THE ROLE OF NITRIC OXIDE AND LIPID PEROXIDATION IN PATIENTS WITH *PLASMODIUM VIVAX* MALARIA

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Summary:

In this study, we investigated the role of nitric oxide metabolism and lipid peroxidation in patients with *P. vivax* malaria. The levels of nitrite and nitrate were analyzed using a procedure based on the Griess reaction and malondialdehyde levels which index of lipid peroxidation was determined by thiobarbituric acid reaction. The levels of nitrite/nitrate and malondialdehyde in patients were higher than controls and found to be statistically significant ($p < 0.001$). We performed this study to determine whether nitric oxide and lipid peroxidation is produced during blood-stage *P. vivax* malaria. This present study shows that lipid peroxidation occurs in *P. vivax* malaria. The levels of nitric oxide are associated with lipid peroxidation in this disease.

KEY WORDS : malaria, *Plasmodium vivax*, nitric oxide, malondialdehyde, lipid peroxidation.

Résumé : LE RÔLE DE L'OXYDE NITRIQUE ET DE LA PEROXYDATION DE LIPIDES CHEZ LES PATIENTS ATTEINTS DE MALARIA À *PLASMODIUM VIVAX*

Les auteurs ont étudié le rôle du métabolisme de l'oxyde nitrique et de la peroxydation de lipides chez des patients atteints de malaria à P. vivax. Les niveaux de nitrite et de nitrate ont été analysés en utilisant un procédé basé sur la réaction de Griess et les niveaux de malondialdehyde index de la peroxydation de lipides ont été déterminés par la réaction à l'acide thiobarbiturique. Les niveaux de nitrite/nitrate et de malondialdehyde des malades étaient plus élevés que ceux des témoins de façon statistiquement significative ($p < 0,001$). Cette étude a été réalisée pour déterminer si le NO et la peroxydation de lipides s'observent au cours du stade sanguin du cycle de P. vivax. Il apparaît que la peroxydation de lipides s'observe dans la malaria à P. vivax ; les niveaux de l'oxyde nitrique étant associés à cette peroxydation.

MOTS CLÉS : *malaria, Plasmodium vivax, oxyde nitrique, malondialdehyde, peroxydation de lipides.*

Malaria is caused by protozoons of four species of the genus *Plasmodium*: *P. falciparum*, *P. malaria*, *P. ovale* and *P. vivax*. *P. falciparum* and *P. vivax* are widespread in many areas of the world (Taylor-Robinson, 1998; Tirasophon *et al.*, 1994; Krogstad, 2000). *P. vivax* is the most frequent and widely distributed cause of malaria (Tirasophon *et al.*, 1994; Aslan *et al.*, 2001). It is less virulent than *P. falciparum* and seldom fatal. It infects only immature erythrocytes (Krogstad, 2000). Most individuals control blood-stage malaria, suggesting that components of the immune system kill replicating parasites in the blood. However, the precise mechanisms by which blood-stage parasites are killed remain to be determined (van der Heyde *et al.*, 2000).

There is increasing evidence of the involvement of free radicals in the aetiology of several diseases (Aruoma *et al.*, 1989; Kasprzak, 1995; Hensley *et al.*, 1995). These radicals, which include the oxide radical ($O^{\cdot}$), superoxide radical ($O_2^{\cdot}$), hydroxyl radical ($HO^{\cdot}$) and the perhydroxyl radical ($HOO^{\cdot}$), are usually products of some *in vivo* metabolic processes (Fridovich, 1995; Nappi & Vass, 1998). The degradative activity of free radicals only results when they are not immediately removed from the cellular milieu by scavenging enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Halliwell & Gutteridge, 1990). The peroxidation of lipid membranes leading to the production of peroxides and other radicals are responsible for the initiation of the free radical pathologies (Halliwell, 1989). The lipid peroxidation is the aerobic free radical-initiated progressive cleavage of a polyunsaturated fatty acid by a radical species. It can be assayed by measuring the quantity of the thiobarbituric acid chromogenic complex of malondialdehyde (MDA). MDA is the major breakdown product of lipid peroxidation (Ohkawa *et al.*, 1979).

Nitric oxide (NO) is known to play a role in the immune system, the nervous system, in inflammation, programmed cell death and host defense against microorganisms (Moncada & Higgs, 1993; Bredt *et al.*, 1990). This free radical has a biological half-life of seconds under normal physiologic oxygen tension. NO diffuses

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freely across membranes to mediate its effects. NO plays a vital role in the supression of the acute parasitemia in experimental *Leishmania* major infections as well as in the prevention of reactivation of chronic low grade infection (Wei *et al.*, 1995; Stenger *et al.*, 1996).
NO is a bioreactive free radical and an important signalling molecule that acts in many tissues to regulate a diverse range of physiological processes (Beckman & Koppenol, 1998). This molecule is produced by a group of enzymes called nitric oxide synthases. These enzymes convert arginine into citrulline and NO in the process (Kasprzak, 1995). NO is able to induce vasodilatation (Moncada & Higgs, 1993; Waldman & Murad, 1988). It is also known to play a role in the immune system, the nervous system, in inflammation and programmed cell death (Moncada & Higgs, 1993; Bredt *et al.*, 1990). It acts as a prooxidant in reacting with superoxide (O_2^-) to form peroxynitrite ($O = NOO^-$). These reactive nitrogen intermediates inactivate key microbial enzymes. NO has a protective function in oxidative stress (Kappenol, 1998). It inhibits free radical-mediated chain propagation reactions such as lipid peroxidation. NO acts as a chain-breaking antioxidant during cellular lipid peroxidation. It is in the propagation and termination process that NO has been proposed to play a crucial role as an antioxidant in lipid peroxidation (Kelley *et al.*, 1999).
The goal of this study was to investigate role of nitric oxide metabolism and lipid peroxidation in *P. vivax* malaria.

MATERIALS AND METHODS

This study was conducted in the South Anatolia region of Turkey. A total of 35 blood samples were obtained from patients with malaria-like symptoms, including fever and/or chills, of several days duration and 23 samples were obtained from healthy persons as a control group from same region. Permission for testing was obtained from patients who were admitted to the Siverek Malaria Eradication Centre. Two millilitres of venous blood were drawn into EDTA-coated syringes, distributed into sterile test tubes, and placed immediately on ice. Thin and thick-smear blood films were prepared on the site at the time of specimen collection. The whole-blood samples were stored at – 80° C until study.

Microscopic determination of the parasite, and counting the parasitaemia

All the blood films were stained with Giemsa and were initially examined at the Siverek Malaria Eradication Centre by a malaria expert. At the Clinical Microbiology Laboratory of Mersin University, two skilled microscopists then re-examined them independently. A slide was considered to be negative when no parasites were detected in 200 WBCs by either of the microscopists (Beadle *et al.*, 1994).
Parasite density (parasites/µl blood) was calculated for each positive thick film by 200 WBCs had been observed, and the parasite count was then multiplied by 40 to give the number of parasites per millilitres of blood. This method does not takes into account the known loss of parasites in the thick film, which suggests that it produces an underestimate, but the "standard" white blood cell value is probably too high, which helps to cancel out this error (Warhurst & Williams, 1996).

Analysis of serum nitrite and nitrate

The levels of nitrite and nitrate were determined using a procedure based on the Griess reaction (Green *et al.*, 1982). Blood samples were immediately centrifuged at 4,000 rpm for 10 min. Serum samples were then separated and stored at – 70°C until used for assay. Equal volumes of serum and potassium phosphate buffer were placed in an ultra filter and centrifuged at 4,000 rpm for 45 min. The ultrafiltrate was collected and used in the test. Nitrates were quantitatively converted to nitrites for analysis. Enzymatic reduction of nitrate to nitrite was carried out using coenzymes (NADPH, FAD) in the presence of nitrate reductase in step of incubation assay. N-1-(naphthyl)ethylenediamine dihydrochloride, sulphanilamide and incubation solutions were mixed at a ratio of 1:1:2 (v/v). These mixtures were incubated for five min at room temperature in dimmed light and measured at 540 nm. Sodium nitrite of 1.00 mM was used as standard for determination of nitrite and potassium nitrate of 80 mM was used as standard for determination of nitrate (Nitric oxide colorimetric assay, 1-756-281, Roche Diagnostics GmbH, Mannheim, Germany).

Determination of MDA

The determination malondialdehyde (MDA) levels an index of lipid peroxidation were determined by thiobarbituric acid (TBA) reaction. The principle of the method depends on measurement of the pink colour produced by interaction of barbituric acid with malondialdahyde elaborated as a result of lipid peroxidation. The colour reaction 1,1,3,3-tetraethoxy propane was used as the primary standard. The determination of MDA levels was performed by method of Yagi *et al.* (Ohkawa *et al.*, 1979; Yagi, 1998).

Statistical analysis

Results were expressed as median serum protein concentration with corresponding 95 % confidence intervals. Differences between groups were calculated using the student' t-test.

RESULTS

Thirty-five patients who had malaria-like symptoms were diagnosed as having *P. vivax* malaria on the basis of thick-film and clinical findings. Table I represent the data of patients and controls. The levels of nitrite/nitrate of patients were higher than controls. In statistical analysis, there was a significance ($p < 0.001$). The levels of MDA in patients were also higher than controls ($p < 0.001$).

Tests	Patients (n: 35)	Controls (n: 23)	Significance
Nitrite/nitrate (µmol/L)	34.47 ± 11.94*	19.22 ± 4.14	$p < 0.001$
MDA (nmol/ml)	20.37 ± 4.12	2.06 ± 0.14	$p < 0.001$

* Mean ± standard error of mean (SEM).

Table I. – The levels of nitrite/nitrate and MDA in patients and controls.

DISCUSSION

Malaria remains one of the major disease of the world. Haematologic changes, which are the most common complications. These changes include red blood cells, leukocytes, and hemostasis. Malaria is a parasite spread by mosquitoes that breaks down haemoglobin in red blood cells (Harinasuta & Bunnag, 1988). The parasite catabolizes haemoglobin, using the amino acids as a source of protein and aggregataing the heme as hemozin, a waste product (Pagola *et al.*, 2000). Free radicals generated by the interaction of hemozoin and oxidant agents may cause oxidative stress.

Lipids are important component of the membrane surrounding cells and cellular organelles. They form a bilayer structure of the membrane (Murray & Granner, 2000). O_2 and radicals can attack polyunsaturated fatty acids. Lipid peroxidation is a free radical related process (Gutteridge, 1995). Oxidants can react with polyunsaturated fatty acids in cell membranes to form toxic metabolites. The cells have a number of ways to protect against the constant threat of the radicals produced in lipid peroxidation (Das & Nair, 1980; Sevinan & Kim, 1985). NO plays a potent oxidant protective role in the vessel wall by inhibiting lipooxygenase dependent lipid oxidation. This process occurs by termination of lipid radical chain propagation reactions catalysed by LO˙ and LOO˙ intermediates of lipid peroxidation (Rubbo *et al.*, 2000). The results of the present study support a role for increased oxidative stress in the pathogenesis of *P. vivax* malaria. We found that patients with *P. vivax* malaria are characterized by enhanced lipid peroxidation in serum. These findings support the notion that enhanced oxidative stress is an important characteristic in these patients.

The serum NO levels are elevated only on a single day during the period of descending parasitemia (Taylor-Robinson *et al.*, 1993). It is reported that high levels of NO are toxic to *Plasmodium falciparum* in vitro (Rockett *et al.*, 1991). Since the discovery that nitric oxide is able to induce vasodilatation a large number of other roles have been described for NO. It can be produced by a number of cells involved in immune responses. In particular cytokine-activated macrophages can produce high concentrations of NO in order to kill target cells such as bacteria or parasites (MacMicking *et al.*, 1997; De Groote & Fang, 1995). NO-mediated cytotoxicity is often associated with the formation of nitrosyl-thiol complexes in enzymes within the target cell. It has been shown to kill cells by disrupting enzymes involved in the Kreb's cycle, DNA synthesis and mitochondrial function (Burgner *et al.*, 1999). NO may act as a mediator of inflammatory processes and its production can be induced, through the upregulation of iNOS, by a number of factors involved in inflammation, including interleukins, interferon-γ, TNF-α and LPS (Jacobs *et al.*, 1995; Jacobs *et al.*, 1996). In the present study, plasma levels of NO were markedly increased in patients. NO is acted as an antioxidant during cellular lipid peroxidation. The elevation of NO levels were also observed with elevation of lipid peroxides. The roles of NO in malaria may explaine by several mechanisms.

The role of NO and lipid peroxidation in the clearance of blood-stage malarial parasites remains uncertain. We therefore performed studies to determine whether NO and lipid peroxidation is produced during bloodstage *P. vivax* malaria. Overproduction of NO is important for killing malarial parasites and consequently alters the course of malaria. Our observation shows that serum NOx levels are 1,7-fold elevated during parasitemia. With the role of NO and lipid peroxidation in the aetiology and pathogenesis of several diaseases, these data present a need for free radical control during malaria. Further studies will be necessary to evaluate these results.

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