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Supplementation of Zinc Sulphate Decrease for Cytokine Levels TNF-A, IL-1β and IL-6 in Multibacillary Leprosy Patients

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ABSTRACT

Background: The role of zinc as an anti-oxidant and anti-inflammatory has been widely proven, this substance is relatively inexpensive and easily available to the public. Serum zinc levels of leprosy patients and patients with leprosy reactions are lower compared to healthy people, and proinflammatory cytokines including TNF-α, IL-1β and IL-6 play a role in the mechanism of leprosy reactions. Research on the effect of zinc supplementation on leprosy sufferers who have not experienced a reaction has not been done. **Objective**: To analyze the effect of 40 mg / day dose of zinc sulphate supplementation for 12 weeks on the levels of proinflammatory cytokines levels TNF-α, IL-1β and IL-6 in type MB leprosy patients. **Method**: This is an experimental study using a randomized control group pretest and posttest design. The study participants were MB patients with 121 patients who did not experience a leprosy reaction, the sample was taken randomly as many as 31 patients as a supplementation group (treatment), and 31 patients as Control group. BMI measurements, food intake by re-call method, Hb level using Hematology Analyzer, serum zinc levels using Atomic Absorption Spectrophotometry (AAS) method, TNF-α, IFN-γ, and IL-1β levels used the ELISA method. Data were analysed using chi-square and t-tests. **Results**: Zinc sulfate supplementation dose of 40 mg / day for 12 weeks in type MB leprosy patients was able to maintain a decrease in zinc levels of 1.22 pg / dL higher than leprosy patients who did not receive zinc supplementation, reduce levels of IL-1β (p=0.032). However, statistically there is insufficient evidence to reduce levels of TNF-α (p=0.063) and IL-6 (p=0.389) and risk factors. **Conclusion**: The study confirmed that 40 mg / day supplementation of zinc sulfate for 12 weeks in type MB leprosy patients able to maintain a decrease in serum zinc levels, reduce IL-1β levels.

Keywords: Supplementation, Cytokines, Type MB leprosy

INTRODUCTION

Background

Leprosy is a chronic disease caused by an infection of the bacterium *Mycobacterium leprae*.[(1)] The disease becomes a major cause of deformity and disability among other infectious diseases that cause a stigma, psychocial disorders and economic problems. [(2,3)]

In 2010, *World Health Organization* (WHO) reported that Southeast Asia was the region with the highest prevalence rate of 120,456 cases per 10,000 population.[(4)] The number of incidence of leprosy reactions both type 1 and type 2 in the world is quite high. Based on studies in some countries such as Indonesia, Brazil, Nepal, Bangladesh and India, the number of leprosy cases ranged from 16-56%, 20 - 60% for the type 1, whereas 19-37% for the type 2 (ENL).[(5–9)] Furthermore, the disability rates due to leprosy are around 20-30% of new cases,

and some of them require rehabilitation. The disability rate in the level 2 of Indonesia leprosy patients in 2015 was 10.07%, especially in Central Java ranged from 8.1%. The Disability rate of leprosy patients in Tegal Regency have tended to increase for five years, respectively from 2011 at 8.12%, 2012 at 7.3%, 2013 at 10.7%, 2014 at 10.2%, and 2015 at 12%.[10–12]

Leprosy is a public health problem in the developing countries and often associated with the poverty and malnutrition.[13,14]. The malnutrition status may be caused by inadequate food intake both in the quantity and quality. Individuals suffering leprosy consume more vegetables and less animal protein. In fact, most of the vegetables are not a good source of zinc because there is phytate. This substance will bind zinc, so it cannot be absorbed by the gastrointestinal tract. Low protein and rich phytate diets play a role in the high prevalence of the zinc deficiency in the developing countries.[15] The zinc deficiency leads to decrease the immune system; for example, a failure of the macrophage cell in the process of phagocytosis and decrease in T-cell's ability to differentiation and proliferation.

Through several studies on the animal experiments and clinical observations to humans, the nutrition acts as the journey and development of leprosy, and among these nutritional elements, zinc is the most important trace element for the growth of thymus organs and lymphoid tissue being as a place to the differentiation and maturation of cells which are involved in cellular immunity.[16] Therefore, assessing zinc status by providing supplementation can improve the nutritional status and fix the oxidative stress which can be an effective strategy as the treatment and control of leprosy.[17]

The decrease of the immune system of its body and increase of the incidence of leprosy infection could be caused by low levels of zinc in its host. Zinc is able to act in increasing both non-specific and specific immune responses. The Macrophage cells in the immune response systems will have obstacles in killing intracellular infectious agents, reduce the cytokine production and disturb the process of phagocytosis. The disturbed immune response causes a resistant changing toward the infection. Thus, the adequacy of zinc mineral needs more attention because of its role to improve our immune system and its effect toward the infection.

Nowadays, there are still not much literature about the risk factors of leprosy reactions such as: Nutritional Status (Body Mass Index (BMI), Anemia Status / Hb Level, Zinc Level), Co-infection, Physical / mental stress, and food intake in MB leprosy patients. The objective of this study is to analyze the effect of zinc sulphate supplementation on the pro-inflammatory cytokines level and the incidence of leprosy reactions by observing the risk factors of Body Mass Index (BMI), Hemoglobin Level, Zinc Level, Co-infectionand food intake history toward type MB leprosy patients. This study is expected to be useful for programs to improve the nutritional status, especially the zinc level of leprosy patients and as an evaluation program for the prevention and treatment toward leprosy reactions.

Purposes

The objective of this study is to analyze the effect of zinc sulphate supplementation on the pro-inflammatory cytokines level and the incidence of leprosy reactions by observing the risk factors of Body Mass Index (BMI), Hemoglobin Level, Zinc Level, Co-infectionand food intake history toward type MB leprosy patients.

METHODS

This study was an experimental study using randomized pre and post test control group design. It started with grouping leprosy patients based on diagnosis: leprosy with reaction and leprosy without reaction. This study was conducted in 11 public health center areas in Tegal Regency on January to May in 2018. The inclusion criteria of the sample in this study were male and female, aged 15-60 years, type MB leprosy in the MDT treatment, without reaction, and willing to participate in the study. The exclusion criteria included pregnant and lactating female patients, leprosy reactions, alcohol consumption, kidney failure, surgery, burns, multitrauma, fractures, diabetes mellitus, autoimmune diseases, inflammatory conditions except leprosy reactions, immune suppressant therapy except corticosteroids, and those who released from treatment (RFT). This study was approved by the Health Research Ethics Committee of the Faculty of Medicine, Diponegoro University.

The subjects of the study were randomly selected including the treatment group was type MB leprosy patients with the clinical signs of reactions, and the control group was type MB leprosy without reactions. The data collection used the questionnaires through the history, interview, clinical examination, and blood serum examination to thesubject and control, There are 72 people as the sample consisting of 36 people as the treatment group and 36 people as the control group. The treatment groups were added zinc sulphate

supplementation with a dose of 40 mg / day for 12 weeks, while the control groups were given the MDT treatment. The data analysis used SPSS for windows program with Chi-Square Correlation test and t test.

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RESULTS

Characteristics of Subjects

At the beginning of the data collection, the subject were 72 participants, but those who fulfilled the criteria to be observed only were 62 people eventually (31 people as the treatment group; 31 people as the control group). The characteristics of the subjects both the treatment and the control groups can be seen in Table 1.

Table 1. The differences in the characteristics of subjects between the treatment and control groups

Variable	Treatment group n : (36)	Control group n : (36)	p-value
Age Category			
- Average	36.81 ± 13.9	42.56 ± 13.9	0.101[b]
- Early adult (16-30 years)	13 (35.1 %)	6 (16.6 %)	0.151[a]
- Middle adult (31-45 years)	13 (35.1 %)	15 (41.6 %)	
- Final adult (46-60 years)	10 (27.8 %)	15 (41.6%)	
Gender			
- Male	23 (63.9 %)	23 (63.8 %)	0.091[a]
- Female	13 (36.1 %)	13 (36.2 %)	
Body Mass Index (BMI)			
- Average of BMI	21.09 ± 2.49	21.47 ± 2.61	0.545[b]
- Thin (< 18)	2 (5.6 %)	4 (11.1 %)	0.910[a]
- Normal	25 (69.4 %)	21 (58.3 %)	
- Fat	9 (25 %)	11 (30.6 %)	
Job			
- Labor/farmhand/pedicap worker	25 (69.4 %)	27 (75.0 %)	0.272[a]
- Farmer/trader	6 (16.7 %)	4 (11.1 %)	
- Student/a civil servant/ Employee	5 (13.9 %)	5 (13.9 %)	

[a)] Chi-square correlation test, [b)] Independent t test

Based on the table 1, it is concluded that the treatment and control groups do not have significant differences in some categories of the age, sex, Body Mass Index (BMI), dwelling conditions and job of the respondents. Furthermore, we can see the data about the frequency and risk factors of leprosy reactions between the treatment and control groups in Table 2 below.

Based on the table 2, the risk factors of reaction towards the subjects are co-infection, food supply, anemia status (Hb level), and daily food intake, both in the treatment and control groups. As the result, there is no significant difference.

Table 2. Comparison of variables between treatment and control groups

Variable	Treatment Group (n : 36)	Control group (n : 36)	p-value
Co-infection			
- Positive	5 (13.8%)	8 (21.6%)	0.880[a]
- Negative	31 (86.2%)	29 (78.4%)	
Food supply			
- Energy Adequacy Ratio	1380 ± 156.80	1406 ±165.47	0.527[b]
- Protein Adequacy Ratio	40.35 ± 5.32	40.83 ± 4.63	0.703[b]
- TKE (%)	59.23 ± 6.97	60.49± 7.54	0.489[b]
- TKP (%)	76.29 ± 9.69	77.53±10.01	0.613[b]
Hb level (anemia status)			
- Average	11.5 (± 1.44)	12.1 (± 1.83)	0.101[b]
- Anemia (<12 g/dL)	19 (52.8 %)	17 (47.2 %)	0.730[a]
- Normal (>12 g/dL)	17 (47.2 %)	19 (52.8 %)	

[a)] Chi-square correlation test, [b)] independent t test

The differences of the average of Hb level, Zinc, TNF-α, IL-1β, and IL-6 before and after adding the zinc sulfate supplementation doses of 40 mg / day toward type MB leprosy are presented in Table 3.

Table 3. The Differences of the Average of Levels of Hb, Zinc, TNF-α, IL-1β, and IL-6 Before and After Receiving the Zinc Sulfate Supplementation of 40 mg / Day Doses, 12 Weeks

Variable / Measurement	Group		p-value
	Treatment group (n:31)	Control group (n: 31)	
Hb level (g/dL)			
- Before	11.4 (± 1.44)	12.1 (± 1.83)	0.095[b]
- After	11.9 (± 1.57)	12.1 (± 1.80)	0.685[b]
p	0.044[a]	0.909[a]	
Zinc Level (µg/ml)			
- Before	95.54 (± 7.41)	95.02 (± 3.91)	0.761[b]
- After	93.67 (± 4.82)	92.00 (± 4.08)	0.044[b]
p	0.218[c]	0.001[c]	
TNF-α level (pg/ml)			
- Before	21.62 (± 21.68)	13.82 (± 13.82)	0.159[b]
- After	15.28 (±14.49)	10.30 (± 7.91)	0.207[b]
p	0.063[c]	0.221[c]	
IL-1β level (pg/ml)			
- Before	10.85 (± 23.64)	3.24 (± 6.82)	0.549[b]
- After	3.81 (± 15.63)	1.20 (± 0.97)	0.788[b]
p	0.032[c]	0.537[c]	
IL-6 level (pg/ml)			
- Before	17.22 (± 26.96)	12.03 (± 23.72)	0.966[b]
- After	7.14 (± 9.65)	8.12 (± 13.07)	0.662[b]
p	0.389[c]	0.784[c]	

Note: [a]) paired t test,[b]) Mann-Whitney test [c]) Wilcoxcon Signed Ranks test

Based on the table 3, it shows that there are significant differences, such as a decline in the Hb and IL-1 β levels to the treatment group, as well as the zinc level in the control group,. It also happens to TNF-αand IL-6 cytokines, but statistically it is not yet significant.

DISCUSSION

It is known that leprosy infection of the host is associated with the quality of the immune response. The main defense mechanism of M. leprae infection involved immune cells, especially macrophages, lymphocytes and cytokines that regulated the production, released and modulated the cellular immunity reactions (Lima et al., 2007; Prabhakar et al., 2012).

Macrophages are the main defense system against M. leprae infection. Phagocyte cells such as macrophages, neutrophils, eosinophils, as well as T and B lymphocytes have the NADPH oxidase enzyme which is responsible for the production of ROS while stimulating the immune response. Macrophages will recognize M. leprae or its cell wall components through TLR, so that this bond will activate the nuclear factor kappa B (NF-κB) pathway which will induce the release of cytokines such as TNF-α, IL-1, IL-6 and IL-12. These cytokines will cause inflammation, and stimulate adaptive immunity. The interleukin-12 will activate T cells, and induce the release of IFN γ, and next it will activate the phagocyte oxidase enzyme (NADPH oxidase), eventually the ROS releases such as superoxides, hydroxyl radicals and hydrogen peroxide. (Prasad et al., 2007; Rahal et al., 2014; Hart and Tapping, 2012; Abbas et al., 2015).

The distribution of the characteristics of the subjects shows that men is greater than women. The result is same with the previous study conducted by Mastrangelo, 2009 in Brazil that the number of male leprosy patients was greater than that of women.[18] Leprosy commonly happened to men with a ratio of 2: 1, whereas according to the age incidence increases around 10 -14 years, then decreases followed by another increases at the age of 30-50 years.[19] The average age of leprosy patients is 45 years. This is in accordance with Mastrangelo's study saying that the average age of leprosy patients is 48.06 years.[18] Leprosy has a long incubation period, so the patients are often found from teenagers to young adults.[20]

Regarding the nutritional status, Singh reported that 40.5% of patients who experienced a decrease in hemoglobin levels having a Body Mass Index (BMI) more than 18.5 (BMI > 18.5) and less than 59.5% of it. On the one hand, BMI > 18.5 shows normal nutritional status, on the other hand BMI <18.5 indicates malnutrition.[21] Some previous studies have found that micronutrient intake affects the immunological response to lepers. In this study, most of the subjects between leprosy patients and healthy people (non-leprosy patients) have normal BMI. This is contrary to Fatimah's study in 2017 which obtains a significant difference in BMI and zinc status toward leprosy and non-leprosy patients as the control group.[22] In fact, most of the subjects worked as laborers/farmhands get sufficient food intake for the energy requirement to do their activities. However, when it is observed from a re-call of 1x24 hours of food intake consumption, most of the leprosy sufferers consume more vegetables and less animal protein. The average of BMI in the treatment and control group are 21.09 and 21.47. This is similar with Rao's study in 2009 in India arguing that the average BMI of leprosy patients is 20.4.[23] The study of Montenegro, 2009 in Brazil showed that there was no significant relationship among the nutritional status to the incidence of leprosy reactions. Therefore, the reaction is not found mostly in the BMI group which is less than the indicator.

Based on the result, there is a relationship between the energy and protein adequacy ratio with the incidence of leprosy reactions. According to a study conducted by Nagari in 2012 that the respondents who are lack of nutrients, especially energy and protein have a poor nutritional status. Theoretically, lepers will have it due to the increasing of the protein catabolism, in case it is not supported by providing sufficient protein. A study conducted by Philani in 2008 toward the subjects who lived with leprosy patients noted that the patients and other people needed high protein food to increase our immune system. (Mustamin. Intake DIIT TKTP and Nutritional Status of Leprosy Patients in Dr. Tadjuddin Chalid Hosiptal, Makassar Media Food Nutrition 2010: 9 (1) The underweight patients does not have an inadequate immunological response due to low levels of energy and protein consumption, then it will effect toward abnormalities in the immunity as indicated by decreasing of T lymphocyte, natural killer cells activities, and the function of interleukin production.

The average of the Hb level in the treatment and control group is 11.5 and 12.1, and both of them are under the normal Hb levels. According to WHO, the normal range of Hb level is 13 g/dl in a man and 12 g/dl in a woman, and anemia is diagnosed if Hb levels is lower than the normal range.[24] Anemia in leprosy is caused by a chronic disease, usually called iron deficiency anemia due to macrophages that destruct the iron substance and iron binding proteins, iron metabolism disorder, as well as release cytokines which suppress erythroprotein production.[25]

In this study, the result of the average of serum zinc levels in leprosy patients is (± 95 µg/ dL) which is lower than healthy people (± 104 µg/ dL). It can be seen that the zinc levels in the treatment group after receiving Zinc sulfate supplementation doses of 40 mg/ day during 12 weeks decrease to be 1.8 µg/ d, and 3.02 µg/ dL in the control group. Thus, it is proved that there is a significant difference between the serum zinc levels before and after receiving zinc sulphate supplementation (p: 0.003). The low zinc level toward leprosy patients can make multiplication of M. leprae bacteria because low cellular immunity which plays a role in eliminating

microbacteria. The zinc sulphate supplementation in patients will have a good effect to increase serum zinc levels which are important for the development of cellular immunity.

The zinc intake requirement for male in a day is 11 mg, and 8 mg for female, then the average of the zinc level of lepers with reactions is lower than lepers without reactions in the control group, but there is no significant difference. Based on the results of this study, there are significant differences between the zinc level that receives zinc supplement intervention doses of 40 mg / day with the control group. This is consistent with a previous study conducted by As'ad in 2003 stating that the supplementation of the intervention group significantly is higher than the control group after 8 weeks.[26]

The results of this study also shows the increase of serum zinc levels and decrease in the TNF-α and IL-6 levels after receiving zinc sulphate supplementation. In fact, this is similar with previous studies that plasma zinc concentrations rapidly decrease during the acute phase response toward the different stimulation, pressure, infection, and trauma. As a result, zinc is dismantled into the cellular compartment, in which it is used for protein synthesis, neutralization of free radicals, and microbial prevention. Redistribution of zinc during inflammation seems to be mediated by cytokines, and some studies have shown how patients suffering an acute disease appearing with hypozincemia and increasing cytokine production.[26,27]

Pro-inflammatory cytokines, including TNF-α, IFN-γ, and IL-1β and IL-6 have been reported to play a role in the mechanism of leprosy reactions in both type I reactions (reversal reactions) and type 2 reactions (including ENL).[12,13]

Chronic inflammation is characterized by increasing the levels of inflammatory cytokine production. Some conditions are associated with chronic inflammation such as obesity that patients with lower zinc food intake appearing to lower plasma and intracellular zinc concentrations, as well as regulated IL-1α, IL-1β, and IL-6 gene expression compared with patients with higher zinc intake.

The increase in TNF-α and IL-6 levels in the serum of leprosy sufferers indicates that cellular immune responses have a major role in the mechanism of ENL which may cause nerve damage and necrosis tissue.[6,8]

In addition to TNF-α inhibiting agents, corticosteroidsare are quite important and reliable in ENL treatments. Besides having anti-inflammatory and immunosuppressant effects, they head off the activities of various cytokines such as IFN-γ, TNF-α, IL-6 , IL-1 and IL-2 receptor expression. Oral corticosteroids recommended by WHO are prednisolone, starting with a dose of 0.5–1 mg/kg/day (40–60 mg) until clinical improvement occurs, then reducing by 5-10 mg every week for 6-8 weeks. This dose treatment of 5–10 mg/day can be given in several weeks to prevent recurrence of ENL.[13,19]

Cytokine production can also be decreased by the MDT treatment, even the same level as healthy people if all M. leprae antigens can be eliminated. The decrease in the TNF-α level is also found in patients after receiving the MDT treatmen showing only few bacteria due to therapy. Clofazimin has an anti-inflammatory effect and inhibit mitogen stimulated by peripheral blood cells. Clofazimin collaborated with dapsone to reduce the production of cytokines, especially pro-inflammatory cytokines. Therefore, the MDT treatment should be done when ENL occurs, because it can also control the episodes of ENL.[13]

Limitation

The variables of pro-inflammatory cytokines as important signs of appearing of leprosy reactions in this study are the TNF-α, IL-1β and IL-6, and Zinc level examination by using only blood serum samples of patients.

CONCLUSION

Zinc sulphate supplementation doses of 40 mg/day for 12 weeks is able to maintain a decrease in serum zinc levels, and reduce IL-1β levels, but there is not enough evidence to reduce TNF-α and IL-6 levels.

Based on the observation toward leprosy patients, it is necessary to concern the frequency and quality of daily food intake by consuming adequate energy and protein, as well as avoiding physical / mental stress, also consuming Zinc Sulphate tablets during the treatment. Furthermore, for the next study, it should add more complete variables of pro-inflammatory cytokines, zinc levels, and cytokine levels using more sensitive method as well.

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