ORIGINAL ARTICLE

Nitric oxide induction as a novel immunoepidemiological target in malaria-infected patients from endemic areas of the Islamic Republic of Iran

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Abstract

Objective. Malaria has been prevalent for a long time in Iran and continues to be a health problem despite substantial control programs. In addition to numerous cytokines, nitric oxide (NO) is thought to be a key molecule and a novel target of malaria immunopathology. ***Material and methods***. The objective of this research was to measure reactive nitrogen intermediates (RNI) as stable metabolites of NO induction in plasma of malaria-infected patients in Iran. In this study, 235 blood samples from malaria patients and 80 blood samples from healthy controls were randomly collected from different malarial endemic provinces of Iran, located in southeastern (Sistan & Balouchestan, Hormozgan, Kerman) and northwestern (Ardabil) areas. The involvement of NO in malaria patients has been investigated by statistical analysis of RNI values. Griess micro assay (GMA) was used during *Plasmodium vivax, P. falciparum* and mixed infections, in order to evaluate whether RNI changes are related to the provincial areas, parasite strains, clinical symptoms and age and gender parameters. ***Results***. The results showed a significant increase of RNI level in malaria patients compared with the control groups of Ardabil ($p < 0.01$), Sistan & Balouchestan, Hormozgan and Kerman ($p < 0.001$) provinces. The level of RNI was higher in mixed plasmodial infection than in single infection. ***Conclusions***. The high level of RNI was dependent on the type of infection, the plasmodia strain, the clinical symptoms, the age groups and the endemic provinces. Although, this study did not clarify the pathogenic and/or protective role of NO in malaria, our findings provide a novel immunoepidemiological aspect of basal NO production in patients with malaria in endemic areas in Iran.

Key Words: *Endemic areas, Iran, nitric oxide, NO, malaria, plasmodium, RNI*

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Introduction

Malaria is one of the most important parasitic diseases in the world [1]. It has been prevalent for a long time in Iran [2] and it is still a problem, despite the national malaria control program [3]. Out of the 23,562 malaria cases that have been reported in 2003, more than 70 % occurred in the southeastern (SE) part of the country [1,4]. The inherent problems are due to the existence of technical and administrative difficulties, drug and vector resistance and complications regarding the importation of malaria from neighboring countries [3,5]. Recently, a new threat of malaria importation emerged from the northwestern (NW) part of the country, the Parsabad area, which was affected by a serious epidemic of *Plasmodium vivax* [5]. Iran was divided into two malaria zones north and south of the Zagros Mountain Range [3] including SE provinces (Sistan & Balouchestan, Hormozgan and Kerman) and the NW province (Ardabil) [6]. To date, the malaria situation in the SE corner is serious [4], but in other endemic areas, malaria is hypoendemic with non-serious cases [7]. *P. vivax* is the most prevalent species, followed by *P. falciparum* [8] and mixed infections [9,10].

Increased drug and insecticide resistance has made vaccine preparation a matter of urgency for malaria, with the focus on new targets in both parasite and host [11,12]. Potential effector mechanisms of immunity against malaria include antibodies, macrophages, T cells, cytokines and a variety of other soluble mediators [13].

Activated phagocytic cells generate large amounts of highly toxic molecules, reactive oxygen and nitrogen intermediates (ROI, RNI) [14], H_2O_2, nitric oxide (NO) and at least 80 cytokines and enzymes [15–17]. NO reacts to form biologically active oxides, including nitrites and nitrates, collectively termed RNI, which react in several ways [18]. In addition to numerous cytokines [13,19,20], the role of NO as a mediator in clinical malaria remains controversial [21,22]. Despite the reported protective effects of NO in the early responses [23,24], some publications do not support a crucial role for NO in immunity against *Plasmodia* [25–27].

In previous reports [18,28–30], NO, RNI and NO synthase (NOS) were investigated in murine *Plasmodia*, less attention being given to their role in immunoparasitology of human malaria. Variation in NO levels is under debate; this might elucidate some of the controversial data obtained with human malaria studies from different locations [29,31,32]. The aim of this novel immunoepidemiological study was to determine RNI as stable metabolites of NO activity during infection in malaria patients of endemic areas of Iran in the eastern Mediterranean region, in order to evaluate whether changes in RNI level are related to parasite strains, clinical symptoms, age groups, gender and geographical parameters.

Material and methods

Malaria patients and control groups

In this report we investigated the possible induction of RNI in patients infected with *P. vivax*, *P. falciparum* or mixed infection. This study was conducted from 2002 to 2004 in the endemic provinces of Hormozgan, Sistan & Balouchestan, Kerman and Ardabil. The pattern of NO induction was studied among the malaria patients and compared with the control groups. Two hundred and thirty-five blood samples (55–60 samples/province) were randomly collected from malaria-confirmed patients (microscopic method) who resided in the above-mentioned endemic areas (Figure 1). In addition, 80 blood samples (20 samples/

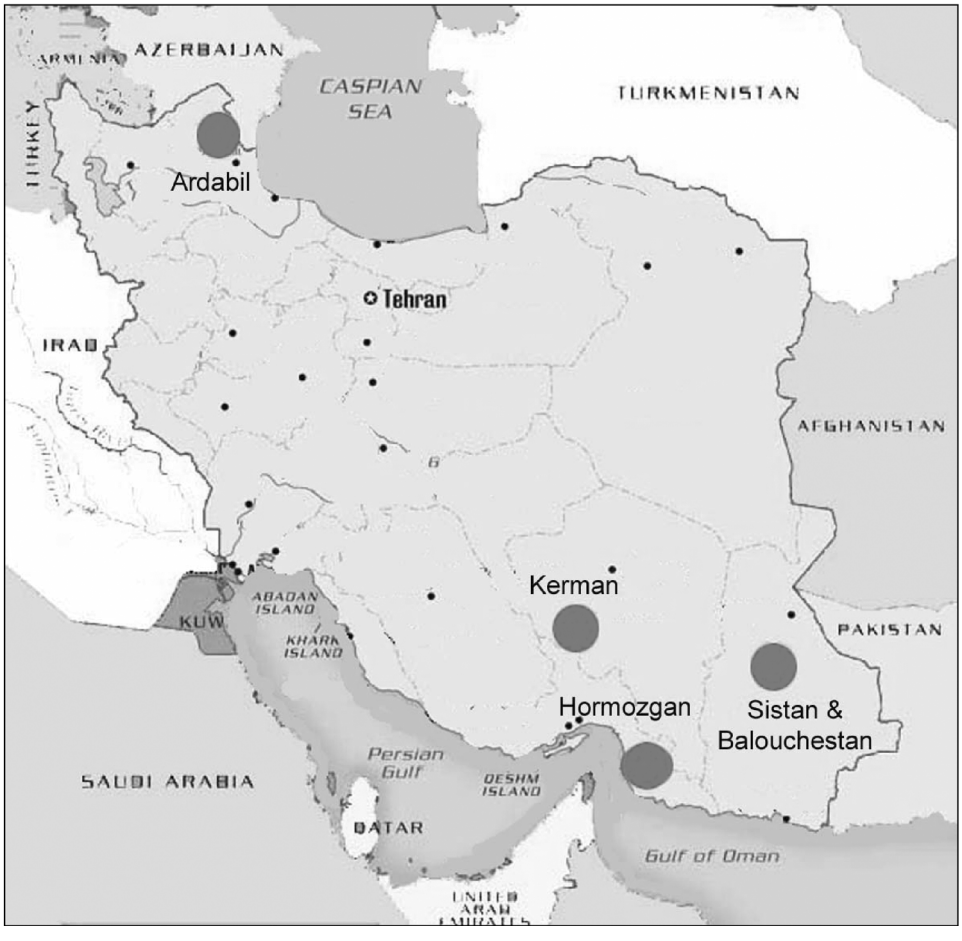

Figure 1. Malaria endemic areas of Iran. The country is divided into two epidemiological zones of malaria in areas north and south of the Zagros Mountain Range: the southeast (Sistan-Balouchestan, Hormozgan, Kerman provinces) and the northwest (Ardabil province).

province) were randomly taken from healthy controls with no new infection and no history of malaria from the same endemic regions. Sample selection in both groups was applied considering all statistical parameters including province, gender and age groups.

Microscopic method

Thick and thin blood smears were produced from finger-prick samples; thin smears were fixed with ethanol, and both smears stained with 10 % Giemsa for 20 min, washed in tap-water, dried at room temperature (22–25°C) and observed under light micropscopy. Up to 200 microscopic fields were examined to establish the diagnosis.

Blood samples

Ten milliliters of circulation blood was collected from malaria patients aliquoted in two tubes; the first tube contained EDTA (Sigma Chemical Co. UK) to extract DNA for

polymerase chain reaction (PCR) assay, and the second tube, containing heparin 50 IU (Monoparin; CP Pharmaceuticals Ltd., UK), was used for plasma preparation for Griess micro assay (GMA).

Nested PCR

PCR assay was used to detect the human malaria parasites [9,10]. Briefly, DNA of all *Plasmodia* was extracted by the boiling method from blood containing EDTA and exposed to first primers including rPLU5 and rPLU6. PCR products were reacted with second primers, which were specific to prevalent species of human malaria in Iran. The primers in this study were FAL1, FAL2 (*P. falciparum*), VIV1, VIV2 (*P. vivax*) and MAL1, MAL2 (*P. malariae*), used according to the method developed by Snounou et al. [33]. DNA was amplified according to the following thermo-cycling program, denaturing 95°C 1 min, annealing 58°C 2 min and extension 72°C 2 min for 35 cycles. PCR products were run in a 1.5 % agarose gel, observed by UV transluminator.

Griess micro assay (GMA)

Plasma was prepared by centrifuging the blood at 1500 RCF (MSE Centaur 2, England) for 10 min and stored at –70°C until use. RNI levels were measured in plasma as nitrites using the modified Griess reaction [34] after first converting nitrates to nitrites with nitrate reductase treatment. Standard curves for sodium nitrite and nitrate (Sigma Chemical Co.) were prepared. Samples (60 μL) were treated with 10 μL nitrate reductase (NAD[P]H *Aspergillus* species 5 U/mL, Sigma Chemical Co.) and 30 μL NADPH β-nicotinamide adenine dinucleotide phosphate (1.25 mg/mL, Sigma Diagnostics, St. Louis, Mo., USA). Griess reagent 200 μL (5 % phosphoric acid, 1 % sulfanilic acid and 0.1 % N (1-naphthyl-1)-ethylendiamine dihydrochloride (NED), all from Sigma Chemical Co., dissolved in 100 mL deionized water) was then added and proteins subsequently precipitated with 200 μl trichloroacetic acid 10 %, (BDH, England). Tube contents were vortex mixed then centrifuged at 13,400 RCF (Model 1-13 Microcentrifuge; Sigma Chemical Co.). Duplicate 200 μL samples of supernatants were transferred to a 96-well, flat-bottomed microplate (Costar, USA) and absorbances read at 520 nm using a microplate reader (MS2 Reader; ICN Flow, UK). Values were calculated from standard calibration plots for $NaNO_2$ and $NaNO_3$ as previously described [18].

Statistical analysis

Values for RNI concentrations are presented as the mean $\pm$ SEM for groups of n samples. The significance of difference was determined by Student's t-test using GraphPad Prism Software (GraphPad, San Diego, Calif., USA).

Results

The results indicated that RNI levels significantly increased in malaria patients living in the Ardabil ($p < 0.01$), Sistan & Balouchestan, Hormozgan and Kerman ($p < 0.001$) provinces. Total data of all malaria-endemic regions of Iran showed an increase in RNI level ($p < 0.001$) in the malarial group compared with the control group (Figures 2, 3).

In each province, the RNI level of malarial patients was compared with that of the related controls. In this study, higher RNI values rather than average control values were

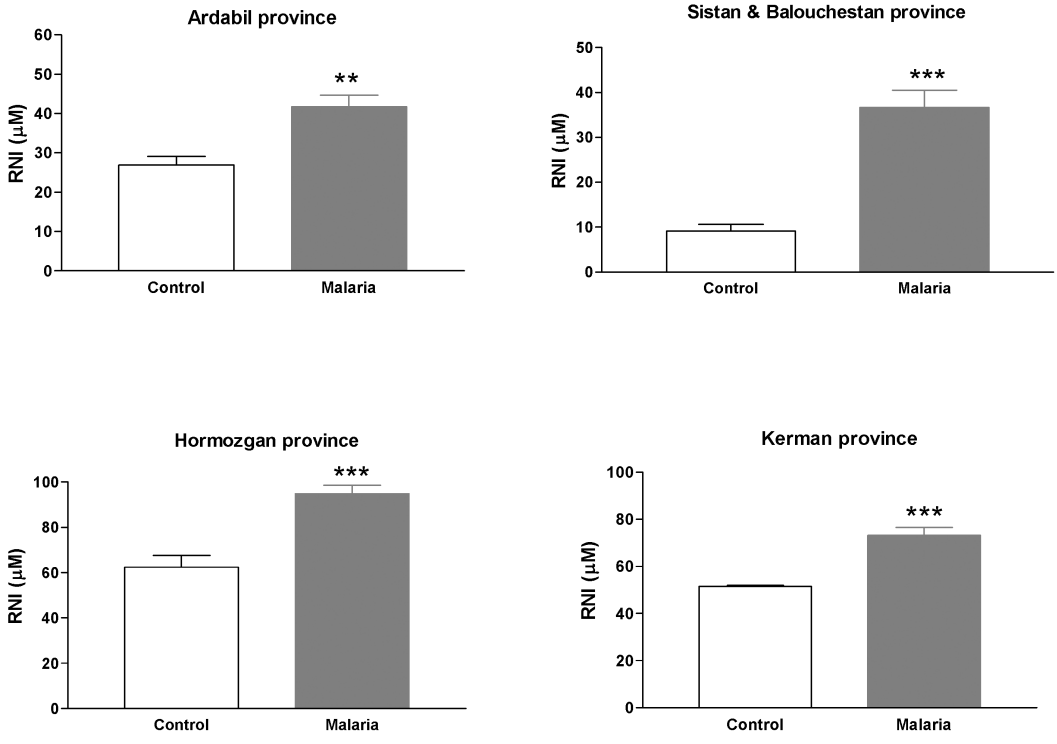

Figure 2. Reactive nitrogen intermediate (RNI) concentrations in control and malaria groups of residents in four endemic provinces of Iran. RNI levels significantly increased in malaria patients of Ardabil (**$p<0.01$), Sistan & Balouchestan (***$p<0.001$), Hormozgan ($p<0.001$) and Kerman ($p<0.001$) provinces in comparison with related controls (mean $\pm$ SEM, n=55–60 malaria, n=20 control samples; samples/province).

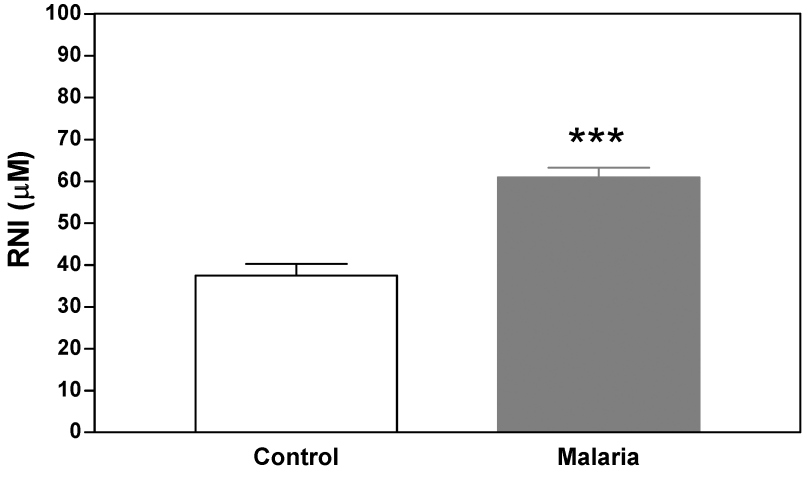

Figure 3. Reactive nitrogen intermediate (RNI) production as nitric oxide (NO) pattern in control and malaria groups of all malaria-endemic areas of Iran. RNI levels significantly increased in all malaria patients of endemic regions of Iran (***$p<0.001$) in comparison with the control group (mean $\pm$ SEM, n=235 malaria, n=80 control samples).

considered as NO positive (NO^+). The data resulting from analyzing the regression line indicated different equation values for control ($Y=0.29X + 26.2$) and malaria ($Y=0.65X + 11.3$) groups. The lowest rate of NO^+ was observed in Ardabil province (63.3 %) and the highest rate was found in Hormozgan province (90.9 %). The NO^+ rates in Sistan & Balouchestan and Kerman were 80 % and 88.3 %, respectively. The average percentage of samples from all malaria patients was 80.4 (Figure 4).

Discussion

In this novel study, the association of NO in malaria by measurement of RNI levels has been investigated. However, indirect determinations assaying NO metabolites as RNI could be misleading. Nitrite and nitrate may not solely represent NO activity in the host, but also that of normal bacterial flora, infections or the nitrate content of the diet [18]. In this instance, to decrease the research bias, using a control group from residents of the same region without any history of malaria could be useful.

Both direct and nested PCR diagnosis revealed a relationship between NO^+ cases and type of infection. The highest NO^+ rates were among mixed-infection cases (94.9 %) and the lowest rate was observed in *P. falciparum* infection (66.9 %). The NO^+ rate for *P. vivax* infection was reported to be 81 %. A relationship was observed between NO^+ cases and clinical symptoms including fever, tremor, anemia and headache. In the majority of symptomatic patients (>80 %), high levels of RNI were observed. Lack of hepatosplenomegaly and brain disturbances in our patients did not help to clarify the actual role of NO in malarial symptoms, which needs further study. Increased NO synthesis appears to have a protective rather than pathological role in malaria. However, pathogenic effects including cerebral malaria [35], severe anemia [36] or even death [37] may be related to toxic metabolites of NO overproduction; therefore, the complex relationship between symptoms, genetic polymorphisms and NO induction in populations requires further study [38]. In order to evaluate any association between NO and age of malaria patients, different groups were studied and the values of NO^+ cases among them were compared. The NO^+

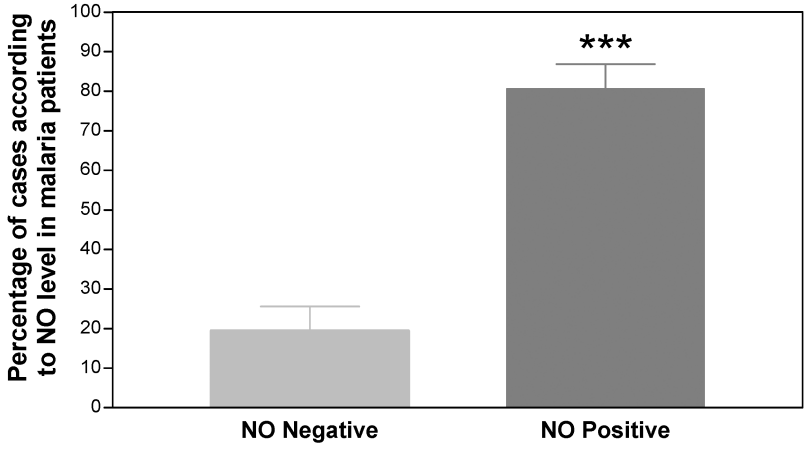

Figure 4. Relative frequency of nitric oxide-(NO) positive and -negative in malarial patients of all endemic areas of Iran. Significant difference between percentages of NO-positive and -negative cases in malarial patients of endemic regions of Iran (***$p<0.001$) (Mean $\pm$ SEM, $n=235$ malaria samples).

cases in malaria patients were 77.9 % (1–15 years), 69.4 % (16–30 years), 82.1 % (31–45 years) and 94.1 % (over 45 years old). In this study, a relationship between NO and age of malaria patients was observed. The majority of NO^+ cases were more than 45 years of age. Meanwhile, this concept was connected to the genetic profiles of population and parasite dominancy. The role of the host's gender and NO induction was also investigated; the percentages of NO^+ cases in male and female patients were different in the four provinces studied. In this study, 83.7 % of male malaria patients and 74.7 % of female malaria patients were NO^+, but no significant difference was observed between the two genders.

The low percentage of NO^+ cases in the NW province and higher values in the SE provinces may emphasize a dependency of NO on the region, which indicates a weak induction role for single infection in the NW region than mixed infection in the SE region. This may indicate a synergistic effect for mixed *Plasmodia* infection to induce more NO in the host, but further investigation is needed to elucidate this concept. The results showed a significant increase in RNI levels and NO^+ cases among malaria patients in Iran. This may clarify the involvement of NO/RNI during malarial infection in this region, but it cannot be confirmed whether changes in NO production are beneficial or detrimental to the host. Notwithstanding the conflicting publications, the role of NO in the immune responses to *Plasmodium* remains uncertain [21,36,37,39,40]. NO involvement in malaria is reported to be varied [29,30], which is related to numerous parameters. Variation in genetic structure and polymorphisms of genes encoding NOS induction could explain the ability of host responses to malaria infection in various regions [31,32,37,41]. These variations in different populations may be consistent with genetic differences in the human profile [42,43]. The protective polymorphisms may undergo selection in populations with a long history of exposure to malaria and other infections. Genetic variation in iNOS or related genes could also contribute to differences in basal NO production in some populations [38] or may influence susceptibility to and severity of malaria [44]. Conclusively, genetic epidemiology or cytokine biology alone is not enough to solve the NO paradox, but together they stand a good chance of doing so [37].

In conclusion, the results of this study indicate that RNI as stable metabolites of NO are produced in malaria patients of all the endemic provinces of Iran. The rate of this production is varied and related to the type of infection, clinical symptoms, age groups and endemic provinces. Although, the present study did not clarify the cytotoxic or protective role of NO in malaria, it provided immunoepidemiological data on basal NO induction in both healthy and malaria groups of endemic areas in Iran.

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