Trace Elements and Anti-Oxidant Status in Gravid BALB/c Mice Infected with Plasmodium yoelii Malaria Parasites at Different Gestational Periods

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Abstract: There are extensive literature on the adverse effects of malaria parasites on pregnancy outcomes, but the basis of this observation is un-conclusive. We investigated the effects of Plasmodium yoelii malaria on the levels of trace elements and total antioxidants in gravid BALB/c mice infected with Plasmodium yoelii at first (early), second (middle) and third (late) trimesters. The mice were grouped into, viz: Gravid mice infected at day 3-post plug (I3PP) representing 1st trimester, gravid mice infected at day 7 post plug (I7PP) representing 2nd trimester, gravid mice infected at day 11-post plug (I11PP) representing 3rd trimester, gravid not infected (GNI), infected non-gravid (ING) and non-gravid non-infected mice (NGNI). The highest % parasitemia was observed in the group of mice infected at 3rd trimester. None of the mice infected at 1st and 2nd trimesters carried the pregnancy to term. The % PCV was lowest in gravid mice infected in 1st trimester compared with other gravid infected mice. At day 5 and day 9 post infection, the levels of Mn, Mg, Zn, total antioxidants, Cu, Fe and Se were raised in all gravid infected mice compared with non-gravid non-infected mice. It may be concluded from this study that P. yoelii malaria is most severe in mice infected at the early stage of gravidity (1st trimester) and that raised levels of trace elements and anti-oxidants in P. yoelii infected pregnant mice could be a result of oxidative stress and free radical burden.

Key words: Trace elements, antioxidants, gravid mice, P. yoelii, malaria and packed cell volume

Introduction
Malaria is a complex disease that varies widely in epidemiology and clinical manifestations in the different parts of the world. In spite of enormous effort to control it, malaria still represents one of the most important parasitic diseases of human worldwide. An estimated population of 700,000 - 2.7 million people die of malaria each year, 75% of them are African children (WHO, 2000). Investigators have provided evidence that cell-mediated and humoral immunity act in concert or sequentially to control and clear a blood-stage malaria infection (Langhorne, 1989). The early role of natural killer cells, gamma delta T-cells and IFN-gamma is essential in the development of protective immunity to the blood stage of Plasmodium infection (Hasib et al., 2000).

Pregnant women are more at risk of Plasmodium infection (Shulman, 2001) and this was related to hormonal changes and parasite sequestrations in the placenta (Serghides and kain, 2001), but the role of antioxidants are yet to be provided. Deficiencies of trace elements like zinc, copper and magnesium have been implicated in various reproductive events like infertility, pregnancy wastage, congenital anomalies, pregnancy induced hypertension, placental abruption, premature rupture of membranes, still births and low birth weight (Pathak and Kapil, 2004). Moreso, antioxidants and trace elements have also been shown to influence host cellular and humoral immunological functions, which are important in the host of Plasmodia infection (Lukasewycz and Prohaska, 1990; Spalholz et al., 1990). The immune system utilizes these essential factors to meet the demands of challenges by infectious agents (Spalholz et al., 1990; Sherman, 1990).

Pregnancy is said to be an immunological balancing act in which the mother's immune system has to remain tolerant to the foetus and yet maintain immune competence for defense against microorganisms. Several studies have documented a decrease of cell-mediated immune responses to malaria parasite antigens during pregnancy (Rasheed et al., 1993, Riche et al., 2000) and early post-partum (Diagne et al., 2000). Studies have shown that oxidative stress (which can be induced by parasitic infection) has been proposed as a key factor involved in the development of a complication of pregnancy (pre-eclampsia) and this was prevented or delayed by antioxidant supplement (Black et al., 2003). An association was established between a reduction in the risk of having a small-for-gestational-age baby and antioxidants supplementation (Rumbold et al., 2006). None of the studies mentioned above discussed the levels of antioxidants in pregnant subjects with malaria infection.

Pregnancy and malaria infection are common in all malaria endemic zones of the world. A related study (Arinola et al., 2004a) showed that resistant C57BL/6
mice infected with *L. major* at different gestational periods had most pronounced adverse pregant-outcome when infected at late gestation (3rd trimester). With dearth of knowledge about antioxidants status and trace elements in pregnant subjects infected with malaria at different gestations, this study is designed to bridge this gap in knowledge.

**Materials and Methods**

**Malaria parasite:** *P. yoelii* was provided by Malaria Research and Reference Repository Center (MR4) of 10801 University Boulevard, Manassas, Virginia, United States of America (Batch number: S0594596).

**Mice and mating:** Sixty-four female BALB/c mice of 8 -12 weeks old (15g-20g each) were used for this study. They consisted of 48 females and 16 males. The mice were divided into 16 groups, having 3 females and 1 male in a cage for mating overnight. The females were separated from the males on the following morning after the location of vaginal plug. The presence of vaginal plug was an indication of conception in mice. The mice were fed with mice pellets produced by Ladokun Feeds Limited, Ibadan, Nigeria and water was given *ad libitum* throughout the period of study.

**Inoculation of mice and preparation of thin blood films:** About 1.5ml - 2.0ml of the malaria parasite inoculum was injected intravenously into the mice through the tail vein using insulin syringe of 28 gauge needle. Thin blood film was prepared on a microscope slide, dried, fix with 96% methanol and stain with Giemsa stain solution.

**Determination of percentage (%) parasitaemia:** *P. yoelii* parasitized and non-parasitized RBC and reticulocytes were identified under the microscope using a high magnification with oil immersion. At least a total of 1000 cells were counted from the blood film to calculate the percentage parasitaemia.

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\text{Calculation} = \frac{\text{Number of parasitized RBC + parasitized reticulocytes}}{\text{Number of unparasitised RBC + unparasitized reticulocytes}} \times 100\%
\]

**Determination of packed cell volume (PCV):** Blood was carefully collected by tail tip amputation directly into a heparinized capillary tube. The capillary tube was spun for five minutes using haematocrit centrifuge that separated plasma and packed cells. The percentage of the packed cells was calculated using a haematocrit reader.

**Collection of blood samples for analyses:** The blood was collected by cardiac puncture into a sterile syringe containing 0.1ml of ACD solution, transferred into EDTA bottle, spun for plasma collection and the plasma was stored -20°C till required for analysis. Three mice were sacrificed in each group at day 5 (D5) and day 9 (D9) post infection.

**Determination of total antioxidants:** This was based on the method described (Arinola et al., 2004b). A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton -type reaction, leading to the formation of hydroxyl radicals. These reactive oxygen species degrade benzoate, resulting in the release of thiobarbituric acid reactive substances (TBARS). The rate of inhibition of colour development is proportional to the concentration of antioxidative activity.

**Determination of plasma trace elements:** Plasma trace elements were determined with Flame Atomic Absorption Spectrophotometer (AAS) using a direct method (Arinola et al., 2004b). The atoms of the element, when aspirated into the AAS vaporized and absorbed light of the same wavelength as emitted by the element when in the excited state.

**Results**

The % parasitemia in all groups of mice increased daily post-infection with *P. yoelii*. The highest % parasitemia was observed in mice infected at 3rd trimester (IIIIP group of mice) (Fig. 1). The % PCV decreased with increasing days of infection and as gestation advances. The least % PCV was observed in infected but non-gravid group of mice and the highest % PCV was observed in gravid non-infected mice. The % PCV were higher in all groups of gravid infected mice compared with non-gravid non-infected mice. Among gravid infected mice, the % PCV was lowest in group of mice infected at 1st trimester (Fig. 2).

At day 5 post infections (D5), the levels of total antioxidants, Cu, Mn, Mg, Fe and Se were raised in all groups of gravid infected mice compared with non-gravid non-infected mice. At day 9 post infections (D6), the levels of Mn, Mg, Zn, total antioxidants, Cu, Fe and Se were raised in all groups of gravid infected mice compared with non-gravid non-infected mice. The levels of Cu, Zn, Fe, Se and total antioxidants were reduced in all groups of mice at D5 post infection compared with D9 post infection (Fig. 3a-3f and Fig. 4).

**Discussion**

Malaria infection during pregnancy is known to be a major public health problem in the tropical and subtropical regions of the world (WHO, 2003). Studies have shown that pregnant women are particularly vulnerable to malaria infection causing severe anemia and death. For the unborn child, maternal malaria
Fig. 1: The course of *P. yoelii* infection in gravid and non-gravid mice.

Fig. 2: The % PCV of gravid and non-gravid mice.

Key for Fig. 1 and 2: NGI = Non gravid infected, 13PP = gravid mice infected at day 3 post plug, 17PP = gravid mice infected at day 7 post plug, 111PP = gravid mice infected at day 11 post plug, PP = Pregnant non-infected.

increases the risk of spontaneous abortion, stillbirth, premature delivery and low birth weight (WHO, 2003). Several studies have documented a decrease of cell-mediated immune responses of pregnant women to malaria antigens (Riche et al., 2000; Diagne et al., 2000; Meeusen et al., 2001). Moreover, direct association had been established between antioxidants, trace elements and cell-mediated immunity. Deficiencies of trace elements do have negative impacts on immune functions, numbers of immunocompetent cells and functions of organs of immune system (Spallholz et al., 1990; Sherman 1990). To the knowledge of the authors, no study had determined the levels of trace elements and antioxidants in pregnant women infected with malaria parasite at different gestations. Such study is not possible in humans because the exact Day 1 of *Plasmodium* parasite infection cannot be ascertain in humans.

An increase in percentage parasitemia as infection progresses was observed in all the groups of mice (either gravid or not). In gravid mice, the severity of malaria infection was highest in mice infected at third trimester (111PP) followed by those infected at first trimester (13PP). Death of mice infected with *P. yoelii* in first trimester (13PP) started on Day 7 post infection as compared with Day 15 post infection in mice infected at second trimester (17PP). Also, none of the 13PP or 17PP group of mice was able to carry the pregnancy to term. This implies that the effect of malaria infection is most severe when contacted at 1st of pregnancy. Similarly, human study carried out in Zimbabwe reported that women are more susceptible to malaria infection was observed during first pregnancy (Harrison and Ibeziako, 1973). Other adverse consequences of malaria infection during pregnancy are low birth weight, intra-uterine and perinatal mortality (Steketee et al., 2001). Low birth weight and loss of pregnancy were also observed in this study.

Animal studies using mice model (Arinola et al., 2004) showed that immune response in the early stage (1-6 days post plug) of pregnancy is biased towards Th 1 (cell-mediated) immunity. The immune response at the late stage (15 - 21 days post plug) is predominantly Th 2 (antibody) mediated immunity. The early stage of malaria parasitemia is cleared from blood circulation by Th 1 immune responses while the late stage is controlled by Th 2 responses (Hasib et al., 2000). It is therefore not surprising that malaria parasitemia is highest in the group of mice infected with *P. yoelii* at late stage of gravidity, when humoral immunity is predominant.

In this study, % PCV was found to decrease with advances in *P. yoelii* infection and gestational periods, but a more noticeable decrease was observed in infected non-gravid mice compared with other groups of mice. Among the pregnant mice, the % PCV decreased mostly in mice infected during first trimester (13PP) compared with those infected in 2nd trimester (17PP) and 3rd trimester (111PP). The implication is that anaemia is most severe in pregnant women infected with malaria parasite at first period of gestation. This is in agreement with previous observation that malaria infection is associated with increased risk of severe anaemia (Menendez et al., 2000).

Reduced % PCV in malaria is caused by RBC haemolysis of parasitized RBC. Higher PCV in infected gravid mice or non-infected gravid mice compared with PCV in infected non-gravid mice may be due to raised number of different blood cells during pregnancy. Previous studies in humans show increased numbers of circulating blood cells in pregnant women (Arinola et al., 2004c). Increases in RBC haemolysis were reported in pregnant humans as a result of *Plasmodium* infection, thus a cause of low PCV. Higher % PCV in gravid infected mice compared with non-gravid infected mice may be a result of increase in the number of RBC of gravid mice that compensated for haemolysed RBC due to *P. yoelii* infection.
membranes, stillbirths and low birth weight (Pathak and Kapil, 2004). The mean levels of Zn, Cu, Se and Fe were highest in mice that were both gravid and infected compared with non-gravid non-infected group. This suggests the production of these trace elements during Trace elements are essential in the maturation, activation and functions of host defense mechanism

Key for Fig 3a, b, c, d and f: NGNI = Non gravid infected, 13PP = gravid mice infected at day 3 - post plug, 17PP = gravid mice infected at day 7 post plug, 111PP = gravid mice infected at day. (Bendich 1990; Romero 2003). Deficiencies of trace elements have been documented to play crucial roles in various reproductive events like infertility, pregnancy
Arinola et al.: Malaria Parasites at Different Gestational Periods

Fig. 4: The plasma level of total antioxidants in gravid and non-gravid mice with or without *P. yoelii* parasitemia.

Key for Fig. 4: NGI=Non gravid infected, 13PP=gravid mice infected at day 3 post plug, 17PP=gravid mice infected at day 7 post plug, 111PP=gravid mice infected at day 11 post plug, PP=Post plug, NGNI=Non gravid non infected.

loss, congenital anomalies, pregnancy induced hypertension, placental abruption, premature rupture of pregnancy, and *P. yoelii* malaria, though the mechanism of production may be different. Damage of RBC by the developmental stages of malaria parasite leads to leakage of Zn and Fe to blood circulation. This will thus increase the plasma levels. Another mechanism for raised level of certain trace elements and antioxidants in *P. yoelii* infection is induction of oxidative stress by malaria parasites as previously found by various authors (Langhorn, 1989; Hasib, 2000). Destruction of malaria parasites by WBCs through oxygen dependent mechanism and engulfing of Kupffer cells by larval stage of malaria parasites induces oxidative stress. This will require antioxidants for removal, thus the need for increased production of antioxidants. Moreso, Cu, Fe, Se, Mn and Zn are integral part of enzymatic antioxidants, hence the increases of these metals during *P. yoelii* infection. Trace elements and antioxidants were low in pregnant mice without *P. yoelii* infection compared with non-pregnant ones; this may be related to haemolysis due to oedema.

The mean levels of the trace elements (Zn, Mn, Cu and Se) were lower in mice infected at first trimester compared with those infected in second and third trimesters. This implies that adverse effects of malaria on levels of trace elements during pregnancy commences early in pregnancy, thus causing reduced cell-mediated immunity (especially lymphocyte and neutrophil functions) necessary for malaria parasite clearance.

The levels of total antioxidants were reduced in pregnant mice infected with *P. yoelii* as parasitemia or gestation increases. Placental macrophages in the presence of infections are sources of nitric oxide (NO), tumor necrosis factor-alpha and other cytokines that induce mitochondrial alterations and the production of free radicals (Romero, 2003). Based on this, it may be inferred that *P. yoelii* malaria in gravid mice could cause increased generation of free radicals thereby leading to excessive production of antioxidants and relevant trace elements as observed in gravid infected mice.

It may be concluded from this study that infection with malaria parasites during pregnancy leads to raised plasma trace elements and total antioxidants as a result of free radical generation.

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