

*Full Length Research Paper*

# Assessment of the level of nitric oxide in the serum of subjects exposed to malarial settings

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Accepted 15 January, 2017

Nitric oxide (NO) has been recognized during the past two decades as one of the most versatile players in the immune system. 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 an important role as a marker for the severity of the disease. In this study we assess the level of nitric oxide in the serum of subjects exposed to malarial settings but who have not become clinically infected by plasmodia parasites. We conclude that NO is in fact a marker of clinical infections but cannot be used as an indicator of the disease's severity.

**Key words:** Nitric oxide, malaria, *Plasmodium falciparum*, imported malaria.

## INTRODUCTION

By the time nitric oxide (NO) was introduced in immunology studies, its characterization was simplistically termed: "NO is produced from L-arginine through the enzyme NOS (nitric oxide synthase) by macrophages when these are activated by cytokines and/or microbial compounds; it has tumoricide and anti-microbial actions *in vivo* and *in vitro*" (Bogdan, 2001). Today, even if this uncomplicated definition is still accepted, it is known that NO has a much wider and complex role in the immune system and is of great importance in cellular signaling (Clarck et al., 2004).

Nitric oxide can be produced by any cell of the immune system (dendritic cells, NK cells, mast cells and all of the phagocytes) as well as by other sort of cells that entail the immune response (Bogdan, 2001). Its has effects not only on tumoricide and anti-microbial levels as described in the original 1987 definition, but it also contributes to the pathogenesis and control of infectious diseases, auto-immune processes, cytokine, chimokine and growth factors modulation, chronic degenerative diseases and

induction and differentiation of T<sub>H</sub> cells (Bogdan, 2001).

On infective diseases, nitric oxide comprise a broad range of activities; in malaria, the NO produced inside the vector (the *Anopheles* mosquito) protects it against the *Plasmodium* parasite, smooth the progress of the mosquito's blood meal and antagonize the human host's haemostatic response (Bogdan, 2001). Another example of the haemostatic role of the nitric oxide regarding *P. falciparum* malarial infections is its ability to inhibit the adhesion of parasitized erythrocyte to the vascular endothelium (Clarck et al., 2004). This adhesion is mandatory to the maximization of tumor necrosis factor (TNF) production and hence, to the development of the disease. Likewise, T-lymphocytes can be suppressed by neighboring NO-producing macrophages (Clarck et al., 2004), which can give explanation to the uncommonness of autoimmune diseases in the regions of the world where malaria is hyperendemic.

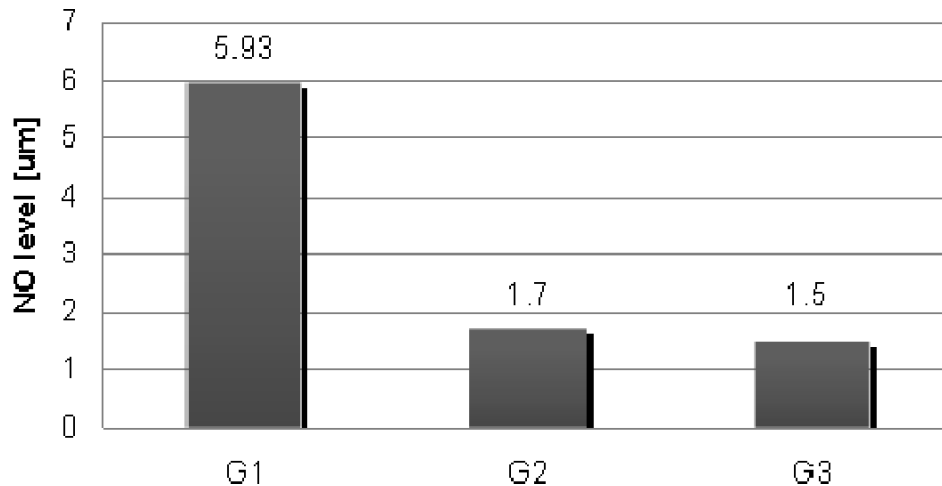
Even though the molecular mechanisms responsible for the naturally acquired immunity against malaria are still to be clarified, the production of NO seems to play a significant role as a marker for the severity of the disease (Keller et al., 2004).

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**Abbreviations:** MCHC, Medium corpuscular hemoglobin concentration; Hgb, hemoglobin; WBC, white blood cells; NO, nitric oxide; Plt, platelets.

## METHODS

Our study population consisted of 140 individuals, 114 recently ex-



**Figure 1.** Nitric oxide levels for each study group. G1: Persons infected with *P. falciparum*; G2, negative tested exposed persons; and G3, negative control persons who had never been exposed to *P. falciparum*.

posed to mosquito bites in endemic regions and 26 (control population) with no previous exposure.

From each person, blood was obtained by venipuncture and blood smears were prepared (thick and a thin blood films). The hematological data were obtained from an automatic Coulter Sysmex K-1000 analyzer (Emílio de Azevedo Campos). Both the thick and thin blood films were stained by Giemsa's staining method and observed on a Leitz Biomed optical microscope. The thick blood film was used to attain a qualitative diagnose for malarial infection and the thin blood film was used to identify the *Plasmodium* species.

Following microscopy detection of malaria parasites in blood, the study population was divided into three groups: Group 1 of persons infected with *P. falciparum*; group 2, negative tested exposed persons; and group 3, negative control persons who had never been exposed to *P. falciparum*.

When infection by *P. falciparum* was established, the thin blood film was used to count the number of parasites in 200 leucocytes and this number was converted to number of parasites in one microliter of blood (WHO, 1985). All of the serum was kept at  $-80^{\circ}\text{C}$  up to its employment.

To investigate the nitric oxide formation, we measured nitrite ( $\text{NO}_2^-$ ), which is one of two primary, stable and nonvolatile breakdown products of NO, using the Promega's Griess Reagent System (Promega Corporation, USA; lot #198593). The method relies on a chemical diazotization reaction that was originally described by Griess in 1879, which uses sulfanilamide and *N*-1-naphthylethylenediamine dihydrochloride (NED) under acidic conditions. We used the same microtiter plates (Maxisorp, Nunca A/S) in all the trials; the final readings were obtained from a Dynatech MR7000 microplate reader (Dynatech) set at 570 nm and the final nitrite concentration was calculated using the Lambert-Beer law for the calibration curves we inserted in every microtiter plate.

## RESULTS AND DISCUSSION

There are four *Plasmodium* species capable of infecting man; of these, *P. falciparum* is the most pathogenic (Ranjit et al., 2005; Dromigny et al., 2005), 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 (Ranjit et al., 2005). The persons belonging to Group 2 were targeted as the main study group, since they have been exposed to the parasite *P. falciparum* but have not become infected; thus raising the question: was nitric oxide accountable for the protection against the infection?

The comparison of nitric oxide levels on the serum samples of the three study groups (Figure 1) shows a 5-fold greater NO concentration for persons infected by *P. falciparum* when compared to the persons of the other two groups. This seems to indicate that NO is in fact a marker of clinical infection by *P. falciparum*.

We could not find a significant variation on nitric oxide levels between group 2 and group 3, possibly meaning that individuals exposed to *P. falciparum* may not produce NO immediately after inoculation of sporozoites by the *Anopheles* mosquito. In fact, some studies (Brunet 2001; James, 1995) suggest that NO intervenes on the response to *Plasmodium* by mediating the intrahepatic killing of parasites. Accounting for the fact that the sporozoites injected by the mosquito vector into the blood stream of the human host mature into schizonts in the liver, it seems reasonable to think that there is no boost on nitric oxide production in the early stages of infection.

Furthermore, since the excessive production of NO is known to be cytotoxic not only to the invading parasites but also to the host's own cells (Brunet, 2001), there is a possibility that the symptoms might be attenuated by killing the limited number of intrahepatic parasites found

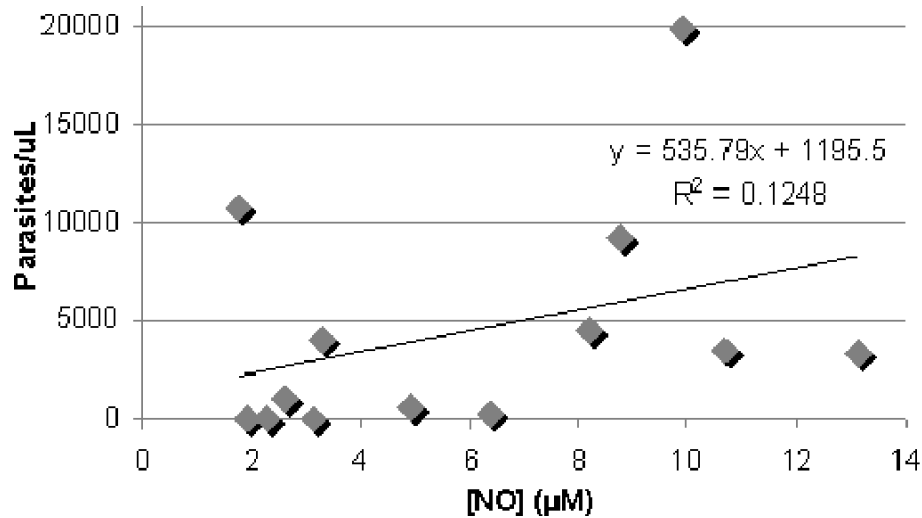


Figure 2. Parasite density versus serum nitric oxide in patients infected with *P. falciparum*.

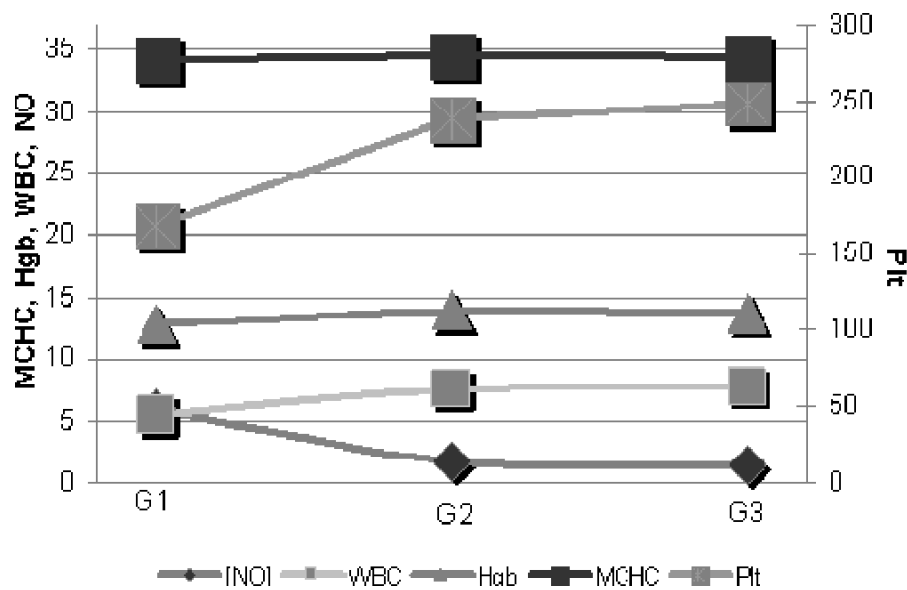


Figure 3. Hematological parameters versus serum nitric oxide for each study group. MCHC (g/dL), Hgb (g/dL), WBC ( $\times 10^3/\mu\text{L}$ ), NO ( $\mu\text{M}$ ), and Plt ( $\times 10^3/\mu\text{L}$ ).

in newly infected hosts, prior to the development to the erythrocytic stages of the parasite life cycle (Brunet, 2001).

A balance between NO-induced anti-parasitic actions, which should result in the elimination of invading parasites, and NO-induced cytotoxic effects, which result in host tissue damage, needs to be achieved for the host's benefit. If NO production fails to be closely regulated and is left unchecked, the host may suffer increased morbidity. Additionally, if the host fails to produce appropriate levels of NO, parasite numbers boost, leading to increased mortality.

The growth of *P. falciparum* can be controlled (*in vitro*) by nitrite, nitrate, and nitrosothiols (James, 1995), implying a protective role for NO in malaria infection. Despite this fact, our observations suggest otherwise, since no correlation between these two factors could be established, pointing out to the fact that there can be a number of other variables entailing the balance between NO production and controlling the malarial infection.

Recent studies (Boutlis et al., 2003) have established that experimentally induced *P. falciparum* infections strongly induce the enzyme NOS (nitric oxide synthase) even among malaria-naïve individuals infected with very

low induced parasitemia.

This agrees with the observations suggesting that asymptomatic individuals with microscopically detectable *P. falciparum* parasitemia produce significantly higher levels of NO than individuals with either PCR (polymerase chain reaction) or microscopy undetectable parasitemia (Anstey et al., 1999). We could not find proof of these observations since there was no correlation between the parasitemia and the serum NO levels (Figure 2).

Regarding the association between serum nitric oxide concentration and hemoglobin levels (Figure 3), the results seem to suggest an inverse proportionality, which is in accordance with other studies (Keller et al., 2004) suggesting that nitric oxide is an inhibitor of erythropoiesis.

However, it seems that individuals with microscopically undetectable parasitemia do not produce enough nitric oxide for it to be associated with protection against *P. falciparum* infection, since their NO concentration resembles that of the negative control group.

All together, these results point out to the fact that nitric oxide is in fact a marker of clinical infections but cannot be used as an indicator for the severity of the disease.

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