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Effect of Vitamin A, Zinc and Vitamin E Supplementation on Immune Response in Seropositive Leprosy Subjects

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Abstract: The immune response plays an important role in leprosy prevention. Here we analyzed the effect of vitamin A, zinc and vitamin E supplementation on the immune response in seropositive leprosy patients by measuring serum levels of retinal, zinc, a-tocopherol, interferon gamma (IFN-y) and interleukin-2 (IL-2). Subjects were randomly divided into either the treatment or control group. The treatment group received high dose vitamin A once and a daily dose of Zn and vitamin E supplementation for 45 days, while the control group received pills that were identical in appearance but lacked supplements. Supplement consumption compliance was recorded weekly. After 45 days of supplementation, IFN-y and IL-2 levels were measured again. Upon study initiation both groups had normal retinal, zinc and a-tocopherol serum levels. After 45 days, serum levels of retinal and a-tocopherol increased only in the supplementation group ($p = 0.046$ and $p = 0.033$, respectively), while zinc serum levels decreased in both the supplementation ($p = 0.001$) and placebo ($p = 0.000$) groups. IFN-y levels decreased slightly in the supplementation group, although the change was not significant ($p = 0.098$). Meanwhile, IFN-y levels in the control group decreased significantly ($p = 0.022$). IL-2 levels decreased slightly in both the supplementation and placebo groups, but the changes were not significant ($p = 0.421$ and $p = 0.556$, respectively). Together our results indicate that supplementation with zinc and vitamins A and E could be a useful alternative therapy for maintaining immune response of seropositive leprosy patients.

Key words: Vitamin A, zinc, vitamin E, leprosy seropositive, interferon-y, IL-2

INTRODUCTION

In 2012 the World Health Organization (WHO) reported that the number of leprosy patients in Indonesia ranked third in the world, behind only India and Brazil (WHO, 2012). The number of new leprosy patients in Indonesia is likely to increase due to household contacts of leprosy patients and the possibility that patients who are for seropositive IgM and anti-Phenolic Glycolipid (PGL)-1, which can be used to detect the presence of *Mycobacterium leprae* infection, will convert into a clinical form of leprosy (Rahfiludin *et al.*, 2011).

Relative to patients with manifest leprosy, the cellular immune response in patients who are seropositive for leprosy remains at normal levels (Pinheiro *et al.*, 2011). The immune response of leprosy patients is affected by several micronutrients, including retinal (vitamin A), a-tocopherol (vitamin E), ascorbic acid (vitamin C), vitamin D, zinc, selenium and magnesium (Varquez *et al.*, 2014). Thus, micronutrients could affect cellular immunity in seropositive leprosy patients and maintenance of adequate levels may improve cellular immune responses (Kassu *et al.*, 2006). A study in an East Java province of Indonesia showed that a single zinc supplementation increased plasma zinc levels and tended to maintain normal IL-2 levels in seropositive leprosy patients (Rahfiludin *et al.*, 2011). However, the effect of vitamin A and vitamin E supplementation on the immune response of seropositive leprosy patients has not been investigated.

Micronutrient supplementation may serve as an important alternative preventative treatment for people who live together in one house with a manifest leprosy patient and could improve the immune response to a greater degree than single supplementation, which may in turn prevent seroconversion of other household members. In this study we investigated whether vitamin A, zinc and vitamin E supplementation could enhance serum levels of zinc, retinal and a-tocopherol in PGL-1 seropositive subjects, as well as the effects of supplementation on the immune response in these patients.

MATERIALS AND METHODS

We conducted an experimental study with pretest/post-test results generated using a control group design with zinc, vitamin A and vitamin E supplementation as the

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intervention. At the beginning of the study, we confirmed that patients had seropositive leprosy by measuring the level of IgM and anti-PGL-1 using ELISA (Polyclonal rabbit anti human IgM/HRP, Dako"). Among 100 subjects, about thirty patients were categorized as seropositive for PGL-1. The IgM Anti-PGL-1 test was conducted at the Tropical Disease Diagnostic Centre (TDDC) Airlangga University, Surabaya, Indonesia.

Criteria for subject exclusion included: symptoms of clinical leprosy either on anamnesis or clinical examination; taking of anti-leprosy drugs; the presence of diseases that can affect zinc status (e.g., diabetes mellitus, HIV, thalassemia treated with DTPA, or long-term continuous diarrhea), ingestion of immunosuppressant drugs in the three months prior to study blood tests, BMI (body mass index) <18 kg/m_2 and lack of willingness to participate in the study as evidenced by refusal to provide written informed consent. Subjects with a supplementation compliance level <80% were also 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.

After seropositivity screening was conducted, subjects were randomly assigned into either the treatment or control group. Each group was contained of 15 seropositive leprosy subjects. The treatment group took supplementation with 10 mg zinc and 40 mg vitamin E tablets were given daily for 45 days, while high dose vitamin A (200,000 IU) was given once during the intervention whether the control group took placebo tablets.

Before beginning the supplementation IFN-y and IL-2 levels of both control and supplement groups were measured with diagnostic ELISA test kits from eBioscience, Bender MedSystens GmbH (CAT: BMS228HS and BMS221HS, respectively) using Microplate Reader 680 series (Bio-Rad Laboratories, Inc., Hercules, CA 94547, USA). Serum retinol and serum a-tocopherol were measured using the HPLC method. Zinc serum levels were measured using Inductively Coupled Plasma-Mass Spectrophotometry (ICP-MS). The same tests were repeated for both groups 60 days after initiating the supplementation. All tests were conducted at the Prodia Laboratory, Indonesia.

Body mass index (BMI) was calculated by determining the ratio of patient body weight (kg) divided by height (rrr). Daily caloric intake was estimated using patient recall for two consecutive days before, during and after the supplementation period. The patient recall results and household size were subsequently converted to a unit weight (gram).

RESULTS

The mean age of the treatment and control groups was 33±7 and 31±12 years old, respectively, such that all subjects could be categorized as being of productive age. All subjects had a BMI that fell into the overweight category and there were no significant differences in the average daily intake of energy, protein, zinc, vitamin A, vitamin E, Cu, phytate and phytate/zn molar ratio between the supplementation and placebo groups (Table 1, both groups p>0.05). All study subjects had a very high phytate/zn molar ratio, suggesting that the study population had a zinc deficiency. Given that the characteristics of the study subjects were essentially equal, we assumed that this deficiency would not confound the results for the effects of supplementation. Before treatment, there were no differences in serum levels of retinol, zinc and a-tocopherol between the treatment and control groups (Table 2, p>0.05). After supplementation, the median values for serum retinol increased for the supplementation group (p = 0.046) and decreased in the control group (change = 44 mg/di, mean±SD = 103.6, p = 0.117). After supplementation, there was no difference between the supplementation and control group, however, the mean values did show that the control groups had a significant decline in retinol levels. Even though after treatment there were no differences in serum retinol between the supplementation and control group (p = 0.316), the mean differences for the groups did show a significant change (p = 0.009).

Table 1: Characteristics of the treatment and control groups

Variable	Suppl. (n = 15)	Control (n =15)	p
Age (years)	33±7	31±12	0.528a
BMI (kg/m_2)	25.3±4.34	23.0±3.83	0.146a
Energy (Calorie)	2.051±890	2.268±657	0.283a
Protein (g)	63.8±24.4	58.2±20.8	0.604a
Zinc (mg)	5.42±4.1	4.82±2.6	0.967a
Vitamin A (IU)	1.012±1.296	5.476±5.541	0.373b
Vitamin E (mg)	0.41±1.05	0.1±0.35	0.587a
Pytate (mg)	10.1±0.8	14.7±0.6	o.124a
Pytate/Zn Mollar ratio	28.0+25.6	44.7±43.55	0.157'

a: Independent t-test, b: Mann-whitney test

After treatment, the mean value for serum zinc levels was significantly different between the supplementation and control group (p = 0.032). Although both groups showed decreased serum zinc levels, the control group had a sharper decline relative to the supplementation group (p = 0.032). The mean values for a-tocopherol after treatment were significantly different (p = 0.004) between the supplementation and control groups, whereas the mean value for a-tocopherol levels increased (change = 3 mg/di, mean±SD = 5.0) in the supplementation group and decreased (change = 0.5 mg/di, mean±SD = 2.3) for the control group.

In terms of the immune response, micronutrient supplements promoted no significant difference in IL-2 levels before and after treatment, as the values were similar for both the control and supplement group (Table 3). Moreover, there was no difference in the

Table 2: Retinol serum, zinc serum and a-tocopherol serum levels before and after treatment in the supplementation and control groups

Variable	Groups	N	Before	After	Δ	p
Retinol serum	vAvEZn	15	468 (125.7)ı	532(160.3)ı	64 (111.6)'	0.046'
(mg/di)	Control	15	554 (154.9)'	510 (114.8)'	-44 (103.6)'	0.117'
	p		0.290'	0.316'	0.009b	
Zinc serum	vAvEZn	15	**68(9.3)'**	**55(7.1)'**	-12(11.0)'	0.001'
(mg/di)	Control	15	69 (9.7)'	49 (6.3)'	-19 (9.2)'	0.000'
	P		QOO~	Qffi~	OM~	
a-Tocopherol	vAvEZn	15	13 (3.4)'	16 (4.9)'	3 (5.0)'	0.033'
(mg/di)	Control	15	12 (3.1)'	12 (2.8)'	-0.5 (2.3)'	0.389'
	p		0.429b	0.004'	0.017'	

-: Paired t-test, ': Independent t-test, ':Wilcoxon sign ranks test, ': Mann Whitney test, -: Mean±SD, ': Median±SD
vAvEZn: supplementation group

Table 3: IF-y and IL-2 before and after treatment in the supplementation and control groups

Variables	Groups	N	Before	After	Δ	P
IF-y (pg)	vAvEZn	15	0.55 (0.3)'	0.44(0.3)'	-0.11(0.2)'	0.098'
	Placebo	15	0.46 (0.9)'	0.43 (0.3)'	-0.31 (0.9)'	0.022'
	p		0.885'	0.836'	0.934'	
IL-2 (pg)	vAvEZn	15	0.44 (3.3)'	0.37 (2.9)'	-0.03(1.6)ı	0.346'
	Placebo	15	0.33 (0.7)'	0.33 (0.4)'	-0.01(0.4)'	0.711'
	p		0.221'	0.442'	0.590'	

a. Paired t-test, ': Independent t-test, ': Wilcoxon sign ranks test,': Mann Whitney test, ': Mean±SD, ': Median±SD
vAvEZn: supplementation group

median IFN-y values between the supplementation and control group before treatment (Table 3, p = 0.885). The median IFN-y levels did decrease for the supplement group, but this change was not significant (p = 0.098). Meanwhile, the median IFN-y levels in the control group also decreased and this change was significant (p = 0.022). The median IL-2 levels decreased slightly in both the treatment and control groups, although this change was not significant (Table 3, p = 0.346 and p = 0.711, respectively).

DISCUSSION

Screening for IgM anti-PGL-1 seropositivity among household contacts showed that 30% residents of Brebes in Central Java were PGL-1 seropositive. Previous studies of seropositive subjects with IgM and anti-PGL-1 levels of more than 0.200 OD and 600 unit/ml, respectively, who had no clinical signs of Hansen disease, can be classified as PGL-1 seropositive (Scollard *et al.*, 2006). This result corresponds with the finding that Indonesia ranks third in the world in terms of the number of leprosy cases and is behind only India and Brazil (WHO, 2012).

In this study we examined whether supplementation with zinc and vitamins A and E for 45 days affected serums levels of micronutrients as well as IFN-y and IL-2 in PGL-1 seropositive subjects.

A previous study by Lima *et al.* (2007) reported that decreases in retinol serum levels are associated with depressed T-helper 1 (Th1) immune response and replication of *M. leprae* in macrophages (Lima *et al.*, 2007). Another study showed that vitamin A deficiencies in clinical leprosy patients affected malondialdehyde status-a marker of oxidative stress-suggesting that vitamin A may reduce malondialdehyde levels and in turn oxidative stress (Trimbake *et al.*, 2013). Oxidative stress-associated lipid peroxidation is often present in clinical leprosy, as evidenced by the finding from a case-control study that lipid peroxidase (LPO) activity is higher in leprosy patients relative to healthy individuals (Schaicher *et al.*, 2014). Lipid peroxidase levels are often not reduced after treatment for leprosy, since increased production of free radicals (ROS) is thought to accompany the bactericidal activity of such treatments toward *M. leprae* (Vijayaraghavan *et al.*, 2005). Here we showed that treatment with a one-time high dose of vitamin A (200,000 IU) could increase retinol serum in the supplementation group, while the serum retinol in the control group decreased (Table 2).

Similar to the effect of vitamin A supplementation, serum levels of a-tocopherol in the supplementation group increased significantly after treatment. Vitamin E (a-tocopherol) is a fat-soluble antioxidant that may prevent oxidation of conjugated double bonds and thus plays an important role in preventing lipid peroxidation in cellular membranes. Furthermore, a-tocopherol is also involved in the activation of T cells and B cells that can affect the immune response (Engin, 2009). Together these attributes could make a-tocopherol an important treatment for *M. leprae* infections in that this vitamin can reduce levels of free radicals that occur in leprosy (Vazquez *et al.*, 2014).

In contrast to the results for vitamin A and vitamin E supplementation, zinc supplementation did not maintain zinc serum levels in either the control or the treatment group. The decline in serum zinc levels seen in both

groups could be due to the high phytate/zn molar ratio, which in this study was more than 12.5 as determined by methods described by IzinCG (IzinCG, 2007, Table 1). These results indicated that all study subjects likely had low zinc intake. The high phytate/zn molar ratio could also be indicative of phytate-mediated zinc absorption inhibition in the intestine. In this study the phytate/zn molar ratio in the control group was higher than the supplementation group, although the mean phytate/zn molar ratio was similar between the two groups ($p = 0.066$).

Zinc supplementation for just three months was demonstrated to increase plasma zinc levels and maintain IL-2 levels in leprosy seropositive patients (Rahfiludin *et al.,* 2011). Production of IFN-y and IL-2 may also be affected by zinc supplementation (Bao *et al.,* 2008), as evidenced by a decrease in cytokine production that can occur at the level of gene transcription. Indeed, several transcription factors require zinc for structural integrity, which may explain the decreased gene expression of IL-2 receptor a and p IL-2 seen in zinc-deficient cells (Prasad, 2007).

Since binding of the transcription factor NF-KB to DNA is also reduced in zinc-deficient cells (Prasad, 2013), gene expression of both IL-2 and IL-2Ra could be attributed to decreased activation of NF-KB in the presence of lower zinc levels. In addition to NF-KB, the activity of transcription factors such as AP-1 and SP-1 is also decreased in zinc deficient states (Chattree *et al.,* 2007). *M. leprae* infection can thus interfere with the host immune response by lowering the activity of molecules that stiumlate immune system activity. Indeed, defective T cell signaling in *M. leprae* leprosy is thought to result from down regulation of B7-1 and CD 28 pathway activity that is induced in response to *M. leprae* antigens (Agrewala *et al.,* 1998). The presence of another trace elements in addition to zinc supplementation suggests that other factors beyond zinc are important for promoting an effective immune response (Cousins *et al.,* 2003).

Conclusion: Together our results suggest that vitamin A, zinc and vitamin E supplementation could be useful for maintaining serum levels of retinol, a-tocopherol, IFN-y and IL-2 in seropositive leprosy patients. Further studies will be needed to identify other nutrients that can increase IFN-y and IL-2 levels in these patients.

Study limitation: This study had the limitation that patient monitoring focused only on whether subjects complied with the supplementation and did not collect information about patient illness during the study.

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