Original Research Article

Evaluation of the relationship between nitric oxide level and severity of *Plasmodium falciparum* malaria

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Abstract

Background: According to WHO's estimated number of cases, India has the largest number of malaria cases occurring outside of Africa. Karnataka has the highest incidence of malaria in south India and in 2015 nearly 100 thousand cases were reported in this state, with 22 deaths. Malaria is a significant and serious health problem in Karnataka state and particularly in Dakshina Kannada (Mangalore) district. **Methods:** Blood samples were collected from both controls and patients for a series of laboratory investigations using standard protocols for estimation of hematological profile, nitrite / nitrate levels. **Results:** Significant decrease in hemoglobin level and thrombocytopenia were the two most important hematological abnormalities seen in our cases of acute malaria infection. The chances of anemia was related more to decrease in hemoglobin level, while, thrombocytopenia was associated with *P. falciparum* infection. **Conclusion:** All together, these results point out to the fact that nitric oxide is in fact a marker of clinical infections can be used as an indicator for the severity of the disease.

Key Words: Malaria, Nitric oxide, Plasmodium falciparum, hematological profile

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INTRODUCTION

Malaria is among the most serious tropical infectious diseases, with *Plasmodium falciparum* malaria being the leading cause of death from a single pathogen[1]. According to the World Health Organization (WHO), approximately 3.3 billion people are at risk, resulting in about 250 million cases every year[2]. Following the industrialization and economic progress in the United States and Europe, the disease was nearly eradicated there by the late 19th century. But, malaria is endemic in many tropical regions with devastating impact in Asia and Africa where P. falciparum dominates and the majority of the malarial deaths occur[3]. Malaria is among the leading infectious causes of death in many of the world's developing countries. This parasitic mosquito-borne illness produces massive hemolysis in many infected human hosts[4]. Malaria is one of the most common parasitic infections in our country and over 1.72 and 1.87 million cases were reported in 2006 and 2007 respectively. Malaria has been a serious problem in some parts of our country due to the slow progress in its control[5]. The largest population at risk of malaria, 1,320 million people, now lives in the South-Eastern WHO Region. India is one of 11 countries in that region with nearly 980 million people at risk. According to WHO's estimated number of cases, India also has the largest number of malaria cases occurring outside of Africa[2]. India's official statistics suggest that P. falciparum accounts for about 50% of the clinical cases in India[5]. Karnataka has the highest incidence of malaria in south India and in 2011 nearly 100 thousand cases were reported in this state, with 22 deaths. Malaria is a significant and serious health problem in Karnataka state and particularly in Dakshina Kannada (Mangalore) district. In the recent years there has been a sharp rise in the incidence of malaria which represents a major challenge for public health in urban areas. There is a rise in the number of malaria cases with the onset of rainy

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season and so is the incidence of Plasmodium Falciparum malaria **(***P. Falciparum)* in the recent years, which is a matter of concern[5]. Nitric oxide, which has become a sort of celebrity molecule, has been found to take part in an extraordinary range of activities, including learning and memory, blood pressure regulation, penile erection, digestion and the fighting of infection and cancer. Laboratory studies have shown that nitric oxide can attack fungi, parasites and bacteria, either killing them or interfering with their ability to multiply[6-8]. Even though the molecular mechanisms responsible for the naturally acquired immunity against malaria are still to be clarified, the production of NO seems to play a significant role as a marker for the severity of the disease. Therefore, we examined the relationship between production of NO and hemoglobin concentration in children suffering from malaria **(***P. Falciparum).* A lack of acquired immunity to *P. falciparum* malaria in young children appears to underlie the high rates of morbidity and mortality from malaria in areas of Asia, sub-Saharan Africa where malaria is endemic [9, 10]. Although the molecular mechanisms responsible for effective malarial immunity remain elusive, production of nitric oxide (NO) appears to be an important marker and potential mediator of disease severity[11]. Also, NO mediates a diverse array of physiologic and pathologic processes, and appears to be an important mediator of the protective immune response to all stages of *Plasmodium* infections[12]. This study has focused on *Plasmodium falciparum* malarial infection and the relationship of nitric oxide with *P. falciparum* malaria.

MATERIALS AND METHODS

Study Design: A total 40 (29 male and 11 female) patients were examined over a period of three months (July-September, 2015), who were attending the hospital, both OP and IP patients and diagnosed for malaria (*P. falciparum)*. Along with 40 age and sex matched healthy volunteers as a control group (32 male and 8 female), with no known history of any disease. Patients were grouped according to the severity of the disease and their NO values have been compared. Severity in malaria patients were determined based on the WHO criteria. The study was conducted after getting the approval from Institutional Ethics Committee. The subjects recruited for the study were the regular attendants of outpatient departments of hospital. An informed Consent from all the participants was taken before the study. Blood samples were collected from both controls and patients for a series of laboratory investigations using standard protocols for estimation of hematological profile, nitrite / nitrate levels.

Hematological investigations: Complete blood picture (white and red blood cell count, hematocrit, hemoglobin and red blood cell indices) and coagulation profile were done immediately on admission. Blood cells, hematocrit, hemoglobin, platelet count were counted with automatic cell counter.

Estimation of Nitric Oxide: We report the presence of Nitric oxide (NO) as measured through its product NO_2 (nitrite) in human serum[6]. Nitrite concentrations were determined by spectrophotometric method at 540 nm. NO_2 measurement by the Greiss reagent (consisting of equal volume of 0-1% N-1- naphthylyl ethyenediamine, HCl and 1% sulfanilamide plus 5% H_3PO_4) was added and incubated for 5 min at room temperature, protected from light. The development of characteristic purple color was then measured in a spectrophotometer at 540nm. Values of nitrites were estimated by comparison with a standard curve of nitrite concentration.

RESULTS

A total of 80 children were recruited for the study. Forty children had *P.falciparum* malaria while the remaining was negative and were used as controls. Table 1 shows the mean value of the general characteristics in subjects of both the group. Table 2 shows hematological parameters in patients and control subjects. There was significant reduction in the haemoglobin and platelet levels in children with malaria compared to the control ($P \leq 0.05$). Red blood cell (RBC) level in the subjects with malaria was lower than the control ($P \leq 0.3$). The white blood cell (WBC) count was low in subjects with malaria but higher when compared with the control ($P \leq 0.05$). Table 2 shows progressive decrease in the platelet count with the severity of malaria ($P \leq 0.05$).

Table 1: Characteristics of malaria patients (*P. falciparum*) when compared with controls. Mean (±SD) of Age, gender, body temperature (^{0}C)

Parameter	Control	Patients	Significance (p)
Age (years)	10.2 ± 4.5	9.3 ± 5.7	<0.1
Gender (Male %)	40 (72.5 %)	40 (80 %)	<0.2
Temperature (^{0}C)	37.6 ± 0.9	38.1 ± 1.3	<0.1

Values are mean ± S.E.M.

Table 2: Mean Value of Hematological Parameters of Children with Malaria and Control Subjects

Parameter (Reference range)	Control	Patients	Significance (p)
Hb(g/dl) (13-18)	12.7 ± 1.4	9.4 ± 0.6	<0.05
RBC (x10^9) (4-5)	4.98 ± 1.02	3.22 ± 0.84	<0.3
PCV (%) (42-16)	36.7 ± 0.9	31.4 ± 1.1	<0.05
WBC (x10^3) (4.3-10.5)	5.8 ± 4.7	11.7 ± 0.3	<0.05
Platelet(x10^6)(150-350)	245 ± 0.2	143 ± 0.1	<0.05

Values are mean ± S.E.M. n=40. Hb- Haemoglobin. PCV- Packed cell volume, WBC- White blood cell.

The comparison of nitric oxide levels on the serum samples of the three study groups Table 3 shows a two-fold greater NO concentration for persons infected by *P.falciparum* when compared to the persons of the control group. This seems to indicate that NO is in fact a marker of clinical infection by *P. falciparum*.

Table 3: Biochemical findings in control group and patients group with malaria (*P. falciparum)* (*n*=40)

Parameter	Control	Patients	Significance (p)
Nitrite (µmol/l)	6.0 ± 2.7	7.8 ± 3.3	<0.01
Nitrate (µmol/l)	13.8 ± 8.6	24.8 ± 10.8	<0.01
Nitrite+nitrate (µmol/l)	19.8 ± 11.3	32.6 ± 14.1	<0.01
Nitrite/nitrate	0.43 ± 0.29	0.31 ± 0.77	<0.01

Values are mean ± S.E.M.

DISCUSSION

Malaria in India is unstable and appears in the form of outbreaks caused mostly by infection due to *P. falciparum*. There is a good malaria control programme in the country. The reasons for such outbreaks have been identified as improper surveillance and untimely anti-vector measures related to spraying activities in rural areas, and anti-larval measures in urban areas[5]. There are four *Plasmodium* species capable of infecting man; of these, *P. falciparum* is the most pathogenic[13], and the one capable to produce several clinical manifestations. The outcome of the infection with this protozoan is the overall result of a number of factors like parasitic density, the accretion of multiple clones of the parasite and their relative virulence; this factor is an intrinsic component of the biochemical cascade of events that lead to the most severe result of malaria[13-15]. The red blood cells (RBCs), hemoglobin (Hb) level and packed cell volume (PCV) with normochromic normocytic features of the RBCs. The reduction was more evident in *P. falciparum* infection. This drop was statistically significant for PCV. Our results coincides with the previous report which stated that in uncomplicated acute malaria the hemoglobin and hematocrit (PCV) are usually normal during the first 24 hours after the onset of fever[15-17]. Malarial anaemia is thought to arise from both decreased red blood cell (RBC) production and increased RBC destruction. Destruction of RBCs can occur as a result of parasite invasion and replications. The pathophysiology of severe anaemia is a complex but relatively neglected area of study. Certainly, malaria gives ample reasons for both increased destruction and reduced production of red cells. Regarding the association between serum nitric oxide concentration and hemoglobin levels, the results seem to suggest an inverse proportionality, which is in accordance with other studies[12] suggesting that nitric oxide is an inhibitor of erythropoiesis. Nitric oxide also alters cellular iron metabolism, and it likely contributes (through its effects on iron metabolism) to the anemia of chronic diseases[9-11]. Iron deficiency itself is a major cause of anemia in malaria-endemic areas[12]. We have demonstrated that NO production is negatively associated with hemoglobin concentration in *P. falciparum* malaria infected children, independent of the known effect of age on hemoglobin concentration. NO is rapidly oxidized to the stable inorganic nitrogen oxides, nitrite and nitrate *in vivo*[10]. Nitrite, rather than nitrite plus nitrate, is believed to be the product of NO in oxygenated water[11]. Hemoglobin possesses anion binding sites that may retain nitrite, raising concerns that the measured NO levels may be overestimated as a result of conversion of hemoglobin-bound nitrite to NO[12]. WBC counts were significantly higher in *P. falciparum* malaria. A mild to moderate thrombocytopenia was demonstrated in our patients who had *P. falciparum* malaria infection. This is in agreement with previous studies which reported frequent incidence of thrombocytopenia in acute malaria[18-20]. The information from this study on Indian vectors of *P. falciparum* malaria from Dakhshin Kannada (Mangalore) district will be useful not only for the Indian subcontinent but also for identifying other important mechanism which can enhance the severity of malaria in varied geographical areas. Future studies are warranted involving bigger sample sizes in wider geographical areas to find out the specific role of nitric oxide synthesis and its role in malaria severity for planning effective and sustainable control measures.

CONCLUSIONS

In conclusion, the pathophysiological processes causing the elevation of nitric oxide level and hematological changes in malaria are complex, multiple and incompletely understood. Significant decrease in hemoglobin level and thrombocytopenia were the two most important hematological abnormalities seen in our cases of acute malaria infection. The chances of anemia was related more to decrease in hemoglobin level, while, thrombocytopenia was associated with *P. falciparum* infection. All together, these results point out to the fact that nitric oxide is in fact a marker of clinical infections can be used as an indicator for the severity of the disease. We also recommend that these nitric oxide synthesis and hematological changes should be further studied in relation to the level of parasitemia. More over, similar multi-center studies should be carried out all over India to define the prevalence pattern of all types of malaria infections.

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