arginine and Nitric Oxide among pre-school children with SCA in UDUTH, Sokoto, North Western Nigeria. Ninety children with sickle cell anaemia and 50 apparently healthy controls were studied. The plasma levels of L- arginine and Nitric Oxide were found to be significantly lower among pre-school children with SCD subjects compared to values among the apparently healthy controls (p=0.05). Our finding is consistent with previous report which indicated that SCD is an arginine deficiency syndrome [14,15]. Our finding is also consistent with previous reports which indicated that adults SCD patients are arginine deficient at steady state [16,17]. An arginine deficiency develops over time and is influenced by acute events [18]. Normal arginine metabolism is impaired through various mechanisms that contribute to endothelial dysfunction, vaso-occlusion and early mortality [19-20]. There is growing advocacy that since low global arginine bioavailability is associated with a growing number of SCD-related complications [21], arginine therapy may represent a promising option for SCD patients [19]. L-arginine is the substrate for NO and NO is the major endothelium-derived relaxing factor in normal physiology. It plays a central role in vascular homeostasis by maintaining vasomotor tone, limiting ischemia-reperfusion injury, and modulating endothelial proliferation [22]. Pre-

vious report indicated that plasma levels of the NO metabolites nitrite and nitrate and plasma arginine levels are depressed in sickle cell patients during vaso-occlusive crisis and the acute chest syndrome (ACS) [23,24].

There are several factors that may be responsible for the low l-arginie and nitric oxide levels among patients with SCD. In patients with sickle cell disease, elevated levels of plasma Hb cause destruction of NO and limit its bioavailability [22] and its ability to cause vasodilatation [25]. Increased plasma Hb concentrations during vasoocclusive crisis and ACS [26] lead to increased scavenging of NO. Thus, NO appears to play a critical compensatory role in maintaining endothelial homeostasis [22]. A deficiency of nitric oxide, a potent vasodilator, has been identified in sickle cell disease and may contribute to episodes of blocked vessels and pain [27].

Vaso-occlusion is one of the hallmarks and major complication of sickle cell disease (SCD), resulting in acute debilitating episodic pain and contributing to infection, acute chest syndrome, splenic sequestration, stroke, acute and chronic multisystem organ damage, and shortened life expectancy. This study demonstrated that both L-arginine and NO levels in SCD children of Nigerian descent with VOC were significantly low compared to those in steady state (p=0.05 and 0.01) respectively. This finding is consistent with previous reports [18, 28] which indicated that there may be a relationship between the L-arginine-nitric oxide pathway and vaso-occlusion in SCD. Low arginine levels during VOC could reflect a state of acute substrate depletion that results in a decrease in nitric oxide production [18].Similarly, a previous report demonstrated that both L-arginine and NO levels in adult VOC subjects are significantly low. It appears logical that there would be a direct connection between low substrate (L-arginine) and lower production of NO. L-arginine supplementation improves small-vessel coronary endothelial function in humans; large doses of L-arginine might overcome the effect of ADMA [29]. At present, the role of ADMA or other endogenous NOS inhibitors in SCD remains to be determined. The role of L-arginine supplementation in adult VOC is unclear. Low arginine levels during VOC could reflect a state of acute substrate depletion that results in a decrease in nitric oxide production [10]. Our finding is also consistent with previous reports which indicated that plasma arginine concentration decreases acutely in both adults and children during episodes of vaso-occlusive pain and is associated with low NO metabolite levels [18]. Our finding is also in agreement with previous report [16] which indicated that L-arginine (the substrate for NO) is deficient in adult steady-state sickle cell disease (SCD). In children, low L-arginine correlated with low NO during the complications of VOC [18]. Findings from this study is consistent with previous report which indicated that L-arginine supplementation can raise nitric oxide levels and may be beneficial in sickle cell disease [30]. In patients with sickle cell disease, endothelial cells become activated and are damaged by neutrophils that release harmful substances. Vaso-occlusive events and tissue damage are mediated by neutrophils. Superoxide anion released from endothelial cells and neutrophils play a role in breaking down nitric oxide. This production of superoxide anion in these cells is enhanced in a state of L-arginine deficiency [28, 31,32].

Children with SCD are at risk for serious complications such as vaso-occlusive pain crises. Abnormalities of nitric oxide metabolism have been reported to be prevalent during the acute illness and baseline health in patients with sickle cell disease [33]. Our finding in this study is also consistent with a previous report which indicated that plasma l-arginine and nitric oxide metabolite (NOx) levels are significantly low compared to steady state control adults [10, 18]. Similarly, a study conducted in Ghana by [34] indicated that L-arginine and NO levels are significantly reduced in children with VOC as compared to patients in their steady state. Nitric oxide (NO) appears to play a significant role in the pathophysiology of sickle cell anaemia [28, 35] and may be important in the treatment of vaso-occlusive crisis (VOC). Additionally, there appears to be a link between low nitric oxide metabolite (NOx) levels and VOC pain [10]. Low arginine and nitric oxide levels observed during VOC could reflect a state of acute substrate depletion. This finding may be a justification to routinely provide nitric oxide generating l-argine supplements particularly for sickle cell disease children of African descent with VOC. Previous report suggests that oral arginine may benefit SCD patients by inducing an increase in NO production during VOC and that there may be a relationship between the L-arginine-nitric oxide pathway and vaso-occlusion in SCD. Low arginine levels during VOC could reflect a state of acute substrate depletion that results in a decrease in nitric oxide production [21]. In the patients with VOC who had ACS develop, L-arginine decreased to the lowest levels at the time of the ACS diagnosis, correlating with decreasing NOx levels [36]. Oral arginine may therefore benefit SCD patients by inducing an increase in NO production during VOC [18, 37]. Previous oral, low dose, chronic supplementation with L-arginine increased plasma arginine concentration, nitric oxide metabolites and heart rate responses to change in posture in sickle cell anaemia subjects [38,39].

Conclusion

This study has shown that that there may be a relationship between the L-arginine-nitric oxide and vaso-occlusion in SCD. Low arginine levels during VOC may be reflection of a state of acute L-arginine substrate depletion that results in a decrease in nitric oxide production. It may be necessary to provide L-arginine supplementation for SCD patients particular those with vaso-occlusion.

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