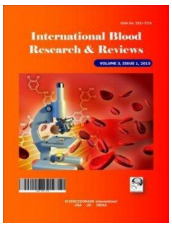

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L- Arginine and Nitric Oxide Levels among Children with Sickle Cell Disease in a Steady State in Federal Teaching Hospital Gombe, Northeastern Nigeria

S. Abubakar[1*], O. Erhabor[2], I. Z. Issac[2], A. B. Ibrahim[2], Van Dyke Knox[3], S. Yuguda[4], A. Girei[4], S. Adamu[4], A. Saidu[4], M. Bello[5] and B. H. Tilako[6]

[1]Women and Children Hospital, Gombe State, Nigeria.
[2]Department of Haematology, Faculty of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria.
[3]Department of Biochemistry and Molecular Pharmacology, West Virginia University Medical School, Morgantown, United States of America.
[4]Department of Haematology, Federal Teaching Hospital Gombe, Gombe State, Nigeria.
[5]Department of Medical Microbiology, Faculty of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria.
[6]Ministry of Health, Gombe State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Authors SA and OE designed the study, performed the statistical analysis. Authors SA, MB and SA wrote the protocol and wrote the first draft of the manuscript. Authors ABI, AG and SA managed the analyses of the study. Authors IZI, VDK, AS, SY and BHT managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Sickle cell disease is a global public health problem. L-arginine is an amino acid that helps in improving blood in the arteries of the heart and improved symptoms of clogged arteries, chest pain or angina and coronary arteries disease. Nitric oxide is a powerful neurotransmitter that helps blood vessels relax and improve circulation. The l-arginine and nitric oxide levels of sickle cell disease

**Corresponding author: E-mail: abuhanif.sa@gmail.com;*

(SCD) subjects with steady stages were also significantly low. The objective of this study was to evaluate L-arginine and Nitric oxide levels in children with sickle cell disease at steady state for 8 weeks. This study included children with a confirmed HbSS electrophoretic pattern aged 1-14 years presented to the sickle cell clinic unit of Federal Teaching Hospital Gombe. The L-arginine and nitric oxide levels were significantly higher post supplementation compared to baseline levels ($p = 0.002$ and 0.000 respectively). It is recommended that L-arginine supplementation be included in the management of patients with sickle cell disease. L-arginine supplement should be made available in the paediatric emergency unit, clinic and pharmacy department as given to patients with sickle cell disease to prevent the adverse effects during the crisis and potentially reduce the length of stay in the hospital.

Keywords: Sickle cell anaemia; Nitric oxide and L-Arginine.

1. INTRODUCTION

Sickle cell disease is a global public health problem. As of 2013 about 3.2 million people have a sickle-cell disease with 176,000 deaths. Sickle cell disease (SCD) is an inherited haemolytic anaemia whose clinical manifestations arise from the tendency of the haemoglobin (HbSS) or sickle haemoglobin to polymerise and deform red blood cell into the characteristic sickle shape. A sickle-cell disease may lead to various acute and chronic complications, several of which have a high mortality rate [1]. It is an autosomal recessive disorder and the most common genetic disease affecting African-Americans [2]. L-arginine is an amino acid that helps in improving blood in the arteries of the heart and improved symptoms of clogged arteries, chest pain or angina and coronary arteries disease. The reduction of L-arginine also occurs following the increased consumption of NO because of the increase in reactive oxygen species (ROS), generated by the presence of free haemoglobin, ischaemic injury of recurrent reperfusion, pro-inflammatory state, and the high autoxidation of haemoglobin S (HbS) [3]. Nitric oxide is a powerful neurotransmitter that helps blood vessels relax and improve circulation. The l-arginine and nitric oxide levels of sickle cell disease (SCD) subjects with steady stages were also significantly low. Nitric oxide is an important cellular signalling molecule involved in many physiological and pathological processes. It is a powerful vasodilator with a short half-life of a few seconds in the blood [4]. An arginine deficiency develops over time in patients with SCD and is influenced by acute events [5]. Normal arginine metabolism is impaired through various mechanisms that contribute to endothelial dysfunction, vaso-occlusion and early mortality [6]. A single dose of arginine given to patients with sickle cell disease and acute pain episodes resulted in a significant dose-dependent increase in plasma nitric oxide concentration [7].

2. MATERIALS AND METHODS

2.1 Study Area

The area selected for this study was Federal Teaching Hospital (FTH), Gombe, Nigeria. Gombe State is in the North- eastern part of Nigeria. As of 2006, it had an estimated population of more than 2,353,000 [8].

2.2 Study Population

This study included 60 children aged 1-14 years with a certain HbSS electrophoretic pattern presented to the sickle cell clinic unit of Federal Teaching Hospital Gombe, Gombe State. Simple random sampling was used to recruit the subjects.

2.3 Study Design

This research is a classical experimental case-control study on children with SCD. Children with SCD, Age and gender-matched children where was monitored as controls. This study included children with SCD aged 1-14 years which were randomly grouped into the treatment and age and gender-matched controls individual with SCA in the non-treatment (As controls) arm was included as controls. Quantitative data was gotten by estimating the serum l-arginine and Nitric Oxide using ELISA techniques.

2.4 Sampling Techniques/Selection of Subjects

2.4.1 Inclusion criteria

This study included confirmed homozygosity for haemoglobin-S, aged (1-14 years) and willingness of parents/guardians who's offered a

written informed consent for their children to participate in this study. The subjects not fulfilling the criteria were excluded from the study.

2.5 Study Instrument

2.5.1 Questionnaire

Questionnaires were distributed to all consenting participants for the collection of socio-demographic and other bio-data such as age, gender, an area of residence, any medication, signs and symptoms of diseases.

2.5.2 Ethical consideration

Ethical approval was obtained from the ethical committee of Federal Teaching Hospital Gombe (FTHG).

2.5.3 Sample size determination

Sample size was calculated using the formula: n=Z2pq/d2

Where n= minimum required sample size in a population >10,000
Z= standard normal deviation
P= proportion of success or prevalence
q = proportion of failure (1-p)
d= precision, tolerable margin of error, expected difference

Thus n =Z2pq/d2

Z = 95% (1.96)
P = 2.3% (0.0239) [9].
Q = 1 – 0.0239 =0.9761
D = 5% (0.05) Therefore n = (1.96)2 x 0.0239 x 0.9761 / (0.05)2
Minimum Sample size = 35

2.6 Supplementary Method

Enrolment= 60 children with SCD
Age eligible for the study = SCD Children (1-14 yrs).
Gender eligible for the study= both male and female
Drugs = L- Arginine supplement 350 mg
Duration = 8 weeks
Dose = the dose administered depended on the weight of the children. Subjects were given 2-10 capsules two times daily.

This study enrolled 60 children with SCD. Participants were randomly assigned to receive twice daily doses of either a low dose of arginine or a high dose of arginine and for 8 weeks. Five millilitres (5 mls) of blood was collected from the patients at baseline before commencement of the treatment. The baseline samples were examined for the following; Nitric oxide and L-arginine levels, also, repeating the laboratory parameters of l-arginine and nitric oxide level that were evaluated at baseline as well as the evaluation of the clinical outcome of the supplementation after 8 weeks of supplementations.

2.7 Sample Collection

About 5 millilitres of blood sample was collected from each subject aseptically using the venepuncture technique. The blood was collected into tubes containing dipotassium ethylenediamine tetra-acetic acid anticoagulant and samples was left undisturbed at room temperature and later centrifuged at 3000 rpm for 5minutes to obtain clear non- haemolysed plasma. The plasma was transferred into sterile labelled test tubes and assayed (in batches) for l-arginine and Nitric Oxide and samples collections was repeated after 8 weeks of supplementations and nitric oxide, l-arginine test was repeated, the results was compared before and after supplementations.

2.8 Laboratory Analysis

2.8.1 Determination of plasma L-arginine level

Plasma L- arginine levels were estimated using the Enzyme Linked Immunosorbent Assay (ELISA) technique (Immundiagnostik, Germany).

2.8.1.1 Principle

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 micro titre plate coated with a 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 micro titre 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 micro titre 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 color 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 450 nm) versus concentration is generated using the values obtained from the standards. L-arginine present in the patient samples is determined directly from this curve.

2.8.1.2 Procedure

Sample preparation procedure

1. The EDTA plasma samples were diluted with reaction buffer by factor 1:40 (25 µl sample + 975 µl sample buffer).
2. 100 µl of diluted samples was added in corresponding vials.
3. 25 µl of freshly prepared derivatization reagent were added into each vial, mixed and incubated on a shaker for 45minutes at room temperature (18-26°C).
4. Afterwards, 1250 µl of diluted assay buffer (ASYBUF) was added into each vial, mixed and incubated for 45 minutes on a shaker at room temperature.

Test procedure

5. Microtiter plates were taken from the kit, each well was washed 5 times by dispensing 250 µl of diluted wash buffer into each well. After the final washing step, the inverted microtiter plate was firmly tapped on absorbent paper to remove excess solution.
6. 50 µl of the samples were taken out of the vial and added into the respective wells of the microtiter plate.
7. 150 µl of diluted anti-L-arginine antibody (AB) was added into each well and the plate covered tightly.
8. The plate was incubated overnight (15-20 hours) at 2-8°C.
9. The contents of each well were aspirated. Each well was washed 5 times by dispensing 250 µl of diluted wash buffer into each well. After the final washing step, the inverted microtiter plate was firmly tapped on absorbent paper to remove excess solution.
10. 200 µl of diluted POD antibody (2. AB) was added into each well.
11. The plate was covered tightly and incubated for 1 hour at room temperature on a horizontal shaker (180- 240 rpm).
12. Step 9 above was repeated.
13. 200 µl of TMB substrate was added into each well.
14. It was incubated for 12-16min at room temperature in the dark.
15. 100 µl of stop solution was added into each well and mixed thoroughly.
16. The absorption was determined immediately with an ELISA reader at 450 nm.

Interpretation of result

The results were extrapolated from a graph that was calibrated in the machine using the standard concentrations.

2.8.2 Determination of plasma nitric oxide level

Plasma Nitric Oxide levels were estimated using Griess Reaction (ENZO Blood Sciences, UK).

Principle

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 NO_2^- produces a nitrosating agent, which reacts with sulphanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric Azo-derivative which absorbs light at 540-570 nm. It was read using an ELISA microtiter plate reader.

Procedure

1. 200 µl of reaction buffer was added into the microtiter wells.

2. 50 µl of standards number 1 through number 6 were added into numbered wells.
3. 50 µl of reaction buffer was added into a numbered well to act as zero standards.
4. 50 µl of each sample was placed into carefully labelled wells.
5. 25 µl of final NADH dilution was added into all zero standard, standard and sample wells.
6. 25 µl of the Nitrate reductase final enzyme dilution was added into all zero standard, standard and sample wells.
7. It was mixed well by tapping the side of the plate and a plate sealer was applied. It was incubated for 30 minutes at 37°C.
8. 50 µl of the Griess Reagent I was added into each well except the blank wells.
9. 50 µl of Griess Reagent II was then added into each well with the exception of the blank wells.
10. It was mixed well by tapping the side of the plate and incubated at room temperature for 10 minutes.
11. The optical density of each well was read at 540-570 nm after blanking against the Blank wells.

Calculation of Results

1. The average net OD bound for each standard and sample was calculated by subtracting the average zero standards OD from the average OD for each standard and sample. Average Net OD = Average OD – Average Zero Standard OD.
2. The average Net OD was plotted for each standard concentration.
3. The average OD for each sample was plotted and the total NO concentration was extrapolated from the graph.

2.9 Statistical Analysis

Statistical analysis was performed using statistical package for social sciences (SPSS) version 20.0. Frequencies and percentages were calculated. Student t- test (independent t test and paired sample t-test) and ANOVA was used for comparison of data. The result was presented as mean ± standard error of mean. A p- value of ≤ 0.05 was considered as significant in all statistical comparisons.

3. RESULTS

This study included children with a confirmed HbSS electrophoretic pattern aged 1-14 years with mean age 7.4500 ±0.50613 years, Presented to the sickle cell clinic unit of Federal Teaching Hospital Gombe, Gombe State.

Table 1. Summary of the physical characteristics of the subjects

Parameter	Mean ± SE
Age (yrs) (1-14)	7.4500 ±0.50613
Height (cm)	131.02 ±2.72854
Weight (kg)	25.2500 ±1.07577

Key: Data are presented as Means ± SEM, Cm=centimetre, Kg=kilogram, Yrs=years
Legend: This table shows the Summary of the Physical Characteristics of the subjects with mean age 7.4500 ±0.50613 years, with Mean height 131.02 ±2.72854 and mean weight 25.2500 ±1.07577

4. DISCUSSION

Sickle cell disease is a global public health problem with a significant number of cases occurring in sub-Saharan Africa. It is an inherited condition in which the body makes red blood cells containing abnormal haemoglobin, the protein that carries oxygen from the lungs to other cells in the body [6]. In this present study, we investigated the effect of 8 weeks, low dose supplementation of sustained-release of nitric oxide generating L-arginine supplement (350mg) given two times daily on children with sickle cell disease attending Federal Teaching Hospital Gombe.

Table 2. Effect of L-Arginine Supplementation on L-arginine and nitric oxide level among the SCD subjects on steady stage

Parameters	Before supplementation (Mean ±SE)	After supplementation (Mean ±SE)	p-value	Remark
L-ARG (umol/L)	6.3620 ±1.01260	45.9400 ±12594	0.002	S
NO (mol/L)	2.04± 0.43004	48.1600± 2.5336	0.000	S

Key: L-ARG = L-arginine, NO = Nitric Oxide, S-Significant

We observed that the L-arginine and nitric oxide levels was significantly higher post supplementation compared to baseline levels (p=0.002 and 0.000 respectively. The l-arginine and nitric oxide levels of sickle cell disease (SCD) subjects with steady stages were also significantly low. Our finding is inconsistent with previous report which indicated that steady state l-arginine levels were normal in children with sickle cell disease [6]. Similarly, [10] observed in a previous report that Nitric oxide (NO) and L-arginine levels were significantly higher among normal control children compared to children with SCD. Our finding is also consistent with previous reports which indicated that adults Sickle cell patients are arginine deficient at steady stages [11]. An arginine deficiency develops over time in patients with SCD and is influenced by acute events [5]. Normal arginine metabolism is impaired through various mechanisms that contribute to endothelial dysfunction, vaso-occlusion and early mortality [6]. There is growing advocacy that since low global arginine bioavailability is associated with a growing number of SCD-related complications [8], arginine therapy may represent a promising option for SCD patients [6]. 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 [12].

5. CONCLUSION AND RECOMMENDATION

L-arginine and nitric oxide are significantly low in children with sickle cell disease in a steady stage. At low dose and elongated duration, arginine supplementation improved plasma arginine and nitric oxide levels. We recommend that L-arginine supplement should be made available in the pediatric emergency unit, clinic and pharmacy department of Federal Teaching Hospital Gombe.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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