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Serum Nitric Oxide Levels among Pre- School Children of African Descent with Malaria in Sokoto, Nigeria

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Abstract

Malaria is the most important tropical parasitic disease affecting about 247 million people each year, resulting in nearly a million deaths. It is the leading cause of under-five mortality. L-Arginine is a precursor for nitric oxide synthesis. Nitric Oxide is a ubiquitous mediator that is formed by a family of enzymes named Nitric Oxide Synthases. In this study, the level of nitric oxide was investigated among pre-school children with malaria presenting to the Pediatric Outpatient Department of Usmanu Danfodiyo University Teaching Hospital Sokoto, Nigeria. A total of 90 pre- school children with malaria aged 3-5 year with mean age of 4.01 ± 0.87 and 50 non-parasitized age and gender- matched pre-school children (control) were recruited for this study. There was a statistically significant difference in nitric oxide level among children with malaria ($p = 0.001$) compared to non-parasitized children. The nitric oxide level of children aged 3 years was significantly lower compared to those who were 5 years old ($p = 0.02$). There was no significant difference in the nitric oxide level among the malaria parasitized children based on gender ($p = 0.25$). The nitric oxide level of children with severe malaria was significantly lower than those with uncomplicated malaria ($p = 0.01$). There was a statistically significant difference in the nitric oxide level of malaria-parasitized children based on ethnicity ($p = 0.001$). This study shows that malaria has a significant effect on the nitric oxide level of children with malaria. It is recommended that nitric oxide generating supplements be prescribed as prophylactic therapy for children with severe malaria. It may also be necessary to routinely monitor the nitric oxide levels of malaria parasitized children during treatment.

Keywords: *Serum Nitric Oxide; Pre- School Children; Malaria; UDUTH; Sokoto; Nigeria*

Introduction

Malaria is the one of the most clinically significant tropical parasitic disease that affects about 247 million people each year, resulting in nearly a million deaths, mostly children under the age of five years [1] (WHO, 2008). Severe malaria due to *Plasmodium falciparum* is responsible for 0.6 - 1.2 million mortalities annually, 86 % of whom are children of African descent [2]. Pregnant women and their unborn children are more vulnerable to malaria, which remain a major cause of maternal anaemia, prenatal mortality and low birth weight. It accounts for 40% of public health expenditure, 30 - 50% of in-patient admissions and up to 50% of out-patient visits and loss of productivity in areas with high malaria endemicity [3].

NO is produced by various group of enzymes termed as nitric oxide syntheses (NOS) which are present normally in the human body [4,5]. The synthesis of NO takes place as a result of the conversion of L-arginine to L-citrulline, a reaction catalyzed by the enzyme nitric oxide synthases (NOS) [6]. NO is a gaseous, lipid-soluble, free radical, produced endogenously from l-arginine and molecular oxygen by members of the nitric oxide synthase family [7]. It regulates a number of physiological and pathological processes, including apoptosis, inflammation vasodilation, platelet aggregation, chemotaxis, neurotransmission, antimicrobial defense including endothelial activation [7]. NO production takes places in various cell types [8], including peripheral blood mononuclear cells (PBMCs) [9]. NO has been shown to have antimicrobial and immuno-modulatory activity during the process of inflammation [10].

Malaria is endemic throughout Nigeria with seasonal variation in different geographic zones of the country. More than 90% of the total population in the country and other sister African countries are at risk of malaria and at least 50% of the population suffers from at least one episode of malaria each year. Malaria not only affect only children and pregnant women but it affects the general population [11]. Despite the use of fast-acting antimalarial such as artemisinins, case mortality rates due to severe malaria infection remain high, underlining the significance of adjunctive therapies [12]. Common manifestations of severe malaria infection include low arginine (hypoargininemia) and significant endothelial dysfunction [13]. As L-Arginine is the substrate of NO synthase (NOS) for the production of NO, hypoargininemia can potentially lead to a state of impaired systemic NO production [14]. The most common life-threatening clinical syndromes associated with *P. falciparum* malaria infection in children are severe malarial-related anaemia, respiratory distress, cerebral malaria (CM) and metabolic acidosis [15]. Critical pathogenic mechanisms in these severe malaria syndromes represent possible potential targets for adjunctive therapies, including nitric oxide. The pathogenesis of CM is not completely understood but may be due to one or more of the following mechanisms: sequestration of parasitized red cells within the cerebral microvasculature [16], reduced microvascular flow [17], associated metabolic alterations including hypoxia and hypoglycemia, inflammatory response in the host [18], dysfunction blood-brain barrier [19] and malaria-associated cerebral oedema [20]. Among African children with severe malaria, lactic acidosis secondary to impaired tissue perfusion is responsible for the respiratory distress and deep respirations observed [21]. Respiratory distress is a clear manifestation of decompensated shock, frequently associated with malaria-related multi-system organ failure and widespread endothelial dysfunction. Severe malarial anaemia is caused by both increased destruction of parasitized and non-parasitized red cells, as well as impaired and suboptimal haematopoiesis [22]. There is a need to determine the nitric oxide levels in uncomplicated malaria to reduce the unacceptably high case-mortality associated with severe malaria (SM). Irrespective of its cause, hypoargininemia may limit NO production and may be associated with mortality seen in cerebral malaria [23]. There is paucity of data on nitric oxide levels among malaria parasitized Nigerian children. No NO-malaria studies have been carried out among malaria parasitized Nigerian children. Findings from this study may potentially be used to justify the need to routinely determine the level of serum nitric oxide among pre-school children with malaria. It may also help to justify the need to prescribe concomitant nitric oxide providing supplements with antimalarials in the treatment of pre-school children with malaria in Usmanu Danfodiyo University Teaching Hospital Sokoto State in particular and Nigeria in general. The aim of the study is to determine the serum nitric oxide level among children with malaria in UDUTH Sokoto, Nigeria.

Materials and Methods

Study Design

The study is a quantitative study to determine the levels of nitric oxide among pre-school children with malaria in UDUTH, Sokoto Nigeria. This study investigated 140 individuals; 90 *Plasmodium* parasitized pre-school children (subjects) and 50 non-parasitized children (controls).

Study Site

The study was conducted in Usmanu Danfodiyo University Teaching Hospital (UDUTH) at the Paediatric Outpatient Department in collaboration with the Department of Haematology and Blood Transfusion in the Faculty of Medical Laboratory Science in Usmanu Danfodiyo

University Sokoto, Nigeria. Sokoto is the capital city of Sokoto State. It is located in the extreme North-Western part of Nigeria. Sokoto city is estimated to have a population of 427,760 people [24] and by the virtue of its origin, the state comprises significantly of mostly of Hausa/Fulani and other groups such as Gobirawa, Zabarmawa, Kabawa, Adarawa, Arawa, Nupe, Yoruba, Ibos and others. Occupation of the majority of inhabitants include trading, commerce, with a proportion of the population working as civil servants in private and public sectors [25]. The Sokoto township is in the dry Sahel surrounded by sandy terrain and isolated hills. Rainfall starts around late June and ends early, in September but can sometimes extend to October. The average annual rainfall is 550 mm peaking around the month of August. The highest temperatures of 45°C during the hot season are experienced in the months of March and April. Harmattan, a dry cold and dusty season is experienced between the months of November and February [26].

Ethical Clearance

The ethical approval for this research was sought from the Ethics Committee of Usmanu Danfodiyo University Teaching Hospital Sokoto. Verbal informed written consent was sought from parents or guardian of all study participants after counseling.

Eligibility Criteria

Eligibility criteria include age (3 - 5 years), confirmed malaria presentation at the paediatric unit and willingness of the parents or guardian to offer a verbal informed consent for their ward to participate in this study.

Exclusion Criteria

The following children who did not meet the inclusion criteria were excluded from participating as subjects in the study; all children < 3 and > 5 years, children negative for malaria and children whose parents have not given an oral informed consent for their ward to participate as subjects in the study.

Sampling Method

Samples was collected from consecutively recruited children attending pediatric unit of Usmanu Danfodiyo University Teaching Hospital Sokoto whose parents gave a verbal informed consent for their wards to participate in the study.

Specimen Collection and Preparation

Five milliliters of blood were obtained from the subjects and control participants by venipuncture into EDTA anticoagulated tubes (2 milliliters) and plain tubes (3 milliliters). The EDTA sample was used for blood smears (thick blood films). The thick blood films will be stained by Giemsa's staining method. The number of asexual parasites among the parasitized subjects was counted and parasite densities computed while the thin blood film was stained using Leishman stain and used for speciation. The sample collected in the plain tube was allowed to clot naturally and centrifuged to obtain serum. Nitric oxide level in the serum, was determine calorimetrically using ENZO Diagnostic (UK) nitric oxide kits.

Statistical Analysis

The data collected was descriptive and analysed using statistical software (SPSS version 20). Results were expressed as mean and standard deviation. Differences in values were determined and compared statistically using analysis of variance and student's t-test. A p-value of ≤ 0.05 was considered as significant in all statistical comparisons.

Result

A total of 90 pre-school children with malaria aged 3 - 5 years with mean age of 4.01 ± 0.87 years and 50 age and gender- matched non- parasitized pre-school children (control) were recruited for this study). Table 1 show the mean Nitric Oxide level among pre-school

children with malaria (test) and non-malaria parasitized subject (control). The Nitric oxide levels was significantly lower among malaria-infected children (366.48 ± 169.45 µmol/l) compared to control (457.76 ± 88.27 µmol/l) (p = 0.001).

Parameter	Group	N	Mean ± SD	Std. Error mean	Df	t-value	p-value
Nitric oxide	Test	90	366.48 ± 169.45	17.86	138	-3.55	0.001
	Control	50	457.76 ± 88.27	12.48			

Table 1: *Nitric Oxide level among pre-school children with malaria (test) and non-malaria parasitized controls.*

N: Number; SD: Standard Deviation; Df: Degree of Freedom; SS: Statistical Significance

Table 2 shows the effect of age on the nitric oxide levels among pre-school children with malaria. There were no significant differences between the Nitric oxide level of malaria parasitized children in the 3 years age group (320.62 ± 131.32 µmol/l) compared to those in the 4 years age group (337.17 ± 163.65 µmol/l) as well as between children in the 4 years age group (337.17 ± 163.65 µmol/l) compared to those in the 5 years age group (419.21 ± 187.22 µmol/l) (p = 0.69 and p = 0.09 respectively). The Nitric oxide level was significantly lower among malaria parasitized children in the 3 years age group (320.62 ± 131.32 µmol/l) compared to those in the 5 years age group (419.21 ± 187.22 µmol/l) (p = 0.02).

Parameter	Age	N	Mean ± SD	Std. Error mean	Df	t-value	p-value
Nitric oxide	3	29	320.62 ± 131.32	24.39	50	-0.41	0.69
	4	23	337.17 ± 163.65	34.12			
Nitric oxide	3	29	320.62 ± 131.32	24.39	65	-2.42	0.02
	5	38	419.21 ± 187.22	30.37			
Nitric oxide	4	23	337.17 ± 163.65	34.12	59	-1.74	0.09
	5	38	419.21 ± 187.22	30.37			

Table 2*: The effect of age on the Nitric Oxide levels among pre-school children with malaria.*

N: Number; SD: Standard Deviation; Df: Degree of Freedom; NSS: Non-Statistical Significance

Table 3 shows the effect of gender on the Nitric oxide level of pre-school children with malaria. There was no statistically significant difference between the nitric oxide level of male children (382.68 ± 156.62 µmol/l) compared to female children (341.02 ± 187.37 µmol/l (p = 0.26).

Parameter	Gender	N	Mean ± SD	Std. Error mean	Df	t-value	p-value
Nitric oxide	Male	55	382.68 ± 156.62	21.12	88	1.14	0.26
	Female	35	341.02 ± 187.37	31.67			

Table 3: *Effect of Gender on the Nitric Oxide levels of pre-school children with malaria.*

N: Number; SD: Standard Deviation; Df: Degree of Freedom; NSS: Non-Statistical Significance

Table 4 shows the effect of ethnicity on the Nitric oxide level of pre-school children with malaria. The nitric oxide was significantly lower among Hausa/Fulani children (373.31 ± 171.03 µmol/l) compared to Yoruba (637.49 ± 106.35 µmol/l) (p = 0.000).

Parameter	Ethnicity	N	Mean ± SD	Std. Error Mean	Df	t-value	p-value
Nitric oxide	Hausa/Fulani	69	373.31 ± 171.03	20.59	76	-4.51	0.000
	Yoruba	9	637.49 ± 106.35	35.45			
Nitric oxide	Hausa/Fulani	69	378.83 ± 170.28	20.50	73	-0.76	0.45
	Igbo	6	433.63 ± 160.98	65.72			

Table 4: *The effect of Ethnicity on the Nitric Oxide level among pre-school children with malaria.*

N: Number; SD: Standard Deviation; Df: Degree of Freedom; SS: Statistical Significance; NSS: Non-Statistical Significance

Table 5 shows the effect of ethnicity on Nitric oxide level of pre-school children with malaria. Nitric oxide was significantly higher among Yoruba children (637.49 ± 106.35 µmol/l) compared to other ethnic groups (334.48 ± 172.99 µmol/l) (p = 0.001).

Parameter	Ethnicity	N	Mean ± SD	Std. Error Mean	Df	t-value	P-value	Remark
Nitric oxide	Yoruba	9	637.49 ± 106.35	35.45	13	4.23	0.001	SS
	Others	6	334.48 ± 172.99	70.62				

Table 5: *The effect of Ethnicity on Nitric Oxide level among pre-school children with malaria.*

N: Number; SD: Standard Deviation; Df: Degree of Freedom; SS: Statistical Significance; NSS: Non-Statistical Significance

Table 6 shows the effect of malaria severity on the Nitric oxide level of pre-school children with malaria. The nitric oxide level was significantly higher among children with severe malaria (390.41 ± 183.92 µmol/l) compared to those with uncomplicated malaria (297.93 ± 124.19 µmol/l) (p = 0.01).

Parameter	Malaria	N	Mean ± SD	Std. Error Mean	Df	t-value	p-value	Remark
Nitric oxide	Severe Malaria	55	297.93 ± 124.19	20.99	88	2.615	0.01	SS
	Uncomplicated Malaria	35	390.41 ± 183.92	24.80				

Table 6*: The effect of malaria severity on Nitric oxide among pre-school children with malaria.*

N: Number; SD: Standard Deviation; Df: Degree of Freedom; SS: Statistical Significance

Discussion

Malaria is the most important tropical parasitic disease affecting about 247 million people each year, resulting in nearly a million deaths, mostly children under the age of five years [1] (WHO, 2008). Nearly 90% of these deaths occur in Africa South of the Sahara where a child dies every 30 seconds [3]. This study investigated the level of l-arginine and nitric oxide among pre-school children with malaria (subjects) and non-parasitized malaria children (control) in UDUTH Sokoto, Nigeria.

We observed that the nitric oxide was significantly lower (p = 0.001) among children with malaria compared to non-parasitized children (control). This finding is consistent with previous reports [10,23] among African children with cerebral malaria which indicated a

reduction in systemic Nitric oxide production and hypo-argininemia. Reduced availability of NO and low plasma arginine levels has been shown to contribute to the pathogenesis of severe malaria. among children of African descent with severe malaria [10,23], the substrate for NO synthesis. A previous report that investigated treatment of malaria-parasitized Indonesian adults with severe malaria with intravenous l-arginine showed increased levels of exhaled NO, and treatment associated reversal in malaria-associated endothelial dysfunction [27]. NO seems to play a role at multiple stages of malaria infection, beginning with the innate defenses of the Anopheles mosquito vector, with NO antagonizing the *Plasmodium* parasite [28]. Several reasons have been postulated for the low nitric oxide levels observed in malaria parasitized children; malaria infection is associated with haemolysis of the RBC which often results in the release of RBC cytoplasmic contents including arginase, lactate dehydrogenase and haemoglobin into the plasma. The released plasma free haemoglobin reduces nitric oxide bioavailability as it binds nitric oxide potentially quenching and scavenging its bioactivity [29]. Plasma arginase also diminishes arginine levels, and thus may reduce production of NO. Also in severe malaria, metabolic acidosis is proportional to disease severity and is an important risk factor for death [30]. Malaria -associated acidosis is thought to result from a combination of obstructed microvascular flow and suboptimal tissue oxygen delivery and/or usage [31]. The mechanism of reduced NO availability has recently been described in disease states with intravascular haemolysis [32]. Erythrocyte rupture results in increased cell-free haemoglobin and plasma arginase [33,34] leading to increased nitric oxide usage, plasma l-arginine catabolism, and an overall significant reduction in nitric oxide bioavailability [27]. Previous report [32] suggests that low nitric oxide bioavailability is a significant characteristic of severe malaria across different age spectrums, ethnicity, transmission intensities and disease manifestations.

In this study, we observed that nitric oxide levels were significantly lower in severely parasitized children compared to children with uncomplicated malaria ($p = 0.01$). Severe malaria patients, supplemented with L-Arginine has been shown to have improved NO bioavailability and reversal of malaria-related endothelial dysfunction [27]. L-Arginine infusion may be safe in malaria patients and there is clinical trial currently undergoing to potentially prevent endothelial dysfunction during severe malaria [27]. As L-Arginine is the substrate of NO synthase (NOS) for the production of NO, hypoargininemia can potentially lead to a state of impaired systemic NO production [14]. The effect of L-Arginine supplement is mostly likely due to enhanced production of NO, and the importance of NO in host defense against a variety of pathogens has been well documented [35]. Nitric oxide (NO) is an attractive, as yet untested, potential adjunctive treatment for severe malaria because it modulates endothelial activation, a critical pathway in the pathogenesis of severe malaria.

Based on promising pre-clinical data from animal models [36] and a human trial using the NO precursor L-arginine [27], together with its established record of safety in clinical practice, a clinical trial evaluating nitric oxide for the adjunctive treatment of severe malaria is warranted. NO plays a role at multiple stages of malaria infection, beginning with the innate defenses of the Anopheles mosquito vector, where NO antagonizes the *Plasmodium* parasite [28].

The reason for the low level of NO among malaria parasitized children in this study is not far-fetched. In malaria parasite infestation, RBC are altered and undergo haemolysis, and these RBC bind to endothelial cells resulting in interference with blood flow [37]. The malaria –related haemolysis is associated with release of RBC cytoplasmic contents such as haemoglobin, lactate dehydrogenase and arginase into the plasma. Plasma free haemoglobin reduces NO bioavailability as its binds NO and quenches/scavenges its bioactivity [38]. Plasma arginase diminishes arginine levels, and thus may reduce production of NO. L-arginine and NO are protective against severe malaria parasites which causes organ/tissue damage. Severe malaria is associated with low L-arginine and by extension nitric oxide production. Hypoargininemia is likely to result in sub-optimal monocyte, endothelial function, and tissue NO production because the intracellular NOS activity is critically dependent on adequate circulating L-arginine. Moreover, hypoargininemia may enhance the deleterious oxidative stress. The use of nitric oxide producing l-arginine supplementation in malaria infection has been suggested [39]. Increase in plasma levels of l-arginine and within and above the normal range will be expected to increase NO production, restores plasma arginine levels to normal, increases systemic and exhaled NO production, reduces oxidant stress, and improves a number of physiological measures of relevance to malaria (including vascular endothelial function and physiological dead space). L-arginine concentrations fall

in the settings of inflammation, haemolysis and malaria infection, and L-arginine levels vary inversely with malaria disease severity. It is hypothesized that the low bioavailability of arginine for NO synthesis contributes to pathogenesis. L-arginine levels are low in patients with CM, moderately low in patients with uncomplicated malaria and normal in healthy controls [23]. Hypoargininemia caused by arginase release from haemolyzed erythrocytes is significantly associated with fatal outcome in patients with CM [23]. Exogenous L-arginine rescues reactive hyperemia-peripheral artery tonometry, a noninvasive measurement of vascular NO production, in patients with severe malaria [27]. Asymmetric dimethylarginine levels are higher in patients with severe malaria than in those with moderately severe malaria and each micromolar increase of this endogenous inhibitor of NOS conversion of L-arginine to NO is associated with an 18-fold increase in mortality [40].

In this study, we observed that the nitric oxide level was only significantly lower among children in the 3 years age group (320.62 ± 131.32 μmol/l) compared to those in the 5 years age group ($p = 0.02$). This finding is consistent with a previous report which suggests that hypoargininemia and low nitric oxide bioavailability are characteristic of severe malaria across different age group [27].

We observed that there was no significant difference in the nitric oxide level based on the gender of the malaria parasitized subjects ($p = 0.25$) respectively. The reason for non-significance is unknown. Previous report indicates that nitric oxide production is gender- related [41-45]. The reason for the differences in the levels of l-arginine and nitric oxide based on gender is unknown. However hormonal differences between male and female gender may play a significant role.

We observed a statistically significant decrease in nitric oxide level of malaria-parasitized children of Yoruba ethnic group and other ethnic groups ($p = 0.001$). The ethnic/racial impact on health has been receiving considerable attention in recent years. The mechanisms underlying these racial disparities may be multifactorial and involve genetic factors, socioeconomic status, nutritional status, psychosocial stressors/risks, and other environmental factors [46].

We observed that mean value of nitric oxide was significantly lower ($p = 0.01$) among children with severe malaria compared to uncomplicated malaria children. Our observation is consistent with the reduction of systemic Nitric oxide production and hypoargininemia found in previous study of African children with cerebral malaria [23]. Finding from this study is justification for the potential use of L-arginine a substrate for synthesis of nitric oxide (NO) as a potential adjunctive therapy in severe malaria. Previous studies have proposed that L-arginine should be used as a potential adjunctive therapy in severe malaria [47,48]. The rationale for this is based on previous findings in severe malaria of impaired NO production [27], hypoargininemia [23], near-universal impairment of NO-dependent endothelial function [27] and a close association between the improvement in endothelial function and recovery of plasma L-arginine concentrations after treatment of severe malaria [27]. NO down-regulates endothelial inflammation [49] and reduces the cytoadherence of parasitized erythrocytes *in vitro* [50]. Endothelial dysfunction, a measure of both impaired endothelial cell NO bioavailability and endothelial cell activation [51], may exacerbate other underlying processes in severe malaria including cytoadherence of parasitized red cells to activated endothelial cells, microvascular obstruction and tissue hypoxia. In severe malaria, impaired endothelial function is associated with markers of impaired perfusion, endothelial activation, and increased parasite biomass [27]. Previous report has demonstrated that L-arginine infusion is able to improve NO bioavailability and endothelial function in patients with moderately severe malaria [27], suggesting the potential for a similar effect if used as adjunctive therapy in severe malaria. Possible causes of hypoargininemia and significantly low nitric oxide in severe malaria include decreased l-arginine synthesis and increased catabolism caused by the increased activity of plasma arginase [52] and/or cytokine-inducible arginase in endothelial cells or immune cells [53], processes that may also occur in other inflammatory conditions. As well as impairing endothelial function, hypoargininemia and NO deficiency may contribute to pathology in severe malaria through other mechanisms. NO inhibits platelet activation [54], and deficiency may therefore exacerbate platelet-mediated processes linked to the microvascular pathology of severe malaria, including endothelial cell activation [55] and apoptosis and platelet-mediated clumping [56].

Conclusion

In conclusion, the findings from this study confirmed that the level of nitric oxide among pre-school children with malaria is significantly lower compared with non-parasitized children. The nitric oxide is lower among children with severe *Plasmodium* parasitaemia compared to those with uncomplicated malaria. Nitric oxide is impaired in malaria parasitized children. It may be necessary to routine provide nitric oxide generating l-arginine supplementation as adjunctive agents along with antimalarial chemotherapeutics in the treatment of malaria parasitized children. There is need to routinely monitor the nitric oxide levels of malaria parasitized children in Sokoto, North Western Nigeria in particular and Nigeria in general. Public awareness and education campaign should be carried out by the State and Federal government to educate parents on the need to provide a balance diet rich in nitric oxide generating l-arginine for their wards.

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