



Journal of Medical Sciences

ISSN 1682-4474

science
alert

ANSI*net*
an open access publisher
<http://ansinet.com>

JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued six times per year on paper and in electronic format.

For further information about this article or if you need reprints, please contact:

Dr. R. Hemalatha
Department of Microbiology,
National Institute of Nutrition,
Jamai-Osmania,
Hyderabad-500 007, India

Tel: 27008921
Fax: 27019074

Cytokines and Micronutrients in *Plasmodium vivax* Infection

¹L. Singotamu, ²R. Hemalatha, ¹P. Madhusudhanachary
and ³M. Seshacharyulu

T helper subset response to *P. falciparum* is well documented, however there is little or no information with respect to *P. vivax*, though it is associated with high rate of morbidity and hospitalisation. In the present study circulating IL-2, IFN γ , IL12, IL10, IL-4 and TNF α concentrations were investigated in patients with mild and severe *P. vivax* infection. Hemoglobin status, Packed Cell Volume (PCV), serum zinc and vitamin A were also evaluated. Hemoglobin concentration was low, as expected and further decreased significantly in 1 week of malaria and was negatively correlated with TNF α , suggesting a role for this cytokine in the pathogenesis of anemia and destruction of RBCs. The initial level of TNF α was significantly correlated with IL10 concentration (regression analysis) thus indicating an anti-inflammatory role for IL10 in the regulation of TNF α . Initial concentration of IL2, IL12 and IL10 were higher in the mild malaria and were associated with low parasite density. In contrast, high concentration of IFN γ was associated with low IL2 levels in severe malaria, suggesting that in severe malaria there is an inability to mount a TH1 response (IL-2) and to maintain an adequate balance of Th1/Th2 response. Both serum zinc and vitamin A were low in mild malaria, however, with increasing severity, serum zinc concentrations increased which could be an adaptive response to potentiate the immune response as reflected by the increase in IFN γ and TNF α . Though there was no significant correlation, higher vitamin A levels were associated with low IL-2 levels implicating vitamin A in down regulating Th1 response.

Key words: IFN γ , IL-2, cytokines, *P. vivax*, micronutrients

INTRODUCTION

Malaria is one of the leading causes of morbidity and mortality worldwide, with an estimated 350 to 500 million new cases each year (World Health Organization, 1997). Of the four species, *P. vivax* has the widest geographic distribution through out the world (Peter, 2000). In India, *P. vivax* is the predominant species, with 70% of the infection due to *P. vivax*, while *P. falciparum* contributes to about 25 to 30%. Hyper parasitemia, severe anemia and cerebral malaria are some of the complications associated with *Plasmodium falciparum* (Mossmann and Coffman, 1989). Despite its wide reputation as the benign parasite, *P. vivax* is nevertheless associated with severe complication and death. Relapses due to *P. vivax* can occur even after 3 to 5 years and may cause death as a result of high parasitemia (2%) after anemia or ruptured spleen and thrombocytopenia or rarely cerebral malaria (Peter, 2000).

Immune response to malaria has been variously described as protective or immune pathogenic (Mossmann and Coffman, 1989; Luty *et al.*, 1999; Stefen *et al.*, 1998). T helper subset response to infections is mediated by Th1 and Th2 cytokines. While Th1 response is associated with up regulation of IL-2 and IFN γ that promotes cell mediated immunity (Mossmann and Coffman, 1989). Th2 response is associated with IL-4, IL-5 and IL-10 and supports antibody mediated immunity. In *P. falciparum* Th1 response has been shown to be protective, particularly IFN γ has been suggested to play a key role in limiting progression from uncomplicated malaria to severe and life threatening complications (Luty *et al.*, 1999; Stefen *et al.*, 1998). Correlates of immunity to *P. vivax* infection are not known though it is associated with high rate of morbidity and hospitalization.

Malaria specific metabolite hemozoin mediates the release of several potent endogenous pyrogens (TNF, MIP-1 α and MIP-1 β) *in vitro* and altered thermoregulation *in vivo*. Knowledge on protective immune response assumes importance in the context of development of novel treatment and intervention strategies to infectious diseases. Identification of cytokines that promote protective immune response may have potential as an adjuvant in vaccines (Sherry *et al.*, 1995; Singotamu, 1996).

The objective of the present study was to study the T helper subset response to *P. vivax*, as it is associated with high rate of morbidity and hospitalization. and also to study the effect of *P. vivax* infection on vitamin A and zinc status, which is known to be altered in infections (Villamor *et al.*, 2002; Zinc Against Plasmodium Study Group, 2002) and their associations with the T helper

subset. Therefore in the present study, circulating IL-2, IFN γ , IL12, IL10, IL-4 and TNF α concentrations were investigated in patients with *P. vivax* infection both in mild and severe conditions. Serum zinc and vitamin A were also measured. As IL12 and IL10 are known to be regulatory cytokines and TNF α is suggested to be associated with anemia in malaria, these three cytokines were determined again in the same patients 1 week after the infection.

MATERIALS AND METHODS

The present study was carried out at National Institute of Nutrition, Hyderabad, India. The Institute's Scientific Advisory Committee approved the study. Patients attending the outpatients clinic, Hospital for tropical diseases, Hyderabad, during the months of August and September, who were diagnosed to be suffering from malaria on the basis of peripheral blood smear examination, aged between 20 to 40 years were enrolled for the study. Patients recruited for the study were classified into 2 groups based on the density of parasites in blood. Patients with 1-2 parasites in occasional High Power Field (HPF) and those with more than 2-10 parasites in all HPF were classified as mild/moderate and those with high density of parasitemia with poor clinical conditions (vomiting, dehydration and temperature, $>39^{\circ}\text{C}$) were classified as severe. Finally, there were 31 mild and 16 severe cases of malaria.

Ten apparently normal subjects were selected randomly for the control group. Patients who received recent antimalarial treatment and those with other systemic infections were excluded from the study. After collecting a venous blood sample, the patients received treatment as outpatients with sulfadoxin and pyrimethamin. Venous blood samples from each were collected for the determination of hemoglobin, packed cell volume, serum vitamin A and zinc and plasma cytokines.

Zinc and vitamin A: Hemoglobin was determined by cyanmethemoglobin method (Dacie and Lewis, 1985). Serum zinc was determined by atomic absorption spectrophotometry (Butrimoritz and Purdy, 1977) and vitamin A was assayed by HPLC (JG Bieri *et al.*, 1979).

Cytokine measurement: Plasma concentrations of IL-2, IFN γ , IL12, IL10, IL-4 and TNF α were determined by quantitative sandwich ELISA technique with commercially available kits (Diaclone) (Bhaskaram *et al.*, 2003). The kits provided, precoated plates with specific monoclonal capture antibody and polyclonal conjugate

antibody specific for IL-2, IFN γ , IL12, IL10, IL-4 and TNF α as detection antibody. Sensitivity of detection for the cytokine assay was as follows: IL-2, 5 pg mL $^{-1}$; IFN γ , 5 pg mL $^{-1}$ and IL-4 0.5 pg mL $^{-1}$.

Log transformed data were compared between groups using students t-test. When data remained skewed after log transformation the Mann Whitney U-test was used.

RESULTS

Mean age of patients was similar and mean number of days the patients had pyrexia before collection of blood was similar in mild/moderate (4.2 \pm 0.5 day) and severe (4.4 \pm 0.4 day) malaria. Mean initial hemoglobin concentration was low (12.23 \pm 0.01 g dL $^{-1}$) in both mild and severe malaria and decreased further to 10.84 and 10.63 g dL $^{-1}$, respectively after 1 week. Packed Cell Volume (PCV) percent showed a similar pattern.

IL2, IL4 and IFN γ , Serum IL-2, IFN γ and IL-4 ranged from 5.8 to 60.8 pg mL $^{-1}$, 15.5 to 1910 pg mL $^{-1}$ and 0.18 to 13.5 pg mL $^{-1}$, respectively in patients, while all the three cytokines were below detectable range in normal controls. The mean concentration of IL2 was higher in mild malaria, however this was not statistically significant, IFN γ was significantly higher in severe malaria (Table 1). IL4 was higher in severe malaria, however it was not statistically significant. In addition, the ratio of IFN γ Vs IL-4 was significantly greater in severe malaria (p<0.025, ANOVA).

IL12, IL10 and TNF α : The IL12 ranged from 85.2 to 464 pg mL $^{-1}$ and the mean was 289.5 \pm 67.94 pg mL $^{-1}$ in mild malaria and was comparable to severe malaria (240.2 \pm 46.24). After one week, with the clearance of parasites, the IL12 decreased by fifty percent in the mild malaria, while same level was maintained in the severe malaria.

The initial plasma level of IL10 was 452.7 \pm 334.33 in the mild malaria that was significantly higher than in the severe malaria (279.1 \pm 121.8). However after 1 week there was a 50% increase in IL10 in the severe malaria while it remained the same in mild malaria. The plasma level of TNF α was 32.6 \pm 3.65 pg mL $^{-1}$, which was maintained even after 1 week in the mild malaria. However, TNF α increased by 3 folds from the initial value of 44.3 \pm 19.365 pg mL $^{-1}$ in the severe malaria (Fig. 1).

Table 1: Cytokine profile in *P. vivax* infection

Cytokines (pg mL $^{-1}$)	<i>P. vivax</i> infection	
	Mild	Severe
IL-2	16.9 \pm 5.19	11.6 \pm 3.33
IFN γ	177.3 \pm 40.98	619.7 \pm 230.7*
IL4	2.4 \pm 1.0	3.8 \pm 1.99
IL-12	289.5 \pm 67.94	240.2 \pm 46.24
IL-10	452.76 \pm 334.33	279.1 \pm 121.8*
TNF α	32.64 \pm 3.65	44.3 \pm 19.3

Values are mean \pm SE * p<0.05

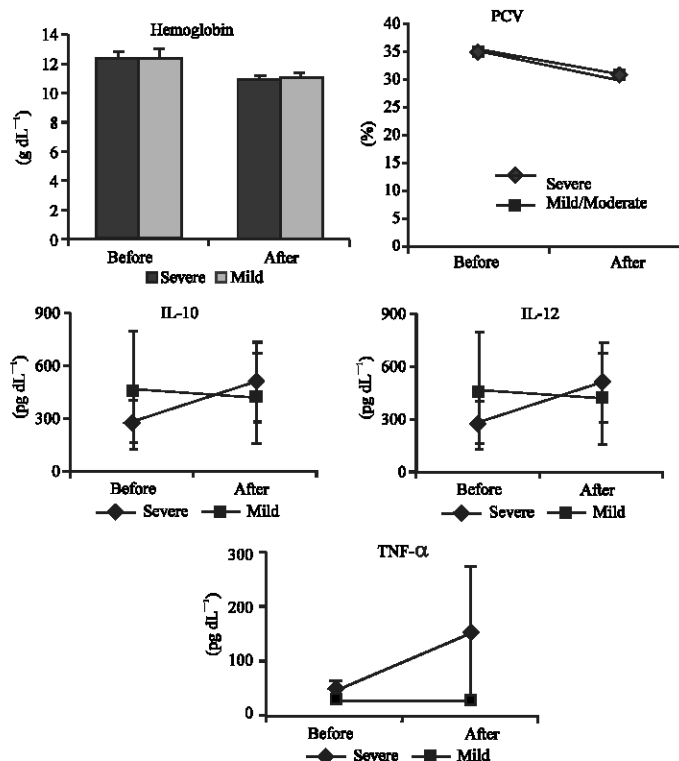


Fig. 1: Hemoglobin, PCV, IL-10, IL-12 and TNF α concentrations 1 week before and after mild and severe malaria

Table 2: *P. vivax* infection and micronutrient status

Micronutrients ($\mu\text{g dL}^{-1}$)	<i>P. vivax</i> infection	
	Mild	Severe
Zn	59.09 \pm 2.73	68.83 \pm 8.88*
Vitamin A	25.10 \pm 3.52	21.24 \pm 5.01

Values are Mean \pm SE *p<0.05

Micronutrients: Serum vitamin A and zinc was done in a sub sample of 36 patients. Zinc ranged from 40 to 100 $\mu\text{g dL}^{-1}$ with a mean \pm SE of 63.8 \pm 2.6 $\mu\text{g dL}^{-1}$, which was much less than the reported normal range (70-120 $\mu\text{g dL}^{-1}$). Out of the 36 patients, 14 had equal to or more than 70 $\mu\text{g dL}^{-1}$ and the rest (22) had low concentration (<70 $\mu\text{g dL}^{-1}$) of zinc (Table 2). Serum vitamin A concentration ranged from 8 to 55 $\mu\text{g dL}^{-1}$ and was less than the observed range (20 to 70 $\mu\text{g dL}^{-1}$) in normal adults. The total mean \pm SE of vitamin A was 29.3 \pm 2.41 $\mu\text{g dL}^{-1}$. Of the 36 patients, 25 had normal vitamin A (\geq 20 $\mu\text{g dL}^{-1}$), While 11 had less than 20 $\mu\text{g dL}^{-1}$. Vitamin A levels did not correlate with severity of malaria.

DISCUSSION

The T cells play a central role in the regulation of immune response and formation of immunologic memory, which has a critical role in control and elimination of established infection. Ever since the T helper subsets with distinct cytokine profile have been described, many researchers have investigated the role of Th1 and Th2 cytokines in various infectious diseases (Mossmann and Coffman, 1989; Stefen *et al.*, 1998; Barnes *et al.*, 1993). It is an established fact that nutrition is a critical determinant of the outcome of host microbe interactions through modulation of the immune response (Bhaskaram, 2001). In malaria infection, *P. falciparum* received the widest attention, with some studies showing a key role for IFN γ and IL12 in the protection from *P. falciparum* infection (Luty *et al.*, 1999; Stefen *et al.*, 1998). Elimination of intracellular pathogens like malarial parasites requires proinflammatory cytokines, such as IL-2, IFN γ and IL-12 etc. that mediate cellular immunity.

In the present study the initial concentration of IL2, IL12 and IL10 were higher in the mild malaria and were associated with low parasite density, suggesting a critical role for IL2 and a fine balance between IL12 and IL10. In contrast, high concentration of IFN γ was associated with low IL2 levels in severe malaria. Low IL2 levels suggests diminished activation of TH 1 cells and further suggests other cell types, such as NK cells (Lilic *et al.*, 1997) and CD8 cells (Mackan and Rosen, 2001) to be the principle sources of IFN γ in those with severe malaria in the

present study. IL2 cytokine has an important role in differentiation and proliferation of T helper and T cytotoxic cells, which have a central role in antigen specific elimination of infected cells. Present study does not support a direct involvement of interleukin-4 (IL-4) in the clearance of *P. falciparum* parasites.

In 1 week of parasitemia, in both mild and severe, there is significant decrease in hemoglobin and PCV. There is a significant increase in IL-10, TNF α , with a slight increase in IL-12 in severe malaria. High density of parasites may lead to increased stimulation of monocytes/macrophages, which is reflected in increased secretion of IL10 and TNF α in the severe malaria after 1 week of parasitemia.

In case of 1 week after mild malaria, there is no change in IL-10 and TNF α and there is a significant decrease in IL-12 concentration which is expected from a decreased load of parasitemia.

With increased parasite load in severe malaria, there is no concomitant increased production of IL-12. It may be envisaged that this inability to mount sufficient IL-12 response may have resulted in poor clearance of the parasites. High parasite load in severe malaria might have stimulated the monocyte/macrophage system resulting in the increased production of IFN- γ . It is reported that a more favorable outcome in animal models of malaria has been associated with increased production of IFN- γ . (Jacobs *et al.*, 1996; Sedegah *et al.*, 1994) and treatment of lethal murine *P. vinckei* infection with IFN- γ enhanced the effect of antimalarial treatment (Kremsner *et al.*, 1991). The increase in IL-12, TNF- α (Th1 response) and IL-10 (Th2 response) after 1 week of severe parasitemia, reflects an adaptive host defense response to increased parasitemia. In mice, the presence of TNF- α and IFN γ are both important early in infection for good granuloma formation and control of infection (Flynn *et al.*, 1995; Caruso *et al.*, 1999).

Thus from our study we may conclude that in severe malaria there is an inability to mount a TH1 response (IL-2) and to maintain an adequate balance of Th1/Th2 response.

Hemoglobin concentration was low in both mild and severe malaria as expected and decreased significantly in 1 week after infection. Furthermore the low hemoglobin concentration was negatively correlated with TNF α , suggesting a role for this cytokine in the pathogenesis of anemia and destruction of RBCs, a finding in agreement with the well-known tissue damaging effects of TNF α . The initial level of TNF α was significantly correlated with IL10 concentration (regression analysis) thus indicating an anti-inflammatory role for IL10 in the regulation of TNF α .

Both serum zinc and vitamin A were low in *P. vivax* infection in the present study, which could be a response to acute phase, however, with increasing severity, serum zinc concentrations increased. Zinc is essential for proliferation and differentiation of immune cells and for various lymphocyte functions implicated in resistance to malaria, including production of IgG, IFN γ and TNF α . Thus the high concentration of zinc seen in malaria in this study could be an adaptive response to potentiate the immune response as reflected by the increase in IFN γ and TNF α .

Though there was no significant correlation, higher vitamin A levels were associated with low IL-2 levels, a finding, in line with our earlier observation in children with acute respiratory infection (unpublished). The findings of this study are also consistent with previous reports that have shown that vitamin A down regulates Th1 response. Vitamin A deficient mice were shown to produce high concentration of IFN- γ and IL12, which are Th1 cytokines that promote the secretion of IL2 cytokine (Carman *et al.*, 1992). In conformity, high dietary vitamin A in mice showed down regulation of Th1 response (Cui *et al.*, 2000)

However, recent studies suggest that micronutrients such as zinc and vitamin A may reduce *P. falciparum* morbidity through immune modulation or by altering oxidative stress (Villamor *et al.*, 2002; Zinc Against Plasmodium Study Group, 2002). Low concentration of serum zinc and reduced morbidity with zinc supplementation have been described with *P. falciparum* infection, however reduction in morbidity was not associated with zinc supplementation in *P. vivax* infection (Shanker *et al.*, 2000). Vitamin A and zinc are important immunomodulators and are critical in determining the outcome of infectious disease (Munoz *et al.*, 1995). However, while the role of zinc has been well defined as protective for diarrhoeal diseases, the precise role of zinc and vitamin A in malaria needs to be explored.

ACKNOWLEDGMENTS

The authors acknowledge the skillful assistance rendered by the nursing staff; Promodini, BV Nancharamma and Santhosamma in sample collection.

REFERENCES

- Barnes, P.F., L.U. Shuzhuang, J.S. Abrams, E. Wang and M. Yamamura, 1993. Cytokine production at the site of disease in human tuberculosis. *Infect. Immunol.*, 61: 3482-3489.
- Bhaskaram, P., 2001. Immunobiology of mild micronutrient deficiencies. *Br. J. Nutr.*, 85: 75-80.
- Bhaskaram, P., R. Hemalatha and B.N. Goud, 2003. Expression of messenger ribonucleic acid and production of cytokines in children with malnutrition. *Nutr. Res.*, 23: 367-376.
- Bieri, G., T.J. Tolliver and G.L. Catignani, 1979. Simultaneous determination of alpha-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am. J. Clin. Nutr.*, 32: 2143-2149.
- Butrimoritz, G.P. and W.C. Purdy, 1977. The determination of Zinc in blood/plasma by atomic absorption spectrophotometry. *Ann. Clin. Acta*, 94: 63-73.
- Carman, J.A., L. Pond and Nashold *et al.*, 1992. Immunity to trichinella spiralis infection in vitamin A deficient mice. *J. Exp. Med.*, 175: 111-120.
- Caruso, A., N. Serbina, E. Klein, K. Triebold, B. Bloom, and J. Flynn, 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J. Immunol.*, 162: 5407-5416.
- Cui, D., Z. Moldoveanu and C.B. Stephensen, 2000. High level dietary vitamins A enhances T-helper type 2 cytokine production and secretory immunoglobulin A response to influenza A virus infection in BALB/C mice. *J. Nutr.*, 130: 1132-1139.
- Dacie, J.V. and S.M. Lewis, 1985. *Practical Haematology*. Edinburgh. Churchill Livingstone, /ELBS.
- Flynn, J.L., M.M. Goldstein, J. Chan, K.J. Triebold, K. Pfeffer, C.J. Lowenstein, R. Schreiber, T.W. Mak, and B.R. Bloom, 1995. Tumor necrosis factor- α is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity*, 2: 561-572.
- Jacobs, P., D. Radzioch and M.M. Stevenson, 1996. *In vivo* regulation of nitric oxide production by tumor necrosis factor alpha in the spleen and gamma interferon, but not interleukin-4, during blood stage malaria in mice. *Infect. Immunol.*, 64: 44-49.
- Kremsner, P.G., S. Neifer, S. Schermick, M. Ferreira Chaves, K. Sliwa and U. Bienzle, 1991. Interferon-gamma enhances the effect of antimalarial chemotherapy in murine *Plasmodium vinckei* malaria. *J. Infect. Dis.*, 163: 1161-1163.
- Lilic, D., A.J. Cant, M. Abinun, J.E. Calvert and G.P. Spickett, 1997. Cytokine production differs in children and adults. *Pediatr. Res.*, 42: 237-241.
- Luty, A.J.F., B. Lell, R. SchmidtOtt, L.G. Lehman, D. Luckner, B. Greve, P. Matousek, K. Herbich, F. Dschmid, P. Migot-Nabias, R.S. Deloron, Nussenweig and P.G. Kremsner, 1999. Interferon- γ responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. *J. Infect. Dis.*, 179: 980-988.

- Mackay, I.R. and F.S. Rosen, 2001. Advances in immunology. *N. Eng. J. Med.*, 344: 350-362.
- Mossmann, T. and R. Coffman, 1989. Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.*, 7: 145-173.
- Munoz, C., L. Schlesinger and J.M. Cavaillon, 1995. Interaction between cytokines, nutrition and infection. *Nutr. Res.*, 15: 1815-1844.
- Peter, J.K., 2000. Malaria (*Plasmodium*). In: Nelson Text book of Pediatrics, Chapter 278. 16th Edn., W.B Saunders Company Philadelphia Pennsylvania, pp: 1049.
- Sedegah, M., F. Finkelman and S.L. Hoffman, 1994. Interleukin 12 induction of interferon gamma-dependent protection against malaria. *Proc. Natl. Acad. Sci. USA.*, 91: 10700-10702.
- Shanker, A.H., B. Genton, M. Baisor, S. Tanja, T. Adiguma, L. Wul Rare, D. Bannon, J.M. Tielsch, K.P. Jr. West and M.P. Alpers, 2000. The influence of zinc supplementation on morbidity due to *Plasmodium falciparum*: A randomized trial in pre school children in Papua New Guinea. *Am. J. Trop. Med. Hyg.*, 62: 663-669.
- Sherry, B.A., G. Alava, K.J. Tracey, J. Martiney, A. Cerami and A.F. Slater, 1995. Malaria specific metabolite hemozoin mediates the release of several potent endogenous pyrogens (TNF, MIP-1 alpha and MIP-1 beta) *in vitro* and altered thermoregulation *in vivo*. *J. Inflamm.*, 45: 85-96.
- Singotamu, L., 1996. Characterization of stable, specific and common antigen for vaccine production against infection of blood feeding parasites pyroplasmids and helminthes by Electron Microscope. 14th International Congress for Tropical Medicine and Malaria held at Nagasaki, Japan, pp: 17-22.
- Stefen, W., M. Willheim, K. Baier, D. Schmd, A. Aichelburg, W. Graninger and P.G. Kremsner, 1998. Reciprocal regulation of Th1 and Th2- Cytokine-producing T cells during clearance of parasitemia in *Plasmodium falciparum* malaria. *Infect. Immunol.*, 66: 6040-6044.
- Villamor, E., R. Mbise, D. Spiegelman, K.E. Hertzmar, M. Fataki, K.E. Peterson, G. Ndossi and W.W. Fawzi, 2002. Vitamin A supplements ameliorate the adverse effect of HIV-1 malaria and diarrheal infections on child growth. *Pediatrics*, 109: E6.
- World Health Organization, 1997. World malaria situation in 1994. *Weekly Epidemiol. Res.*, 72: 269-279.
- Zinc Against Plasmodium Study Group, 2002. Effect of zinc on the treatment of *Plasmodium falciparum* malaria on children: A randomized controlled trial. *AJCN.*, 76: 805-812.