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Immunobiochemical Study of Host During Malaria Infection via Nitric Oxide Pathway after Modulation by Lipopolysaccharide and Dexamethasone in Mice Model

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ABSTRACT

Malaria is a parasitic disease which is common in tropical and subtropical regions of the world. Lipopolysaccharide (LPS) as an inducer and dexamethasone (DEX) as an inhibitor of the immune system are used in this study. The LPS and DEX effects on the level of nitric oxide (NO) of plasma, liver and spleen, hepatomegaly, splenomegaly, survival rate and the degree of parasitaemia on malaria infected mice were investigated. In this study, 24 outbred mice were randomly divided into 4 groups of 6 animals. Entire mice were infected by the murine malaria parasite *Plasmodium berghei*. Two groups of mice were selected as controls, which were injected with intraperitoneal (ip) injection of normal saline. The two DEX and LPS groups of mice had received concentrations of 4 and 1 mg kg^{-1} treatment respectively by 8 times ip injection every other day. Their body weight, survival rates and parasitaemia were monitored during the study. Finally, mice were euthanized by terminal anesthesia and cardiac puncture and the entire liver and spleen were removed for hepatosplenomegaly. Plasma and liver/spleen suspensions were assessed for immunobiochemical alterations of NO levels. The results indicated an increase in hepatomegaly of test group compared to control mice. The LPS mice represented splenomegaly more than DEX one. Nitric oxide in plasma, liver and spleen in both DEX and LPS group was changed, however, the only significant difference was observed in the liver of LPS. It is concluded that LPS and DEX can be applied as a standard and medically approved inducer and inhibitor of the immune system respectively, for experimental immunomodulation studies in animal models. In this study, LPS induced and DEX reduced immune responses in mice during malaria infection by alterations and manipulation of immunobiochemical factors via NO pathway. This may lead to an immunotherapy trial of malaria by control the pathophysiology and degree of parasitaemia in mouse model.

Key words: Malaria, lipopolysaccharide, dexamethasone, nitric oxide

INTRODUCTION

Malaria is an infection disease which is caused by protozoan parasites in genus *Plasmodium*. It is transmitted by a species of female Anopheles mosquitoes. It is one of the most important infection diseases in tropical and subtropical regions (Evans *et al.*, 2007). Limitation of experimental studies has paved on human malaria for testing on rodent malaria. Biological characteristics are somehow similar to small mammals and rodents with humans and the

convenience and low cost of maintenance, feeding and reproduction are the advantages of rodent malaria in malaria research (Nahrevanian and Eskandarian, 2005). It has long been known that malaria infection causes host immune modulation by various mechanisms (Coban *et al.*, 2007).

The nitric oxide (NO) molecule including oxygen and nitrogen atoms, bound by a double bond (Kroncke *et al.*, 1997) has a less half-life in biological environments and in the presence of H_2O and O_2 is converted to nitrite and nitrate (Porsti and Poakkari, 1995; Synder and Bredt, 1992). The primary source of NO is the NO synthase (NOS) enzyme, which has three isoforms; two are constitutively expressed and one is inducible (Griffith and Stuehr, 1995). Moreover, NO and superoxide, which are produced by several cell types, rapidly combine to form peroxynitrite (Pryor and Squadrito, 1995).

There is increasing evidence that NO is an important molecule involved in a wide variety of biological processes (Iadecola *et al.*, 1994; Amini *et al.*, 2009). Studies have shown that severe cases of human malaria are accompanied by increased iNOS activity and the subsequent production of NO (Anstey *et al.*, 1996; Perkins *et al.*, 1999).

There are several stimuli were reported to activate the immune system, especially to induce cytokines and NO (Gharavi *et al.*, 2011). The LPS is in the outer membrane of negative bacteria and composed of two main parts lipid and polysaccharide and his name is bacterial endotoxin. Immunogenicity of LPS is due to its polysaccharide form (Gunnett *et al.*, 1998). The LPS has a molecular weight of 2-20 kDa used generally as a "Standard stimulus" for the induction of inflammation or fever in laboratory animals (Abbas and Lichtmn, 1997). A number of factors, including cytokines and immune intermediates are produced with lipopolysaccharide (LPS) as an inducer stimulation in animal models and acts as an inducer of reactive nitrogen (RNI) and oxygen (ROI) intermediates in macrophages (Nahrevanian *et al.*, 2005).

Dexamethasone (DEX) is from glucocorticoid hormone families that have anti-inflammatory properties (Reynold and Prasad, 1996). Glucocorticoids are potent drugs that can be used widely inflammatory disorders such asasthma, allergies, infections, autoimmune diseases (Rhen and Cidlowski, 2005). They have spread rapidly in the circulatory system and when they are passing through the cell membrane they will bind themselves to cytoplasmic receptors which cause regulated transcription for some genes that disabled such as inflammatory cytokines and enzymes including NOS (Barnes *et al.*, 2006).

Plasmodium berghei is a similar malarial species, which is reported to be susceptible to NO mediators in rodents (Luckhart *et al.*, 1998). In addition, a study showed that manipulating NO levels in the mosquito's gut could provide a method for controlling *Plasmodium* transmission (Ali *et al.*, 2010). In current immunobiochemical study, the effect of immunomodulation of host during malaria infection was investigated via NO pathway after modulation by LPS and DEX in mice model.

MATERIALS AND METHODS

Animals: Outbred Naval Medical Research Institute (NMRI) 4-6 weeks mice (supplied by the Karaj Animal Production Unit, Pasteur Institute of Iran, Karaj, Alborz Province, Iran) were used in this study. Their body weight was 21-30 g, when initially measured on day zero. Experiments with animals were applied according to the ethical standards formulated in the Declaration of Helsinki and measures taken to protect animals from pain. It has been approved by "Ethical Committee of the Pasteur Institute of Iran", in which the work was done according to the "Guide to Working with Animals themes conveyed by the Ministry of Health and Medical Education of Iran" and a "Checklist about experimental animal" (signed by corresponding author).

Malaria parasites: The rodent malaria parasite used in this study was the *Plasmodium berghei* which represents a third of rodent malaria species. The infectivity of the parasites was maintained by regular passage in susceptible NMRI mice. Briefly, mice were infected by intra-peritoneal injection of *P. berghei* and the parasitaemia percentage was monitored from every other day post-challenge by calculation of infected RBC in Giemsa-stained thin blood smears.

Bacterial lipopolysaccharide and dose of injection: Ten milligram lipopolysaccharide (LPS) from *Salmonella abortus equi* species (Sigma Chemical Co. UK) was dissolved in sterile 0.9% normal saline and the standard inoculation (0.5 mg kg^{-1} b.wt.) was used for injection into mice. The LPS was used to increase the production of immune mediators and cytokines especially used as a NO inducer in animal models. The LPS groups of mice had received LPS treatment by 8 times i.p injection every other day.

Dexamethasone and dose of injection: Dexamethasone (DEX) (4 mg kg^{-1}) from Daroupakhsh Co., Tehran, Iran was used as NO inhibitor in this animal model. The DEX groups had received DEX treatment by 8 times i.p injection every other day.

Body weight: Body weight was measured initially and at different times of experiment using a top pan balance (OHAUS Scale Corp., USA).

Assessment of hepato/splenomegaly: Body weight of mice was measured by a digital scale. Entire liver and spleen were removed post mortem at the end of the experimental period from mice after induction of terminal anaesthesia by inhalation of diethyl ether (Sigma Co., Germany). Organ wet weights were measured and compared with controls as indices for degree of hepatomegaly and splenomegaly (Nahrevanian *et al.*, 2007).

Preparation of plasma: Plasma was prepared from blood taken by cardiac puncture into a 1 mL syringe containing 50 i.u. heparin (Sigma Chemical Co., Germany), from mice terminally anaesthetized by inhalation of diethyl ether. Plasma was prepared by centrifuging blood at 1500×g for 10 min, collected and stored at -70°C until assayed with Griess microassay (GMA) (Nahrevanian and Dascombe, 2001).

Preparation of tissue suspension: Liver and spleen tissue samples were removed and 0.1 g of spleen and 0.2 g of liver tissues were dissected into eppendorf tubes, weighed and homogenized using Pellet Pestle Motor, UK, then 1 mL Distilled Water (DW) was added to it, the resulting suspension was supposed into the Shaker (Heidolph Co. Gemany) to be completely smooth.

Griess microassay: Griess microassay (GMA) was applied to determine the effect of LPS and DEX on NO concentration in plasma and tissues suspensions with modifications to measure nitrite (Nahrevanian *et al.*, 2007). NaNO$_2$ directly were measured by Griess solution and trichloroacetic acid (TCAA). The standard solution of NaNO$_2$ was prepared with 10 different concentrations and the standard curve was drawn. Then after 100 µL DW was poured into 10 eppendorf tubes (1.5 mL), followed by adding 100 µL of NaNO$_2$ solution (1 mM) in the first eppendorf tube, since then, 100 µL of the first mixed solution was replaced with the same amount of solution in the later eppendorf and finally 100 µL of solution was removed from the last tube. Final concentrations of

NaNO$_2$, were 0.9-500 µM. Then 100 µL of Griess solution was added into each tube and was placed in shaker (5 min) to be well mixed. Final solution was centrifuged at RCF 13000 (Model 1-13 Microcentrifuge, Sigma, UK) for 5 min. Duplicate 100 µL samples of supernatants were transferred to a 96-well flat-bottomed microplate (Costar, USA) and absorbances read at 480 nm using a microplate reader (Bio-Tek-Powerwave XS, USA). Values for the concentration of nitrite assayed were calculated from standard calibration plots for NaNO$_2$. Thus, the standard curve was used to determine nitrite in serum samples and liver/spleen suspensions (Nahrevanian and Dascombe, 2002).

Evaluation of NO in plasma and tissue suspensions: To determine NO levels, 100 µL of plasma or liver and spleen tissue suspension were transferred into tubes and 100 µL of Griess reagent added to them, mixed and the proteins were sedimented by adding 100 µL of 10% trichloroacetic (TCAA) with shaking and incubation for 5 min at room temperature. Contents were centrifuged at 13000×g for 5 min and 100 µL of supernatants were removed and inserted in duplicate into a flat-bottomed microplate, the absorbances measured at 480 nm and the NO values determined using the standard curve. Since the first amount of liver and spleen tissue were respectively, 0.2 and 0.1 g, to determine NO concentrations in liver and spleen in terms of micromolar per gram (µM g^{-1}) weight, NO concentration were calculated by multiplication in 5 and 10 times, respectively. Nitrogen oxide concentrations were expressed as micromolar per milliliter (µM mL^{-1}) (in plasma) and micromolar per gram (µM g^{-1}) wet weight of tissue (in liver/spleen suspensions).

Statistical analysis: Values for NO are presented as the Mean±SEM for group samples. The significance of differences was determined by Analysis of Variances (ANOVA) and Student's t-test using Graph Pad Prism Software (Graph Pad, San Diego, California, USA). A minimum $p<0.05$ was considered statistically significant.

RESULTS
Effects of LPS and DEX on parasitaemia: Although, DEX as inhibitor decreased the parasitaemia in comparison to LPS and control groups, however, no significant results were observed (Fig. 1).
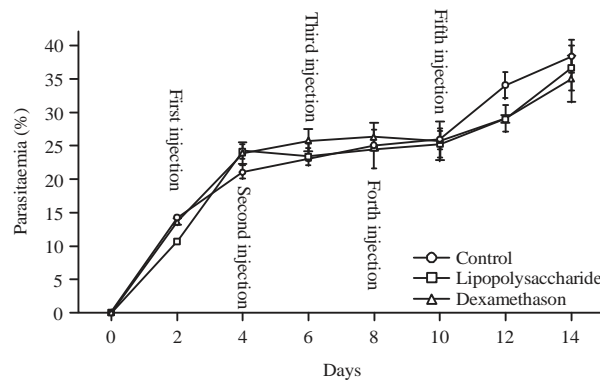

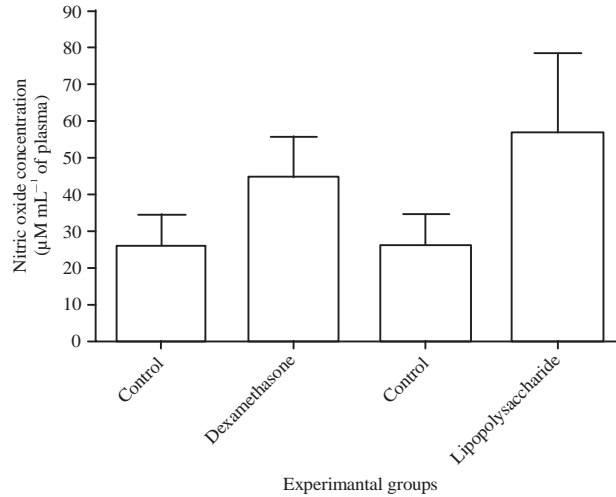Fig. 1: Percentage of parasitaemia after treatment with DEX and LPS

Fig. 2: Amount of NO in the plasma after treatment with DEX and LPS
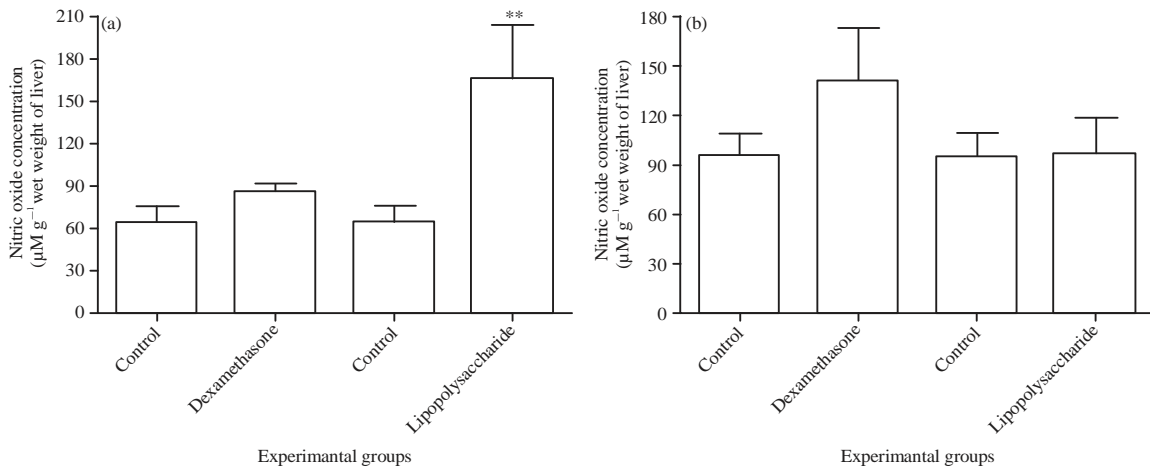

Fig. 3(a-b): Nitric oxide levels in suspensions of (a) Liver and (b) Spleen after treatment with DEX and LPS

Evaluation of NO in plasma, liver and spleen suspensions: No significant difference in NO concentration was observed between the control and tests groups in plasma. An increase in NO level with significant difference was associated with liver test group after LPS treatment. Moreover, no significant difference was found between the control and test groups in spleen (Fig. 2 and 3).

Hepato/splenomegaly: Data of whole liver and spleen weight in both control and tests groups to assess the hepato/splenomegaly obtained and were expressed as gram. No significant difference in hepato/splenomegaly values were not found between the control and test groups (Fig. 4).

DISCUSSION

In this study partially, LPS induced and DEX reduced immune responses in mice during malaria infection by alterations and manipulation of immunobiochemical factors via NO
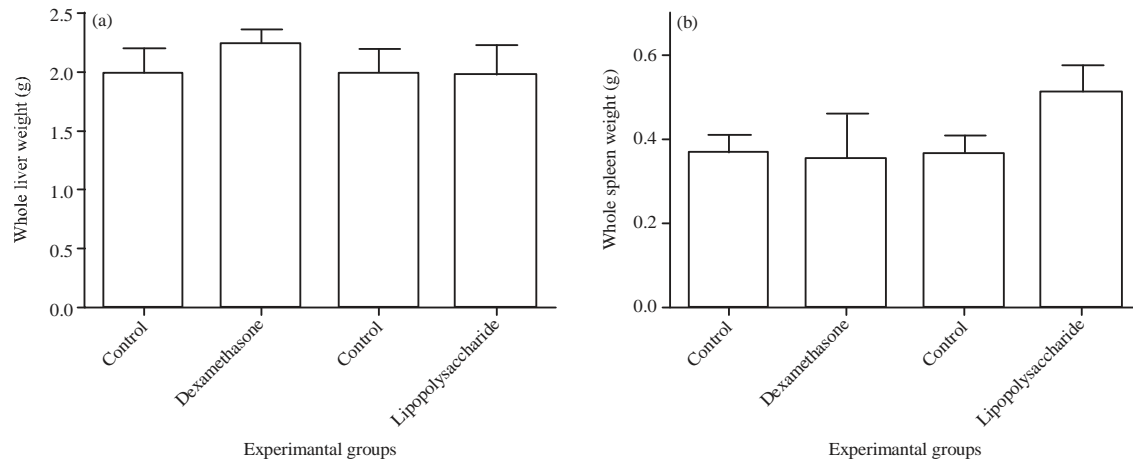
Fig. 4(a-b): Assessment of (a) Hepatomegaly and (b) Splenomegaly after treatment with DEX and LPS

pathway. The LPS stimulation increased NO and DEX stimulation decreased NO activity in NMRI mice.

There are several stimuli were reported to activate the immune system, especially to induce cytokines and NO (Gharavi *et al.*, 2011). Nitrogen oxide has complex and diverse functions in physiological and pathophysiological phenomena; accumulated evidence suggests that NO and oxygen radicals such as superoxide are key molecules in the pathogenesis of various infectious diseases (Akaki *et al.*, 2000). Studies have shown that severe cases of malaria are accompanied by increased iNOS activity and the subsequent production of NO (Bryk *et al.*, 2000). Increased levels of NO were found to be beneficial, as NO was shown to kill parasites (Gyan *et al.*, 1994; Rockett *et al.*, 1991). The LPS are believed to be potent inducers of macrophage ROI and RNI against a variety of intracellular and extracellular pathogens and to be potent stimulators of NOS isoforms (Nahrevanian, 2006). The synthetic glucocorticoid DEX has been reported to inhibit iNOS induction, which may contribute to its inflammation-reducing effects (Orazizadeh *et al.*, 2010). Najafi *et al.* (2015) used the same LPS to stimulate the immune system in inbred balb/c mice and they found that LPS stimulates the immune system did not increase NO levels in this species of mice (Najafi *et al.*, 2015). Nahrevanian (2006) applied the same LPS to stimulate the immune system in outbred NMRI mice and they observed that LPS stimulates the immune system and increase NO levels in this species of mice (Nahrevanian *et al.*, 2005). Scheller *et al.* (1997) and Klotz *et al.* (1995) used DEX in several studies as a specific inhibitor of the immune system by reducing of cellular and humoral defense in preventing various diseases (Klotz *et al.*, 1995; Scheller *et al.*, 1997).

CONCLUSION

It is concluded that LPS and DEX can be applied as a standard and medically approved inducer and inhibitor of the immune system, respectively, for experimental immunomodulation studies in animal models. This may lead to an immunotherapy trial of malaria by control the pathophysiology and degree of parasitaemia in mouse model. It can be said that the level of

immune response in mice depends on the genetic profile of hosts for NO induction and malaria tolerance (Souza *et al.*, 2012).

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