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RESEARCH ARTICLE

The Role of Zinc Supplementation on the level of MDA and the number of *Mycobacterium tuberculosis* colonies in male tuberculosis Wistar rats

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ABSTRACT:
Objective: to examine the role of zinc supplementation on the level of MDA and the number of *Mycobacterium tuberculosis* colonies in male tuberculosis Wistar rats **Methods**: This is a pure experimental study (true experimental laboratory) which was carried out in a laboratory in vivo by using a post-test control group design with the subjects were male Wistar (Rattus norvegicus) rats aged 2-3 months, obtained from the PUSVETNA unit (Surabaya). **Results**: Significant differences were found in levels of MDA and the number of Mycobacterium tuberculosis colonies in male tuberculosis male wistar rats compared with the control group. **Conclusion**: This study found the ability of Zinc in reducing levels of oxidative stress in patients with tuberculosis and the function of Zinc in modulating the immune system. However in the actual clinical situation it is necessary to consider other comorbid conditions.

KEYWORDS:

INTRODUCTION:
Tuberculosis is a chronic disease caused by infection of *Mycobacterium tuberculosis* (Pratomo et al., 2012). This disease is closely related to poverty and is a problem throughout the world. Although there has been a decrease in the incidence of tuberculosis in the last 10 years, 8.6 million new cases and 1.3 million cases of tuberculosis occurred in 2012 in Indonesia (Sulis et al., 2014), tuberculosis mortality in 2016 reached 1,020,000 cases with an average of 391 cases / 100,000 population (WHO, 2016). Various treatment strategies have been used to reduce the incidence and mortality rate due to tuberculosis, but the cases and mortality rates remain high and even tend to increase even more with the presence of human immunodeficiency virus (HIV) infection. The number of tuberculosis sufferers who also suffer from HIV is 27% in the world, whereas in Indonesia it is 2.8%. In patients with acquired immunodeficiency syndrome (AIDS) tuberculosis is the number one cause of death. This data shows that the body's immune response plays an important role in tuberculosis infection (WHO, 2016).

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Oxidative stress is a condition caused by free radicals due to an imbalance between the production of reactive oxygen compounds and antioxidant capacity. Free radicals can cause lipid degradation (peroxidation) in plasma and membrane organelles. Oxidative damage begins when the double bonds in unsaturated fatty acids from lipids in cell membranes are attacked by free radicals especially by OH. Oxidative damage to polyunsaturated fatty acids by lipid peroxidation is very dangerous because it can change the integrity of the cell membrane (Rao S, 2011). The oxidative stress index increases significantly in untreated tuberculosis (TB) patients and will decrease due to antioxidant supplementation (Kondaveeti et al., 2012). Oxidative stress in tuberculosis can increase reactive oxygen species (ROS) through several pathways such as increased expression of CCL2 mRNA, activated signal kinase-1 (ASK-1), release bonds between Nrf2 from KEAP1, and increase mitogen activated protein kinase (MAPK) (p38) pathway so that it can increase NF-Kβ which then triggers inflammation (Armstrong and Stratton, 2016; Samperio et al., 2010; Chulsu et al., 2008). Oxidative stress is a phenomenon that can cause immunosuppression. Oxidative stress will cause T-cell-related immune defects, which will accelerate tuberculosis infection (Zhang et al., 2012). High ROS

can cause lipid peroxidation. The end result of lipid peroxidase is malondialdehyde (MDA) which is a marker of oxidative damage and MDA levels correlate with the progression of pulmonary lesions (granulomas) (Palasinamy et al., 2011).

Study Method:

This is a true experimental study (true experimental laboratory) which was carried out in a laboratory in vivo by using a post-test control group design with research subjects as male Wistar (Rattus norvegicus) rats aged 2-3 months, obtained from the PUSVETNA unit (Surabaya) . Dependent variables in this study are levels of MDA and the number of Mycobacterium tuberculosis and control variables in this study are temperature, humidity, close body, age, physical activity, and stress.

The induction of tuberculosis rats refers to previous studies (Mustika et al., 2014; Rodrigueset al., 2009; Heng et al., 2011; Kumar et al., 2014). Mice were infected with *Mycobcterium tuberculosis* through the trachea. The rat was anesthetized before being infected with *Mycobacterium tuberculosis*. Anesthesia is performed by injection with ketamine HCl 50 mg / kg weight and Xylazine 0.2 ml intramuscularly or subcutaneously. The mice were fixed supine and incised in the median line of the cervical area so that the trachea was seen. *Mycobacterium tuberculosis* dose was 10^8 / ml injected into the trachea as much as 50 µl with a vertical position tuberculin needle, then stitched again. On the 29th day (week 4) after infection with *Mycobacterium tuberculosis*, mice in the negative control group (K1) were killed to prove the existence of tuberculosis infection in rat lung tissue. The left lung tissue was taken aseptically and cultured on Middlebrook 7H10 media, while the right lung was put in 10% formalin buffer for MDA examination.

After the mice were adapted 7 days before treatment, 5 rats were set aside as a negative / healthy control group (K0), and the remaining 30 mice were infected with *Mycobacterium tuberculosis* H37Rv and waited for 29 days. Rats were divided into 6 groups randomly namely Group 1 (K0) as a negative control group / healthy group of 5 animals, group 2 (K1) as a positive control group 1 / group of TB rats as much as 5 animals, group 3 (K2) as a control group positive 2 received INH dose of 6 mg / day and rifampicin 10 mg / day for 5 heads, group 4 (P1) as a treatment group 1 received INH dose of 6 mg / day, rifampicin 10 mg / day and zinc 50 mg / kg / day for 5 tails. group 5 (P2) as treatment group 2 received INH dose 6 mg / day, rifampicin 10 mg / day and zinc 100 mg / kg weight / day as many as 5 tails and group 6 (P3) as treatment group 3 received INH 6 mg / day, rifampicin 10 mg / day and zinc 200 mg / kg / day as many as 5 tails. Groups K0 and K1 were euthanized on the 30th day to prove that K1 had been infected with TB. The left lung tissue was taken aseptically and cultured on Middlebrook 7H10 media, while the right lung was put in 10% buffered formalin to be tested for MDA. Research for groups K1, and P1-P3 continued with treatment according to the group for 8 weeks. After 8 weeks of treatment of rats in euthanasia for their organ harvesting for MDA and the number of *Mycobacterium tuberculosis* colonies. MDA measurements were performed using pulmonary tissue by the TBARS method using the rat Malondialdehyde kit.

RESULT:

In this study there were 2 variables observed, namely MDA leveland the number of *Mycobacterium tuberculosis* H37Rv. While there are 6 observation groups, each consisting of 5 male Wistar rats (*Rattus norvegicus*) aged 2-3 months. Table 1 shows the average data values and standard deviations. Of the 6 groups of samples the most prominent was the number in the positive control group (K1), namely the group of mice infected with H37Rv bacteria. On MDA levels showed the highest value compared to the other observation groups. Likewise in the variable number of *Mycobacterium tuberculosis* H37Rv, the positive control group (K1) (mice infected with H37Rv bacteria) showed the only group found to be as many as *Mycobacterium tuberculosis* H37Rv while in the other group no *Mycobacterium tuberculosis* H37Rv was found.

Table 1. Comparison of MDA levels and bacteria count amoung groups.

Kelompok Pengamatan	Rerata ±SD	
	Kadar MDA	Jumlah bakteri
Kontrol (-)	166 ±21	0
Kontrol (+) (bakteri)(K1)	356 ± 18	83.2 ± 4.56
Kontrol (+) (bakteri+INH)(K2)	308 ± 16	0
P1	272 ± 16	0
P2	232 ± 14	0
P3	195 ± 13	0

After the Shapiro-Wilk test on 2 variants in 6 groups, all data have met the parametric prerequisite test, which is the data proven to be spread following the normal distribution. Furthermore, the data is ready to be further analyzed by parametric statistical tests. One way Anova test was then performed between the control groups namely positive control (K1) (mice infected with H37Rv bacteria), positive control group (K2) (mice infected with H37Rv bacteria and received INH treatments 6mg / day + Rifampicin 10mg / day), to prove the effect of the treatment of *Mycobacterium tuberculosis* H37Rv and the combination of *Mycobacterium tuberculosis* H37Rv + INH treatment at a dose of 6 mg / day, Rifampicin 10 mg / day. Significant differences were found in levels of MDA, IL-10 and IL-17 between control groups.

In the one way ANOVA test between treatment groups namely group P1 (mice infected with H37Rv bacteria and receiving INH treatment 6mg / day + Rifampicin 10mg / day + Zinc 50mg / kg weight / day), P2 group (mice infected with H37Rv bacteria and receiving H37Rv bacteria) INH treatment 6mg / day + Rifampicin 10mg / day + Zinc 100mg / kg weight / day), and group P3 (mice infected with H37Rv bacteria and receive INH treatment 6mg / day + Rifampicin 10mg / day + Zinc 200mg / kg weight / day).

Table 2. Duncan test results among groups

Kelompok pengamatan	Rerata±SD	p-value
Control(+) (bakeri)	356±18[a]	0.000<∝
Control (+) (bakteri+INH)	308±16[b]	
P1	272±16[c]	
P2	232±14[d]	
P3	195±13[e]	

Table 2 shows the results of the Duncan test between the positive control group and the three treatment groups. The apparent value of MDA levels in the positive control group (K1) is greater than the three treatment groups P1, P2, or P3. This means that the combination treatment of *Mycobacterium tuberculosis* H37Rv + INH treatment 6mg / day + Rifampicin 10mg / day + Zinc in male wistar rats is proven to reduce MDA levels when compared with mice infected with H37Rv bacteria. There was also a significant difference in the mean MDA level between the positive control group (K2) (308 ± 16b nmol / mL) and the P1 group (272 ± 16c nmol / mL), with the P2 group (232 ± 14d nmol / mL), and also different from the P3 group (195 ± 13e nmol / mL). The apparent value of MDA levels in the positive control group (K2) is greater than the three treatment groups P1, P2, or P3. This means that the combined treatment of *Mycobacterium tuberculosis* H37Rv + INH treatment 6mg / day + Rifampicin 10mg / day + Zinc in male wistar rats is proven to reduce MDA levels when compared with mice infected with H37Rv bacteria and receive INH treatments 6mg / day + Rifampicin 10mg / day proven to reduce MDA levels when compared with mice infected with H37Rv bacteria and receive INH treatments 6mg / day + Rifampicin 10mg / day /day. So it was proven the first sub-hypothesis, there were differences in MDA levels between the control group compared with the tuberculosis group in male wistar rats that had been given zinc supplementation

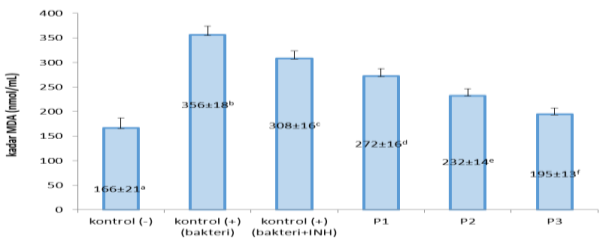

Figure 1.

Figure 1 shows the highest MDA levels in the control group (+) (bacteria) compared to the other groups. This means that the treatment of *Mycobacterium tuberculosis* H37Rv in male wistar rats will cause high MDA levels, or in other words mice will become tuberculosis. While the average stem MDA levels were slightly lower than the average stem of the control group (+) (bacteria) was the control group (+) (bacteria + INH + Rifampicin). This means that the treatment of INH treatment dose of 6 mg / day + Rifampicin dose of 10 mg / day can suppress the increase in MDA levels in male Wistar tuberculosis rats. Also visible rods and MDA levels in group P1, group P2, and group P3 showed lower than the control group (+) (bacteria) and the control group (+) (bacteria + INH + Rifampicin). Means that there is an effect of zinc supplementation can reduce MDA levels in tuberculosis male wistar rats. The higher the dose given, the lower the MDA level in tuberculosis male wistar rats.

On the lowest mean bar of MDA level is the control group (-) (healthy mice). Whereas the closest bar of MDA level to the control group (-) which is (166 ± 21a nmol / mL) the mean bar of group P3 (195 ± 13f nmol / mL). So the zinc dose of 200mg / kg / day is considered as the most optimum dose in reducing MDA levels in tuberculosis male Wistar rats.

Table 3. Mean number of *Mycobacterium tuberculosis* among groups

Kelompok pengamatan	Rerata ± SD
Control (-)	0
Control (+) (bakteri)	83.2±4.66
Control (+) (bakteri+INH)	0
P1	0
P2	0
P3	0

Table 3 shows that in male Wistar rats with tuberculosis a number of *Mycobacterium tuberculosis* were found with an average ± standard deviation of 83.2 ± 4.66. Whereas in the positive control group K2, a number of *Mycobacterium tuberculosis* was not found. It is suspected that the administration of INH treatment dose of 6mg / day + Rifampicin 10mg / day can suppress the growth of *Mycobacterium tuberculosis* in male Wistar tuberculosis rats. Likewise in the P1 group, P2 group, and P3 group there were no Mycobacterium tuberculosis found. It is suspected that the administration of a combination of 6 mg / day INH treatment + 10 mg / day Rifampicin + zinc dose 50 mg / kg / day, 100 mg / kg / day dose, and 200 mg / kg / day dose can suppress *Mycobacterium tuberculosis* growth in male wistar rats tuberculosis . So the dose of zinc supplementation that can reduce the number of bacteria is a dose of 50 mg / kg / day, a dose of 100 mg / kg / day, and a dose of 200 mg / kg / day. In other words, all these doses are able to suppress the growth of *Mycobacterium tuberculosis* in male tuberculosis wistar rats. So the twelfth sub-

hypothesis is proven, that is suspected to be found the optimum dose of zinc supplementation able to reduce the number of *Mycobacterium tuberculosis* in male Wistar tuberculosis rats.

DISCUSSION:

In this study, Zinc was given a dose of Zinc at a dose of 50 mg / kg / day, a dose of 100 mg / kg / day, and a dose of 200 mg / kg / day proved to be able to reduce levels of MDA and the number of *M tuberculosis* bacteria in male wistar rats infected with *Mycobacterium tuberculosis* H37Rv + INH treatment 6mg / day + Rifampicin 10mg / day. Provision of Zinc in TB patients has been shown to reduce levels of oxidative stress in TB patients in several studies. In another study conducted by Suparno et al, a comparison of MDA and Zinc levels was performed in multi drug resistant tuberculosis and sensitive tuberculosis patients. In this study, there were no significant differences in MDA levels in the MDR-TB group and the TB-sensitive group. In this study, MDA levels were probably influenced by high zinc levels in patients with MDR-TB, where Zinc inhibits the nicotinamide oxidase of Adenine Dinucleotide Phosphate (NADPH). Zinc acts as an antioxidant that stops free radical reactions (Suparno, et al., 2018). Different results obtained in the study conducted by Visser et al in 2010, found no significant difference in the success of the treatment of TB patients. This study provides Zinc and vitamin A to TB patients, then monitors the progress of treatment for 8 weeks. However, the researchers stated that there were many confounding factors in this study, especially in the severity of patients and when uncontrolled sampling (Visser, et al., 2011).

The number of tuberculosis bacteria is one of the parameters used in research to assess the response of treatment. This study showed that in male Wistar rats with tuberculosis, a number of Mycobacterium tuberculosis was found with an average ± standard deviation of 83.2 ± 4.66. Whereas in the positive control group K2, a number of Mycobacterium tuberculosis was not found. It is suspected that the administration of INH treatment dose of 6mg / day + Rifampicin 10mg / day can suppress the growth of Mycobacterium tuberculosis in male Wistar tuberculosis rats. Likewise in the P1 group, P2 group, and P3 group there were no *Mycobacterium tuberculosis* found. It is suspected that the administration of a combination of 6 mg / day INH treatment + 10 mg / day Rifampicin + zinc dose 50 mg / kg / day, 100 mg / kg / day dose, and 200 mg / kg / day dose can suppress Mycobacterium tuberculosis growth in male wistar rats tuberculosis. So the dose of zinc supplementation that can reduce the number of bacteria is a dose of 50 mg / kg / day, a dose of 100 mg / kg / day, and a dose of 200 mg / kg / day. In other words, all these doses are able to suppress the growth of Mycobacterium tuberculosis in male tuberculosis wistar rats. So the twelfth sub-hypothesis is proven, that is suspected to be found the optimum dose of zinc supplementation able to reduce the number of *Mycobacterium tuberculosis* in male Wistar tuberculosis rats.

These results are consistent with research conducted by Knegt et al in 2017, where experimental animals in the form of Wistar BALB / c rats given OAT showed a lower number of *Mycobacterium tuberculosis* colonies than those without. Identification of *Mycobacterium tuberculosis* subpopulation is one of the crucial parameters that shows the success of treatment and is widely used in research on the management of *Mycobacterium tuberculosis* infection (Knegt, et al., 2017). In this study, the result of a significant decrease in the number of *Mycobacterium tuberculosis* colonies in experimental animals given Zinc supplementation. It can be concluded that zinc supplementation can increase the immunity of experimental animals and decrease the number of *Mycobacterium tuberculosis* bacteria.

CONCLUSION:

In this study, there was a significant difference in MDA levels as a parameter of oxidative stress in TB infected mice in both the control group and the treatment group. Lower MDA levels in rats with treatment groups showed the ability of Zinc to reduce levels of oxidative stress in patients with tuberculosis.

The results of this study are in accordance with previous studies that show the function of Zinc as an antioxidant that can reduce oxidative stress in infections. This research also shows the function of Zinc in modulating the immune system. In this case, there is a response that shows an increase in the immune system in the body, thus helping in healing tuberculosis infections. However, in actual clinical settings, other comorbid conditions such as immunodeficiency, severity of tuberculosis infection and the age of the patient should be considered in determining the prognosis for successful therapy.

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