THE EFFECT OF ZINC AND VITAMIN C SUPPLEMENTATION ON HEMOGLOBIN AND HEMATOCRIT LEVELS AND IMMUNE RESPONSE IN PATIENTS WITH *PLASMODIUM VIVAX* MALARIA

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Abstract. *Plasmodium vivax* infection in humans can relapse and is associated with iron deficiency. The immune response plays an important role in preventing relapse. In this study we analyzed the effect of zinc and vitamin C supplementation on hemoglobin and hematocrit levels and immune response in patients with *P. vivax* malaria. We measured immune response by examining interferon gamma (IFN-γ) and interleukin-10 (IL-10) levels. Subjects were divided into either treatment or control groups. The treatment group received daily zinc and vitamin C supplementation for 45 days. Compliance with supplement consumption was recorded weekly. After 45 days of supplementation, IFN-γ and IL-1 levels were remeasured. All study subjects in both groups had normal hemoglobin and hematocrit levels. The hemoglobin levels increased only in the supplementation group (p=0.011), while hematocrit levels increased in both the supplementation (p=0.001) and control (p=0.023) groups. IFN-γ decreased slightly in the supplementation group, but the change was not significant (p=0.688). IL-10 increased slightly in both the supplementation and the control groups, but the change were not significant (p=0.421 and p=0.556, respectively), suggesting the elevated hemoglobin and hematocrit levels were unrelated to immune response.

Keywords: *Plasmodium vivax*, IFN-γ, IL-10, hemoglobin, zinc, vitamin C

INTRODUCTION

Plasmodium vivax malaria is widely distributed, threatening approximately 3 billion people in 95 countries globally (Guerra *et al*, 2010). A study by Elyazar *et al* (2012) in Indonesia found an estimated 129.6 million people are at risk of contracting vivax malaria. Of these, 79.3% live in unstable transmission areas and the rest live in stable transmission areas. Over 70% of the population in Java and Bali are at risk of contracting vivax malaria (Elyazar *et al*, 2012). These figures are disconcertingly high for a preventable and treatable disease.

Iron (Fe) deficiency is prevalent in malaria endemic areas (Awah and Kaneko, 2012). However, iron supplementation remains controversial because it may have detrimental effects during plasmodium infections (Pasricha *et al*,

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2013). Conflicting outcomes have been reported in different studies. Some studies have found Fe supplementation increases the risk of severe illness and death during malaria infection (Sazawal *et al*, 2006) while others have not (Ojukwu *et al*, 2009). We hypothesized Fe absorption from food is better during malaria infection. Therefore, we studied vitamin C supplementation instead of Fe supplementation since vitamin C increases Fe absorption (Naidu, 2003) and the vitamin C might enhance the immune response (Failla, 2003).

The clinical presentation of malaria depends on several factors, including patient immunity. Infection with *Plasmodium vivax* produces high levels of interleukin 10 (IL-10) and interferon gamma (IFN-γ) (Praba-Edge *et al*, 2003; Medina *et al*, 2012). IFN-γ is a pro-inflammatory cytokine and IL-10 is an anti-inflammatory cytokine (Murphy, 2012). A recent study of vivax malaria showed a cytokine imbalance between interleukin 10 and IFN-γ is associated with more severe vivax malaria (Andrade *et al*, 2010). IFN-γ causes infected hepatocytes to produce nitric oxide that kills the intrahepatic parasite and plays a central role in liver stage protective immunity (Taylor-Robinson, 2003), helping to prevent relapse of vivax malaria due to the dormant liver-stage of the *P. vivax* hypnozoite (Krotoski *et al*, 1986; Vichchathorn *et al*, 2006).

Micronutrients play an important role in enhancing immune response (Wintergerst *et al*, 2006). In this study we evaluated the effect of zinc and vitamin C supplementation on enhancing hemoglobin levels and immune response in patients with *Plasmodium vivax* malaria.

MATERIALS AND METHODS

We conducted an experimental study with pretest/posttest results using a control group design; the study was double blinded. Zinc and vitamin C supplementation was the intervention. To determine the minimum sample size, we used a mean significant difference between the treatment and control group of 80, a standard deviation of 89, $Z_{1-\alpha} = 1.645$ for a one tail test, $\alpha = 0.05$ and $Z_{1-\beta} = 1.285$, for a $\beta = 0.10$; we obtained 10.6 as the minimum number of subjects. In this study we recruited 13 patients for the treatment group and 14 patients for the control group.

Subjects were patients diagnosed with *Plasmodium vivax* malaria, as evidenced by detection of *Plasmodium vivax* on a thick or thin blood smear. Other inclusion criteria for subjects were not taking immunosuppressants during the previous three months before the study, having a minimal body mass index of at least 18 kg/m^2 and being willing to participate in the study as evidenced by giving written informed consent. Subjects complying with the supplementation less than 80% of the time were excluded from the study. Ethical clearance was obtained from the Commission of Ethics of Medical and Public Health Research, Faculty of Public Health, Diponegoro University.

Supplementation consisted of zinc tablets 10 mg daily for 45 days and vitamin C tablets 40 mg daily for 45 days. The cellular immune response was tested by measuring IFN-γ and IL-10 levels using an ELISA. At the beginning of the study, prior to the intervention (supplementation), we measured hemoglobin, hematocrit, IFN-γ and IL-10 levels on all subjects and controls. After 45 days supplementation in the study group, we again measured these levels in both groups.

IFN-γ levels were measured with a diagnostic test kit from eBioscience (San

Table 1
Variables in treatment and control groups.

Variables	Zn + VC (n = 13)	Control (n =14)	p-value
Age (years)	35 ± 15	36 ± 10	0.758[a]
BMI (kg/m^2)	19.3 ± 0.93	19.7 ± 0.96	0.315[a]
Energy (calories)	1,531 ± 376	1,316 ± 309	0.103[a]
Protein (g)	60.2 ± 29.8	58.6 ± 43.4	0.882[a]
Iron (mg)	5.3 ± 2.3	7.2 ± 3.8	0.138[a]
Zinc (mg)	5.32 ± 1.6	4.6 ± 1.6	0.357[a]
Vitamin C (mg)	37.8 ± 38.0	19.3 ± 18.8	0.145[b]

[a]Independent t-test; [b]Mann-Whitney test.

Diego, CA) (CAT: BMS228HS) and IL-10 levels were measured with the Quantikine® R&D System (CAT: HS100C, Minneapolis, MN). Hemoglobin and hematocrit levels were measured using spectrophotometry. All tests were conducted at The Prodia Laboratory, Indonesia.

Daily food intake was determined by two 24 hour recall periods during 2 nonconsecutive days. From the recall we obtained household food intake, which was converted into grams of food. We used Nutrisoft software (from The Nutritional Research and Development Center, Ministry of Health, Indonesia) to measure the daily intake of energy, protein, zinc, iron and vitamin C. Body mass index (BMI) was measured as a ratio of body weight in kilograms divided by height in meters squared.

RESULTS

Table 1 shows the variables in the treatment and control groups did not differ significantly from each other. The average daily intake of energy, protein, iron, zinc and vitamin C in the supplementation and control groups were not different from each other (p=0.118, 0.168, 0.688, 0.160 and 0.196, respectively). The mean of age of participants was 35±15 years in the treatment group and 36±10 years in the control group. All subjects had a normal body mass index and similar daily intake. Since the characteristics were similar, we assumed the characteristics would not confound the results.

All treatment and control subjects had normal hemoglobin and hematocrit levels. The mean hemoglobin level increased in the supplementation group only (p=0.011), while the mean hematocrit levels increased in both the supplementation (p=0.0001) and control (p=0.023) groups. The median IFN-γ levels decreased in the treatment group but the change was not significant (p=0.688). The median IL-10 levels increased slightly in both the treatment group and control group, but the change was not significant (p=0.421 and p=0.556, respectively) (Table 2).

DISCUSSION

Zinc and vitamin C supplementation increased hemoglobin levels, similar to several previous studies (Shankar *et al*, 2000; Makonnen *et al*, 2003; Alarcon *et al*, 2004). Zinc and iron supplementation

Table 2
Variables before and after treatment in the treatment and control groups.

Variables	Zn + VC (n = 13)			Control (n = 14)		
	Before	After	p-value	Before	After	p-value
Hb (g/dl)[c]	14.42 ± 0.61	15.04 ± 0.81	0.011[a]	14.80 ± 1.30	15.08 ± 1.22	0.345[a]
Ht (%)[c]	42.66 ± 1.72	45.53 ± 2.54	0.001[a]	43.36 ± 3.44	45.44 ± 3.38	0.023[a]
IFN gamma (pg/ml)[d]	0.20 ± 1.59	0.15 ± 0.18	0.688[b]	0.08 ± 0.95	0.08 ± 0.13	0.529[b]
IL-10 (pg/ml)[d]	0.38 ± 0.77	0.52 ± 0.60	0.421[b]	0.37 ± 6.86	0.47 ± 1.94	0.556[b]

[a]Paired t-test; [b]Wilcoxon signed ranks test; [c]mean ± SD; [d]median ± SD.
IFN, interferon; IL, interleukin.

of 3 mg/kg/day for 18 weeks in children resulted in an increase of hemoglobin levels, compared to iron supplementation alone (Alarcon *et al*, 2004). Zinc supplementation of 10 mg/day for three months also resulted in an increase in hemoglobin levels up to 12 g/l (Makonnen *et al*, 2003). Our results are similar to those of Shankar *et al* (2000) from Papua New Guinea who found less anemia in the zinc supplementation group (19%) than in the placebo group (23%). Zinc supplements may provide hematological benefits in populations that have increased requirements for zinc due to a higher underlying prevalence of diarrheal, malaria or other infections (Dekker and Villamor, 2010).

Zinc and vitamin C both indirectly influence hemoglobin synthesis (Smith and Bidlack, 1980; Kelada *et al*, 2001; Naidu, 2003). Vitamin C may improve red cell production by mobilizing stored iron, especially the portion of iron that accumulates as hemosiderin (Smith and Bidlack, 1980). Vitamin C also helps iron absorption, which leads to increased hemoglobin levels (Naidu, 2003). Vitamin C is positively associated with hemoglobin levels (Finkelstein *et al*, 2011). Zinc influences the activity of δ-aminolevulinic acid dehydratase (ALAD), an enzyme that catalyzes heme synthesis (Kelada *et al*, 2001). Malaria-induced inflammation may be associated with impaired release of storaged iron from hepatocytes (Verhoef, 2010), which leads to anemia.

We suggest supplementation with zinc and vitamin C is more suitable for anemia in malaria, compared to iron supplementation. Iron supplementation may have detrimental effects in plasmodium infections (Oppenheimer, 2001; Pasricha *et al*, 2013), increasing the risk of severe illness and death during malaria infection (Sazawal *et al*, 2006). Iron combined with zinc may provide protection against anemia in *Plasmodium vivax* malaria (Richard *et al*, 2006). No previous studies have shown zinc to cause problems during malaria infection (Shankar *et al*, 2000; Richard *et al*, 2006; Dekker and Villamor, 2010). However, zinc should not be used to treat severe anemia (Zlotkin *et al*, 2003). In sumary, zinc and vitamin C supplementation increased hemoglobin levels in *Plasmodium vivax* malaria patients.

Our study found zinc and vitamin C supplementation had no effect on IFN-γ. A previous study among cancer patients showed a decrease in IFN-γ levels after

high-dose vitamin C intravenous supplementation (Mikirova *et al*, 2012). Sanstead *et al* (2008) found zinc supplementation for 10 weeks increased IFN-γ levels. A difference in our study could be the minerals and vitamins used for supplementation, since Sanstead *et al* (2008) also supplemented with other minerals and vitamins (zinc, copper, selenium, iodine, fluoride, manganese, molybdenum, chromium, vitamins A, D, E, K, B complex, niacin, and folic acid). However, the attenuation of the inflammatory response may be advantageous, since it prevents potentially deterious effects of systemic inflammation in the host (Opal *et al*, 1998).

Our results show zinc and vitamin C supplementation had no effect on IL-10 levels. Our results showed a nonsignificant increase in IL-10 levels in the supplementation group. Zinc and vitamin C supplementation may stabilize conditions in malaria, since severe malaria is associated with high IFN-γ levels and low IL-10 levels. A previous study showed different clinical presentations of vivax malaria infection had strong associations with activation of pro-inflammatory responses and cytokine imbalances, since IFN-γ/IL-10 ratios were higher in patients with more severe disease (Andrade *et al*, 2010). Although IFN-γ is involved in resistance to malaria (D'Ombrain *et al*, 2008), it also contributes to disease immunopathology (Wroczynska *et al*, 2005). Zinc and vitamin C supplementation may balance the IFN-γ/IL-10 ratio.

There were several limitations of this study. The subjects were not randomly allocated to the two groups due to the conditions at the study site. Patients were only asked about underlying illness at the beginning of the study. Monitoring only focused on compliance with supplementation, without asking about other illnesses during the study.

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