Zinc supplementation ~~in~~in ~~normal~~ conditions ~~could~~ increase~~s~~ the pro-inflammatory cytokines

Abstract

Background

Zinc supplementation in young adults has been found to increase~~s~~ dose-dependent pro-inflammatory cytokines. Increased pro-inflammatory cytokine have been found in severe sepsis and~~dan~~ shock condition~~s~~.

Objective

To determine the effect of zinc administration on normal conditions on proinflammatory cytokines compared to sepsis and normal conditions.

Methods

A total sample of 40 rats were randomized into four~~4~~ control groups, LPS (lipopolysaccharide), LPS-Zinc, and Zinc. Placebo normal saline ~~intravenous~~ was given intravenously to the control and Z~~z~~inc groups, whereas in the LPS and LPS-Zinc groups, intravenous *E. coli* LPS ~~*E coli*~~ was given. Blood collection was carried out at the second hour after administration to measure zinc levels, and ~~the~~ an oral distilled water~~aquabidest~~ placebo was given to the control group and LPS, while the LPS-Zinc and Zinc groups were given ~~oral~~ zinc supplementation orally for 3 days. At 8, 24 ~~hours~~ and 72 hours, blood was collected ~~again~~ to measure TNF-α and IL-6 levels. At the 72nd hour, blood was also taken to measure zinc levels. Examination of TNF-α and IL-6 levels use~~ds~~ the ELISA Sandwich technique, while zinc levels used atomic absorption spectroscopy ~~AAS~~.

Results

The TNF-α level in the Zinc group at 72nd hour was higher than the control group, while the Z~~z~~inc ~~IL-6~~ group's IL-6 level was higher than the control group at 8th and 24th hours. The levels of ~~TNF-α and IL-6 Zinc group~~ TNF-α and IL-6 in the Zinc group were lower than LPS and LPS-~~Seng~~ Zinc groups

Conclusion

Zinc supplementation under normal conditions increases the cytokines of TNF-α and IL-6.

Keywords: *Zinc, normal conditions, TNF-α, IL-6*

Background

Zinc supplementation has been~~is~~ used as an ~~an~~ additional ~~to the~~ therapy for~~in~~ certain conditions, namely sepsis, diarrhea, pneumonia, often resulting in ~~and~~ a decrease in the incidence and mortality rate of these~~at~~ disease~~s~~~~e.~~ (Black 2003; Fischer Walker et al. 2009). Zinc ~~as~~ supplementation has been used~~given in~~ several conditions, such~~namely~~ as sepsis, malnutrition, diarrhea, sickle c~~s~~ell disease, ~~and~~ in the elderly (~~Ganatra et al. 2017;~~ As'ad & Yusuf 2003; Bao et al. 2008; Ganatra et al. 2017; Prasad et al. 2007). Zinc supplementation in sickle cell~~s~~ patients can reduce levels of TNF-α~~:~~ ~~whereas~~ in the elderly ~~there is~~ a decrease in TNF-α, oxidative stress, and increased IL-2 were seen (Bao et al. 2008; Prasad et al. 2007). ~~Zinc supplementation in normal conditions or in healthy subjects can be used as suppression of cytokines when monocytes from the subject are exposed to~~

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~~LPS~~Further,~~.~~ z~~Z~~inc supplementation in healthy older subjects can increase immune reactions to invading pathogens. Mild zinc deficiencies~~y~~ can be found in the elderly~~–~~ (Kahmann et al. 2008). In healthy young subjects, z~~Z~~inc~~—~~ supplementation ~~to healthy young subject~~ can increase the expression of TNF-α and IL-1β when monocytes~~—~~ of subjects are exposed to lipopolysaccharides (LPSs)~~–~~ (Aydemir et al. 2006).

The p~~P~~ro-inflammatory effects of zinc are mediated through tyrosine phosphorylation and induction of protein kinase C. Tyrosine phosphorylation is used for signal induction of TLR-4 by ~~lipopolysaccharide (~~LPSs. Tyrosine phosphorylation is performed by protein tyrosine kinase (PTK) and is degraded by protein tyrosine phosphatase (PTP)~~; and zz~~inc is a potent inhibitor of PTP~~–~~ (Haase & Rink 2007). Zinc also has the direct effect of stimulating monocytes to secrete IL-1, IL-6, TNF-α and IFN-γ~~–~~ (~~Rink & Kirchner 2000;~~ Haase & Rink 2007~~; Rink & Kirchner 2000~~).

TNF-α and IL-6 are pro-inflammatory cytokines that can distinguish survivors and non-survivors at 28 days and as predictors of shock sepsis, MOF and DIC~~—~~ (Pierrakos & Vincent 2010). Increased IL-6 in severe sepsis causes increased capillary leakage and decreased intestinal contraction~~–~~ (Krüttgen & Rose-John 2011; Nullens et al. 2016).~~—~~ TNF-α has an important role in inflammation, and administration of rh-TNF in experimental animals can cause symptoms of hypotension, metabolic acidosis, massive pulmonary bleeding, acute tubular necrosis in the kidneys and gastrointestinal bleeding lesions (Bauss et al. 1987; Spooner et al. 1992).

Zinc supplementation in sepsis induced by intravenous *E. coli* LPSs ~~*E coli*~~ can reduce levels of pro-inflammatory cytokines TNF-α and IL-6, ~~so it can~~ improving~~e~~ the condition of sepsis ~~by comparing pro-inflammatory cytokines in the sepsis group and~~in the sepsis group given zinc~~—~~ (Utomo et al. 2019). Zinc administration in healthy young adults can increase pro-inflammatory cytokine ~~that depend on~~in a dose-dependent manner ~~that was given~~ (Foster & Samman 2012). The purpose of this study was to examine the effect of zinc supplementation in normal subjects~~conditions~~ on proinflammatory cytokine levels~~s~~.

Materials and methods

Preparation of animal study

This study was given ethical clearance~~, proven with~~ number 700-KEP-UB. This study used 40 Dawley spra~~g~~que rats aged 10~~–~~12 weeks, which ~~ands~~ were acclimatized for 2 weeks at the University of Brawijaya Bioscience Institute before being used as experimental animals. Rats we~~a~~re kept in a wire-covered, ~~-~~ventilated plastic enclosure, ~~ventilated with~~on a 12-hour bright lighting~~/ system and~~ 12 hours dark cycle and equipped with a place to eat and drink. The rats were ~~and lay the husks~~ fed standard pellets and drink water ad libitum and husk bedding~~s~~ ~~are~~ was replaced every 2 days.~~,~~ Prior to treatments, the rats~~then they~~ were allocated randomly to 4 groups~~,:~~ namely control, LPS, LPS-Zinc and Zinc, with 10 animals each. The same method was also used in a~~in~~ previous study~~ies~~ (Utomo et al. 2019).

Provision of LPS *E. coli* serotype O11: B4 Sigma at a dose of 10 mg~~-~~/~~-~~kg intravenously was done by means of a 500 mg preparation~~; was taken~~ 100 mg of the preparation was~~ then~~ diluted with 10 mL ~~aquabidest~~ distilled water and injected into rats in the LPS and LPS Zinc groups in~~of~~ 0.2 ml doses. The control group and zinc group were given normal saline 0.2 mL intravenously. To facilitate intravenous injection, a 0.1 mL intramuscular ketamine injection was given. After two hours, blood from 5 rats were collected to measure the zinc serum concentration, using the measuring technique described below.

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Oral administration of zinc was done by mixingmixing zinc drop with a content of 1 mL of 10 mg / mL of zinc sulfphate and , taken 1 mL and then added aquabidest 9 mL distilled water to make the content of 1 mg/mL. Zinc was is given at a dose of 2 mg/kg body weight (which is comparable with 4.65 mg / kg BB rats through the sonde in the 1 mL LPS zinc and Zinc group), while the control group and LPS are were given 1 mL aquabidestdistilled water. At the 8th hours post administration, bloods was were collected and measured the level of TNF- α and IL-6 were measured using ELISA as described below. The previous steps were done again after 24 hours and 48 hours. At the 48th hours, bloods were collected to measure the zinc, TNF- α and IL-6 levels.

Serum Collection

As noted above, aAt hour 2, 2 mL of blood was withdrawnm through the veins of rats tail veins of the rats in first 5 rats in each group to check the zinc levels. At hour 8 and hour 24, 2 mL of blood was withdrawn in the same manner the same procedures were done in the second 5 rats of each group to check the TNF-α and IL-6 cytokine levels. The groups were split to avoid

Based on the ethical feasibility of taking blood should not be more than 1 x in 24 hours to avoid the shock conditions in the animal from too-frequent blood withdrawals. so that each group is divided into 2, namely for taking zinc serum in the second hour and the 8th hour for examination of cytokines TNF-α and IL-6, at 24 hours taken from 2nd 5 rats for equality of cytokine data from the same sample. At hour 72 at the end of the experiment, blood was taken from the heart and the aorta after the rats were killed and check for zinc level, TNF-α an IL-6 levels.

The withdrawn blood the was inserted in tube without EDTA and then centrifuged 6,000 x g for 10 minutes., Tthe serum was removed and taken then leaftve at 25 °C in 30 minutes then stored in the refrigerator freezer at -20 °C.

Serum zinc level

Zinc content was examined by thae Atomic aAbsorption sSpectrophotometry (AAS) in accordance with the previous method (Smith et al. (1979). For AAS, first we made the standard 1000 mg of zinc per liter standard were made: Dilute 10 mL of nitric acid was diluted to 50 mL, into which dissolve 1.000 g of zinc metal was added and dissolved in this and further diluted to 100 mL. (b) Working standards, 100, 200, 300, and 400 µg of zinc per liter were prepared; Deliver 1 mL of 1000 mg/L zinc standard were added into a 100-mL volumetric flask and dilute to volume with a glycerol/water solution (5/95 by vol), and mMixed by inverting at least 16 times. Place Aaliquots of this intermediate stock (1, 2, 3, and 4 mL) were placed into four 100-mL volumetric flasks and dilute to volume with the glycerol/water mixture. The standards (0.1, 0.2, 0.3, and 0.4 mg of zinc per liter) correspond to apparent plasma zinc concentrations of 500, 1000, 1500, and 2000 µg of zinc per liter. APrepare a working curve was prepared daily from fresh standards, and Calculate the concentration of zinc in the plasma was calculated directly from the curve.

To determine serum zinc levels,Collect 2 mL of whole blood was collected in a tube by cutting the tip of rat tail. Add Ttwo drops (~50 µL) of a 300 g/L sodium citrate solution was added to the tube before collecting the specimen of blood. The blood was cCentrifuged the blood promptly at 6,000 x g for 10 minutes.

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A total of~~Deliver~~ 0.5 mL of plasma sample was delivered with a serological pipette into a 16~~-~~mm plastic test tube. Next, ~~Add~~ 2.0 mL of de-ionized water was added and immediately mixed into the solution for 30 s. The zinc levels were t~~T~~hen read with the spectroph~~f~~otometry to compare with the standard.

Cytokine analysis

Pro-inflammatory cytokine concentrations of TNF-α and IL-6 were carried out by the sandwich-ELISA method in accordance with the manuals of the FineTest ELISA Manual Kit Kit instruction from Wuhan Fine Biological Technology Co., Ltd. The ELISA kit of Rat IL-6 code ER0042 size 96 T batch R0042C046~~–~~ and Rat TNF-α ELISA Kit code ER1393 size 96 T batch R1393C064 were used in this study.

Assay procedure

Before adding reagents into wells, ~~equilibrate~~ TMB s~~S~~ubstrate was equilibrated for 30 min at 37 °C. ~~Set~~ S~~s~~tandard, test sample and control (zero) wells were set on the pre-coated plate with antibody anti TNF-α and IL-6 respectively, and then, ~~record~~ their positions recorded. Each standard and sample was~~ere~~ measured in duplicate. The ~~Wash~~ plate was washed two~~2~~ times before adding standard, sample and control (zero) wells. Next,~~Aliquot~~ 0.1 ml of ~~,~~1000 ~~pg/ml~~, 500 ~~pg/ml~~, 250 ~~pg/ml~~, 125 ~~pg/ml~~, 62.5 ~~pg/ml~~, 31.25 ~~pg/ml~~, and 15.625 pg/ml~~,~~ standard solutions were added into the standard wells.~~–~~ Then,~~Add~~ 0.1 ml of Sample/Standard Dilution Buffer was added into the control (zero) well. Finally, ~~Add~~ 0.1 ml of properly diluted rat serum was added into the test sample wells.~~—~~ T~~Seal t~~he plate was sealed with a cover and incubated at 37 °C for 90 minutes.~~—~~ ~~Remove~~ T~~t~~he cover was removed and ~~discard~~ the plate contents discarded, and the ~~wash~~ plate was washed two~~2~~ times with w~~W~~ash b~~B~~uffer.

~~Add~~ Next, 0.1 ml b~~B~~iotin-labeled antibody working solution was loaded into the ~~above wells~~ ~~(~~standard, test sample & zero wells~~)~~. ~~Add~~ T~~t~~he solution was added to~~at~~ the bottom of each well without touching the sidewalls.~~—~~ T~~Seal t~~he plate was sealed with a cover and incubated at 37 °C for 60 min.~~—~~ ~~Remove~~ T~~t~~he cover, was removed and the plate ~~and~~ washed plate three~~3~~ times with w~~W~~ash b~~B~~uffer, with ~~and let the~~the ~~wash~~ buffer allowed to stay in the wells for 1 minute each time. A total of ~~Add~~ 0.1 ml of SABC w~~W~~orking s~~S~~olution was added into each well, ~~cover~~ then the plate was covered and incubated at 37°C for 30 minutes.~~—~~ The plate was ~~Remove the cover and~~ washed five~~plate 5~~ times with wa~~W~~ash b~~B~~uffer, and ~~let~~ the wash buffer allowed to stay in the wells for 1~~–~~2 minutes each time.

A total of ~~dd~~ 90 µl TMB Substrate was added into each well, ~~cover~~ the plate covered and incubated at 37°C in dark for~~within~~ 15~~–~~30 minutes. It will turn blue in the first 3~~–~~4 wells. ~~Add~~ Next, 50 µl s~~S~~top s~~S~~olution was added into each well and mixed ~~them~~ thoroughly. The color changed~~s~~ to yellow immediately.~~—~~ ~~Read~~ T~~t~~he O.D. absorbance at 450 nm in a Microplate Reader was read immediately after adding the stop solution.~~—~~ ~~Regarding calculation,~~ T~~(~~the relative O.D. at 450 nm was~~)~~ calculated as~~=~~ ~~(~~the O.D. 450 nm of each well~~)~~ – ~~(~~the O.D. 450 nm of the z~~Z~~ero well~~)~~. The standard curve can be plotted as the relative O.D. 450nm of each standard solution (Y) vs. the respective concentration of the standard solution (X). The TNF-α and IL-6 concentration of the samples can be interpolated from the standard curve.

Statistical analysis

Data is presented as mean ± SD. Data in the four~~4~~ groups were analyzed by ~~the~~ one-way

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ANOVA ~~method~~ and post -hoc tests if the distribution was normal;~~,~~ whereas if the data~~it~~ w~~a~~ere~~s~~ not normal then Kruskal-Wallis test was used. Data at 8th, 24th and 72nd hours were analyzed by ANOVA ~~and subjects~~ if ~~the~~ normally distributed~~ion~~ and the Friedman test if~~were~~ not normally distributed. Zinc level data at 2 and 72 hours were analyzed by t-~~-~~dependent test. SPSS v. 21 was used ~~to calcaulate~~for the analyse~~i~~s.

Results

In this study, each group of samples ~~was~~ contained ~~of~~ five rats and was done in duplicate~~o~~ to obtain~~et~~ enough blood for the analyses.

~~In this study the minimal sample for each group was 5 and duplicated to 10 because of blood sample collection more than 1 times in 24 hours.~~ The 2-~~-~~hour~~s~~ samplesing ~~to~~ reflected the condition of zinc serum after LPS and placebo administration, the 8-hour~~s~~ samplesing ~~to~~ showed the effect of zinc o~~i~~n the pro-inflammatory cytokine levels. These groups ~~i~~are~~s~~ comparable as ~~the result of the t~~Table 1 shows that there were ~~that~~ ~~showed~~ no differences in the body weight of the rats in the four~~4~~ groups.

Table 1. Mean r~~R~~at ~~'s~~ body weights in the four treatment ~~4~~ groups

		Body weight (g)				
Group	**n**	**Mean**	**SD**	**Minimum**	**Maximum**	**p**
Control	5	221.~~,~~40	6.~~,~~50	213	229	
LPS	5	240.~~,~~80	10.~~,~~50	225	253	0.~~,~~336
LPS-Zinc	5	226.~~,~~60	19.~~,~~46	208	256	
Zinc	5	232.~~,~~80	24.~~,~~81	201	263	

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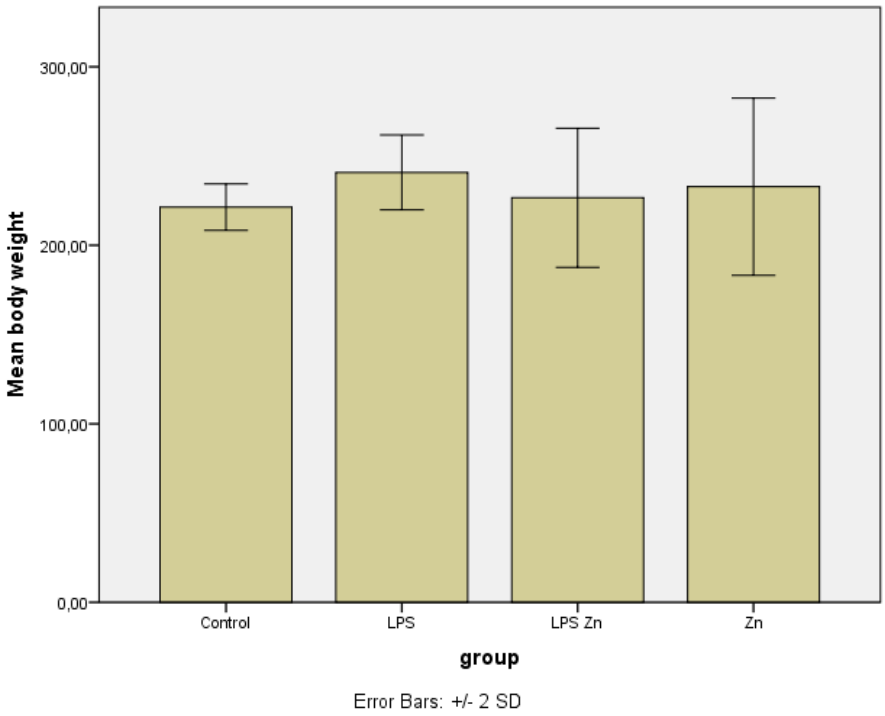

Figure 2. Mean b~~B~~ody weights of the ~~subject~~treatment groups.

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~~A~~ The three~~3~~-day zinc supplementation significantly increased zinc serum levels in the z~~Z~~inc group. From Table 2 and Figure 2, the results showed that zinc levels at~~in the second~~ hour 2 were not~~had no~~ differen~~t~~ce between the four~~4~~ groups, but~~and~~ found an increase in zinc level at~~in the 72nd~~ hour 72 compared to the 2nd hour in the LPS, LPS-Zinc, and Zinc groups. At ~~t~~The 72 hour 72, z~~Z~~inc levels in the Zinc group was significantly increased compared to the other three groups, which showed that administering~~giving~~ zinc for three~~3~~ days was able to significantly increase zinc levels.

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~~This study showed that administration of zinc under normal conditions represented in the zinc group. Zinc levels in zinc gruop did not differ from the control group at 2 hours after administration of normal saline intravenously. In this study the zinc level in the 72nd hours of zinc group increased significantly compared to the 2nd hour and at the 72nd hour the zinc group zinc level was higher than the control group which showed zinc administration for 3 days could increase zinc levels~~

Table 2. Plasma Zinc levels at 2 hours and 72 hours after *E. coli* LPS ~~*E coli*~~ administration.

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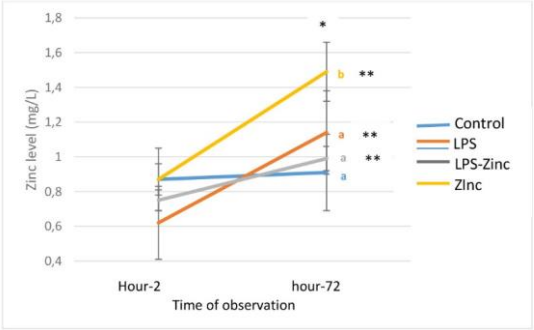
Group	n	Plasma zinc (mg/L)		p
		2 hours	72 hours	

Control	5	0,87 ± 0,09 (0,75–0,97)	0,91 ± 0,22[a] (0,57–1,19)	0,670
LPS	5	0,62 ± 0,21 (0,42–0,97)	1,14 ± 0,24[a] (0,94–1,54)	0,039**
LPS-Zinc	5	0,75 ± 0,06 (0,71-0,86)	0,99 ± 0,07[a] (0,94–1,11)	0,043**
Zinc	5	0,87 ± 0,18 (0,62–1,12)	1,49 ± 0,17[b] (1,33–1,74)	0,001**
p		0,073	0,001*	

*– Significant at α=0,05

**– Significant at α=0,05 (Paired t-test/Wilcoxon Signed Rank Test)

[a,b] *Same superscript* in one column showed no difference between groups *(multiple comparisons* LSD)



*– Significant at α=0,05

a,b Same letters in same hour showed no difference between groups (*multiple comparisons* LSD)

**– Significant at α=0,05 (Paired t-test/Wilcoxon Signed Rank Test)

Figure 2. Serum zinc levels at hours 2 and 72 after administration.

From Ttable 3 and Ffigure 3 show, it was found that in the ZZinc group and the control there wasas no difference– of TNF-α level at the 8th and 24th hours, whereas atin the 72nd hour there was a difference in the levels of TNF-α, which wereas higher in the Zinc group compared to the controls, but the levels were still lower than in the LPS group and LPS-zinc.

Table 3. TNF-α plasma concentration at 8, 24, and 72 hours

Group	n	TNF-α plasma concentration (pg/mL)			p
		8 hours	24 hours	72 hours	
Control	5	79,95 ± 2,04[a] (76,55–81,55)	81,55 ± 1,30[a] (80,55–84,05)	76,35 ± 3,19[a] (71,55–80,55)	0,029**
LPS	5	122,55 ± 11,40[c]	110,05 ± 5,48[c]	108,05 ± 4,87[d]	0,034**

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		(111,55–136,55)	(106,55–119,05)	(101,55–114,05)	
LPS-Zinc	5	92,75 ± 1,25[b] (91,55–94,55)	95,35 ± 2,17[b] (91,55–96,55)	93,35 ± 2,49[c] (91,55–96,55)	0,211
Zinc	5	77,85 ± 4,15[a] (71,55–81,55)	76,55 ± 6,12[a] (71,55–86,55)	82,35 ± 4,27[b] (76,55–86,55)	0,101
p		0,000*	0,001*	0,001*	

Note:

*– Significant at α=0,05 (Brown–Forsythe/Kruskal–Wallis)

**– Significant at α=0,05 (Same-subject ANOVA~~nova~~/ANOVA~~nova~~ Friedman)

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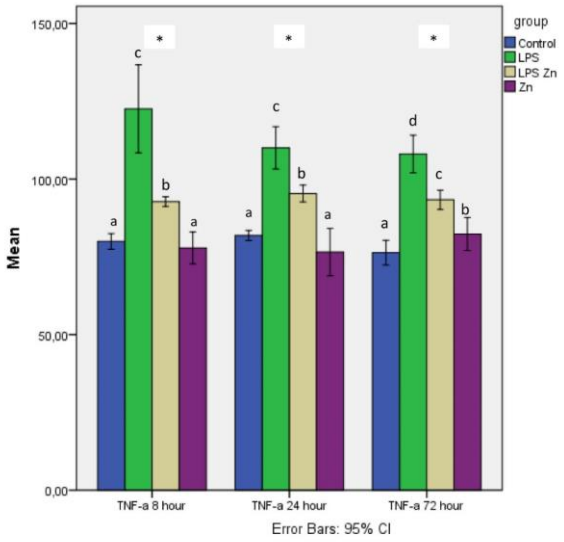

*significant at α = 0,05

[a,b,c,d] Same letters in the same hour revealed no difference between groups (*multiple comparisons* Games–Howell/Mann–Whitney)

Figure 3. TNF-α levels in treatments after 8, 24 and 72 hours.

IL-6 levels in the Zinc group were higher than the control group at 8th and 24th hours, but lower than the LPS and LPS-zinc groups. At 72 hours, the IL-6 level in the Zinc group did not differ significantly from the control group. This showed the effect of zinc administration in normal conditions, in that there was an increased in levels of IL-6 cytokines at 8 and 24 hours, but the effect disappeared at 72 hours.

Table 4. IL-6 plasma concentration at 8, 24, and 72 hours

Group	n	IL-6 plasma concentration (pg/mL)	p

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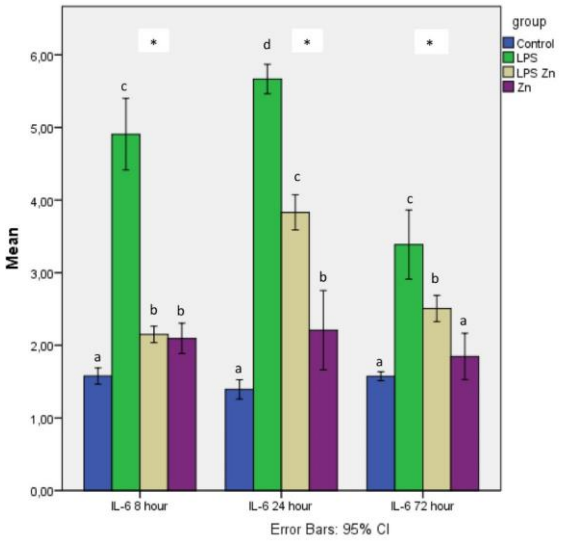
		8 hours	24 hours	72 hours	
Control	5	1,58 ± 0,09[a] (1,42–1,64)	1,39 ± 0,11[a] (1,31–1,55)	1,57 ± 0,05[a] (1,53–1,63)	0,091
LPS	5	4,91 ± 0,40[c] (4,25–5,21)	5,57 ± 0,16[d] (5,54–5,93)	3,39 ± 0,38[c] (3,09–4,05)	0,000**
LPS-Zinc	5	2,15 ± 0,09[b] (2,06–2,25)	3,83 ± 0,19[c] (3,64–4,11)	2,51 ± 0,15[b] (2,34–2,74)	0,000**
Zinc	5	2,10 ± 0,17[b] (1,91–2,27)	2,21 ± 0,44[b] (1,81–2,72)	1,84 ± 0,26[a] (1,55–2,14)	0,304
p		0,000*	0,000*	0,001*	

Note:
*– Significant at α=0,05 (Brown–Forsythe/Kruskal–Wallis)
**– Significant at α=0,05 (Same subject ANOVAnova)
[a,b,c,d] Same superscript in a column revealed no difference between groups (*multiple comparisons* Games–Howell/Mann–Whitney)



*significant at α = 0,05
a,b,c,d Same letters in the same hour revealed no difference between groups (*multiple comparisons* Games-Howell/Mann-Whitney)

Figure 4. IL-6 levels in treatments at 8, 24 and 72 hours.

Discussion

Fortification of Zinc supplementation on a diet for three days can increase zinc levels in rats previously given a diet that is deficient in zinc (Bao et al. 2010). Increased

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plasma zinc levels ~~also occur~~was also seen in other studies but with a longer duration of administration of two to four~~2~~ months ~~to 4 months (Bhandari et al. 2002(;~~ As'ad & Yusuf 2003; Bhandari et al. 2002; Bao et al. 2008; Sandstead et al. 2008). ZnT lowers intracellular zinc by efflux from cells or influx to intracellular vesicles so that extracellular or plasma levels increase, while ZIP (Zrt-and Irt-ike protein) promotes zinc transport from extracellular fluid or from intracellular vesicles to the cytoplasm so that cytoplasmic levels increase. ZnT1 mRNA levels in leukocytes increased significantly after Zn supplementation, resulting in an increase in serum zinc because ZnT promotes cell efflux from cells to extracellular or to blood circulation~~—~~ (Cousins et al. 2006). Zinc can stimulate monocytes directly to secrete TNF-α (Haase & Rink 2007).~~—~~ Zinc exposure of more than 100 µM in monocyte cells can increase levels of TNF-α (Chang et al. 2006).

Pro-inflammatory cytokine levels of TNF-α in the Z~~z~~inc group were higher than the control group at 72 hours while IL-6 cytokines in the Z~~z~~inc group were higher than controls at 8th and 24th hours, whereas at 72nd hours there were no differences between controls and Z~~z~~inc group for IL-6 levels. TNF-α levels and IL-6 Zinc groups were lower than LPS and LPS-Zinc groups.

In this study zinc administration under normal conditions could increase the levels of proinflammatory cytokines TNF-α and IL-6. TNF-α has an important role in inflammation and administration of rhTNF in experimental animals can cause symptoms of hypotension, metabolic acidosis, massive pulmonary bleeding, acute tubular necrosis in the kidneys and gastrointestinal bleeding lesions (Bauss et al. 1987; Spooner et al. 1992).~~—~~ Zinc supplementation can increase TNF-α levels. In a study conducted by Chu et al. (2015) ~~Chu 2015~~ in DM2 patients, it was found that TNF-α mRNA increased. The same thing was also found in the study conducted by Meksawan et al. (2014)~~Meksawan 2014~~, ~~which was~~ i.e., an increase in TNF-α in monocyte and lymphocyte transmembrane, which was useful as an immune response to cancer, but plasma TNF-α did not increase ~~(Chu et al. 2015; Meksawan et al. 2014)~~. Zinc supplementation can increase NFkb activation through tyrosine phosphorylation and induction of protein kinase C. Tyrosine phosphorylation is used for signal induction of TLR-4 by LPSs~~lipopolysaccharide~~. Tyrosine phosphorylation is performed by protein tyrosine kinase (PTK) and is degraded by protein tyrosine phosphatase (PTP) and zinc is a potent inhibitor of PTP (Haase & Rink 2007). Activation of NFkB after administration of zinc then downstream inflammatory mediators TNF-α, IL-6, IL-1β, and IL-8~~–~~ (Freitas & Fernandes 2011).

The increase in the IL-6 levels of the Zinc group in this study occurred at the 72nd hour. Increased IL-6 can determine SIRS due to infection and is associated with mortality (Ma et al. 2016; Remick et al. 2005). IL-6 increases also occur in severe sepsis and cause an increase in capillary leak and decrease in intestinal contractions (Krüttgen & Rose-John 2011; Nullens et al. 2016). In this study there was an increase in IL-6 in the Zinc group compared to controls, but it was still lower than the LPS and LPS-Zinc groups, so that severe symptoms also did not appear.

Low zinc levels and increased IL-6, IL-8, IL-1β, and TNF-α cytokines occur in sepsis compared to healthy individuals (Besecker et al. 2011). Low plasma zinc levels in sepsis are caused by the influence of pro-inflammatory cytokines including IL-1β and IL-6, which activate STAT-mediated signals and upregulation of ZIP14 and ZIP6, which trigger plasma zinc influx into the intracellular~~–~~ (Cousins et al. 2006).

LPS enters the system~~ic~~ and then is recognized by TLR4 of monocytes and is transmitted through signal transduction, which ~~and~~ activates Nuclear Factor kappa B (NFκB) (Schulte et al. 2013). Activated NFκB induces the expression of cytokines IL-6 (Brasier 2010), IL-8 (Elliott et al. 2001), and~~dan~~ TNF-α (Dong et al. 2010).~~–~~ LPS is bound by LBP

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(LPS Binding Protein) which is captured by TLR-4 on cell membranes then through signal MyD88, IRAKs, and recruitment of adaptors TRAF6— then recruit IKK— complex lead to phosphorylation and degradation of IkB inhibitors. NFkB is active and translocated to the nucleus to activate the pro-inflammatory cytokine gene (Alexander & Rietschel 2001).

In a review conducted by Foster & Samman (2012)~~Foster~~ of ~~in~~ several studies, it was found that zinc supplementation in normal adult conditions for 8 weeks increased IL-2, TNF-α, and IFN-γ whereas in elderly it was found that zinc supplementation for 48 days increased plasma IL-6 levels. ~~(Foster & Samman 2012).~~ In this study, the administration of zinc for ~~3~~ three days ~~turned out to~~ increased TNF-α cytokines and IL-6 levels.

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Conclusion

Zinc supplementation under normal conditions increases proinflammatory cytokines TNF-α and IL-6 so it needs a caution and need to monitor clinically for zinc supplementation under normal conditions.

Research Article

Zinc supplementation in normal conditions increases the pro-inflammatory cytokines

Martono T. Utomo[1]*, Subijanto M. Sudarmo[1], Ketut Sudiana[2]

ABSTRACT

Zinc supplementation in young adults has been found to increase dose-dependent pro-inflammatory cytokines. Increased pro-inflammatory cytokine has been found in severe sepsis and shock conditions. To determine the effect of zinc administration on normal conditions on pro-inflammatory cytokines compared to sepsis and normal conditions. A total sample of 40 rats was randomized into four control groups, lipopolysaccharide (LPS), LPS-zinc, and zinc. Placebo normal saline was given intravenously to the control and zinc groups, whereas in the LPS and LPS-zinc groups, intravenous *Escherichia coli* LPS was given. Blood collection was carried out at the 2nd h after administration to measure zinc levels, and an oral distilled water placebo was given to the control group and LPS, while the LPS-zinc and zinc groups were given zinc supplementation orally for 3 days. At 8, 24, and 72 h, blood was collected to measure tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels. At the 72nd h, blood was also taken to measure zinc levels. Examination of TNF-α and IL-6 levels used the enzyme-linked immunosorbent assay Sandwich technique, while zinc levels used atomic absorption spectroscopy. The TNF-α level in the zinc group at 72nd h was higher than the control group, while the zinc group's IL-6 level was higher than the control group at 8th and 24th h. The levels of TNF-α and IL-6 in the zinc group were lower than LPS and LPS-zinc groups. Zinc supplementation under normal conditions increases the cytokines of TNF-α and IL-6.

KEY WORDS: Interleukin-6, Normal conditions, Tumor necrosis factor-alpha, Zinc

INTRODUCTION

Zinc supplementation has been used as an additional therapy for certain conditions, namely, sepsis, diarrhea, and pneumonia, often resulting in a decrease in the incidence and mortality rate of these diseases.[1,2] Zinc supplementation has been used several conditions, such as sepsis, malnutrition, diarrhea, sickle cell disease, and in the elderly.[3-6] Zinc supplementation in sickle cell patients can reduce levels of tumor necrosis factor-alpha (TNF-α); in the elderly, a decrease in TNF-α, oxidative stress, and increased interleukin-2 (IL-2) were seen.[4,6] Further, zinc supplementation in healthy older subjects can increase immune reactions to invading pathogens. Mild zinc deficiencies can be found in the elderly.[7] In healthy young subjects, zinc supplementation can increase the expression of TNF-α and IL-1b when monocytes of subjects are exposed to lipopolysaccharides (LPSs).[8]

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The pro-inflammatory effects of zinc are mediated through tyrosine phosphorylation and induction of protein kinase C. Tyrosine phosphorylation is used for signal induction of toll-like receptor (TLR)-4 by LPSs. Tyrosine phosphorylation is performed by protein tyrosine kinase (PTK) and is degraded by protein tyrosine phosphatase (PTP); zinc is a potent inhibitor of PTP.[9] Zinc also has the direct effect of stimulating monocytes to secrete IL-1, IL-6, TNF-α, and interferon gamma (IFN-γ).[9,10]

TNF-α and IL-6 are pro-inflammatory cytokines that can distinguish survivors and non-survivors at 28 days and as predictors of shock sepsis, MOF and DIC.[11] Increased IL-6 in severe sepsis caused increased capillary leakage and decreased intestinal contraction.[12,13] TNF-α has an essential role in inflammation, and administration of recombinant human (rh-TNF) in experimental animals can cause symptoms of hypotension, metabolic acidosis, massive pulmonary bleeding, acute tubular necrosis in the kidneys, and gastrointestinal bleeding lesions.[14,15]

Zinc supplementation in sepsis induced by intravenous *Escherichia coli* LPSs can reduce levels

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Received on: 14-02-2019; Revised on: 26-03-2019; Accepted on: 29-04-2019

of pro-inflammatory cytokines TNF-α and IL-6, improving the condition of sepsis in the sepsis group given zinc.[16] Zinc administration in healthy young adults can increase pro-inflammatory cytokine in a dose-dependent manner.[17] The purpose of this study was to examine the effect of zinc supplementation in normal subjects on proinflammatory cytokine levels.

MATERIALS AND METHODS

Preparation of Animal Study

This study was given ethical clearance, number 700-KEP-UB. This study used 40 Dawley Sprague rats aged 10–12 weeks, which were acclimatized for 2 weeks at the University of Brawijaya Bioscience Institute before being used as experimental animals. Rats were kept in a wire-covered, ventilated plastic enclosure, on a 12-h bright lighting/12 h dark cycle and equipped with a place to eat and drink. The rats were fed standard pellets and drank water *ad libitum*, and husk bedding was replaced every 2 days. Before treatments, the rats were allocated randomly to four groups, namely, control, LPS, LPS-zinc, and zinc, with ten animals each. The same method was also used in a previous study.[16]

Provision of LPS *E. coli* serotype O11: B4 Sigma at a dose of 10 mg/kg intravenously was done using a 500 mg preparation; 100 mg of the preparation was diluted with 10 mL distilled water and injected into rats in the LPS and LPS zinc groups in 0.2 mL doses. The control group and zinc group were given normal saline 0.2 mL intravenously. To facilitate intravenous injection, a 0.1 mL intramuscular ketamine injection was given. After 2 h, blood from five rats was collected to measure the zinc serum concentration, using the measuring technique described below.

Oral administration of zinc was done by mixing 1 mL of 10 mg/mL of zinc sulfate and 9 mL distilled water to make the content of 1 mg/mL. Zinc was given at a dose of 2 mg/kg body weight (which is comparable with 4.65 mg/kg BB rats through the sonde in the 1 mL LPS zinc and zinc group) while the control group and LPS were given 1 mL distilled water. At the 8th h postadministration, blood was collected, and the level of TNF-α and IL-6 was measured using enzyme-linked immunosorbent assay (ELISA) as described below. The previous steps were done again after 24 h and 48 h. At the 48th h, blood was collected to measure the zinc, TNF-α, and IL-6 levels.

Serum Collection

As noted above, at 2nd h, 2 mL of blood was withdrawn through the tail veins of the rats in the first five rats in each group to check the zinc levels. At h 8 and h 24, 2 mL of blood was withdrawn in the same manner in the second five rats of each group to check the TNF-α and IL-6 cytokine levels. The groups were split to avoid shock conditions in the animal from too-frequent blood withdrawals. At the end of the experiment (72 h), blood was taken from the heart and the aorta after the rats were killed and check for zinc, TNF-α IL-6 levels.

The withdrawn blood was inserted in the tube without EDTA and then centrifuged 6,000 x g for 10 min. The serum was removed and left at 25°C in 30 min then stored in the freezer at −20°C.

Serum Zinc Level

Zinc content was examined by atomic absorption spectrophotometry (AAS) by Smith *et al*. For AAS, 1000 mg of zinc per liter standard were made: 10 mL of nitric acid was diluted to 50 mL, into which 1.000 g of zinc metal was added and dissolved and further diluted to 100 mL. (b) Working standards, 100, 200, 300, and 400 µg of zinc per liter were prepared: 1 mL of 1000 mg/L zinc standard were added into a 100-mL volumetric flask and diluted to volume with a glycerol/water solution (5/95 by vol), and mixed by inverting at least 16 times. Aliquots of this common stock (1, 2, 3, and 4 mL) were placed into four 100-mL volumetric flasks and dilute to volume with the glycerol/water mixture. The standards (0.1, 0.2, 0.3, and 0.4 mg of zinc per liter) correspond to apparent plasma zinc concentrations of 500, 1000, 1500, and 2000 µg of zinc per liter. A working curve was prepared daily from fresh standards, and the concentration of zinc in the plasma was calculated directly from the curve.

To determine serum zinc levels, 2 mL of whole blood was collected in a tube by cutting the tip of the rat tail. Two drops (~50 µL) of a 300 g/L sodium citrate solution were added to the tube before collecting the specimen of blood. The blood was centrifuged promptly at 6000 × g for 10 min. A total of 0.5 mL of plasma sample was delivered with a serological pipette into a 16 mm plastic test tube. Next, 2.0 mL of de-ionized water was added and immediately mixed into the solution for 30 s. The zinc levels were then read with the spectrophotometry to compare with the standard.

Cytokine Analysis

Pro-inflammatory cytokine concentrations of TNF-α and IL-6 were carried out by the sandwich-ELISA method by the manuals of the FineTest ELISA Manual Kit Kit instruction from Wuhan Fine Biological Technology Co., Ltd. The ELISA kit of Rat IL-6 code ER0042 size 96 T batch R0042C046 and Rat TNF-α ELISA Kit code ER1393 size 96 T batch R1393C064 were used in this study.

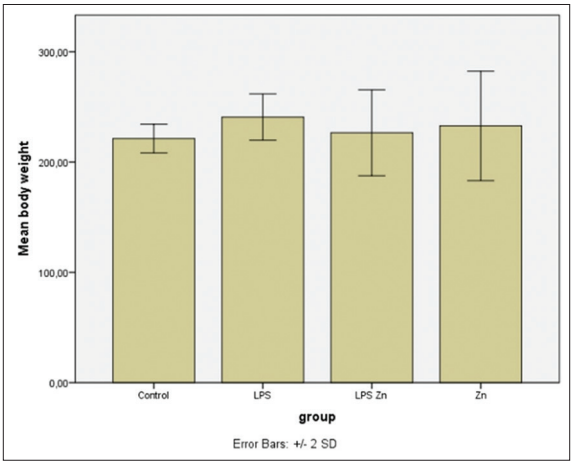
Assay procedure

Before adding reagents into wells, TMB substrate was equilibrated for 30 min at 37°C. Standard, test sample AQ2

and control (zero) wells were set on the pre-coated plate with antibody anti-TNF-α and IL-6 respectively, and then, their positions recorded. Each standard and sample was measured in duplicate. The plate was washed 2 times before adding standard, sample, and control (zero) wells. Next, 0.1 ml of 1000, 500, 250, 125, 62.5, 31.25, and 15.625 pg/mL standard solutions were added into the standard wells. Then, 0.1 ml of Sample/Standard Dilution Buffer was added into the control (zero) well. Finally, 0.1 ml of properly diluted rat serum was added to the test sample wells. The plate was sealed with a cover and incubated at 37°C for 90 min. The cover was removed and the plate contents discarded, and the plate was washed 2 times with wash buffer.

Next, 0.1 ml biotin-labeled antibody working solution was loaded into the standard, test sample, and zero wells. The solution was added to the bottom of each well without touching the sidewalls. The plate was sealed with a cover and incubated at 37°C for 60 min. The cover was removed, and the plate washed plate 3 times with wash buffer, with the buffer allowed to stay in the wells for 1 min each time. A total of 0.1 ml of SABC working solution was added into each well; then, the plate was covered and incubated at 37°C for 30 min. The plate was washed 5 times with wash buffer, and the wash buffer allowed to stay in the wells for 1–2 min each time.

A total of 90 µl TMB substrate was added into each well, the plate covered and incubated at 37°C in the dark for 15–30 min. It will turn blue in the first 3–4 wells. Next, 50 µl stop solution was added into each well and mixed thoroughly. The color changed to yellow immediately. The O. D. absorbance at 450 nm AQ2 in a microplate reader was read immediately after adding the stop solution. The relative O. D. at 450 nm was calculated as the O. D. 450 nm of each well – the O. D. 450 nm of the zero well. The standard curve can be plotted as the relative O. D. 450 nm of each standard solution (Y) versus the particular concentration of the standard solution (X). The TNF-α and IL-6 concentration of the samples can be interpolated from the standard curve.



AQ3 **Figure 1:** Mean body weights of the treatment groups

Statistical Analysis

Data are presented as mean + standard deviation. One-way ANOVA analyzed data in the four groups and *post hoc* tests if the distribution was normal; whereas if the data were not normal then the Kruskal–Wallis test was used. Data at 8th, 24th, and 72nd h were analyzed by ANOVA if normally distributed and the Friedman test if not normally distributed. The t-dependent test analyzed zinc level data at 2 and 72 h. SPSS v. 21 AQ5 were used for the analyses.

RESULTS

In this study, each group of samples contained five rats and was done in duplicate to obtain enough blood for the analyses. The 2-h samples reflected the condition of zinc serum after LPS and placebo administration; the 8-h samples showed the effect of zinc on the pro-inflammatory cytokine levels. These groups are comparable, as Table 1 shows that there were no differences in the body weight of the rats in the four groups.

The 3-day zinc supplementation significantly increased zinc serum levels in the zinc group. From Table 2 and Figure 2, the results showed that zinc levels at h 2 were not different between the four groups, but found an increase in zinc level at h 72 compared to the 2nd h in the LPS, LPS-zinc, and zinc groups. At h 72, zinc levels in the zinc group were significantly increased compared to the other three groups, which showed that administering zinc for 3 days was able to increase substantially zinc levels.

Table 3 and Figure 3 show that in the zinc group and the control there was no difference of TNF-α level at

Table 1: Mean rat body weights in the four treatment groups

Group	*n*	Body weight (g)			*P*
		Mean±SD	Minimum	Maximum	
Control	5	221.40±6.50	213	229	0.336
LPS	5	240.80±10.50	225	253	
LPS-Zinc	5	226.60±19.46	208	256	
Zinc	5	232.80±24.81	201	263	

SD: Standard deviation, LPS: Lipopolysaccharide

the 8th and 24th h, whereas at the 72nd h there was a difference in the levels of TNF-α, which were higher in the zinc group compared to the controls, but the levels were still lower than in the LPS group and LPS-zinc.

IL-6 levels in the zinc group were higher than the control group at 8th and 24th h, but lower than the LPS and LPS-zinc groups. At 72 h, the IL-6 level in the zinc group did not differ significantly from the control group. This showed the effect of zinc administration in normal conditions, in that there was an increase in levels of IL-6 cytokines at 8 and 24 h, but the effect disappeared at 72 h.

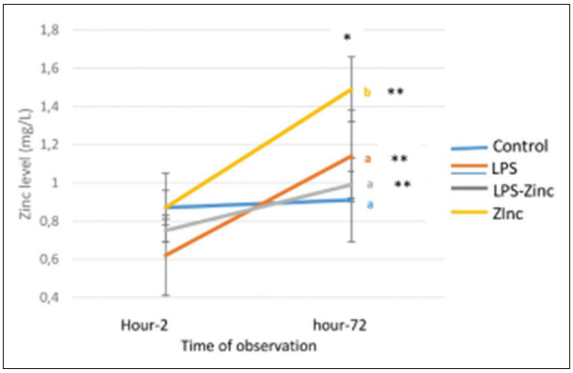


Figure 2: Serum zinc levels at h 2 and 72 after administration. *Significant at a = 0.05; [a,b]Same letters in same hour showed no difference between groups (multiple comparisons least significant difference); **Significant at a = 0.05 (Paired *t*-test/Wilcoxon signed-rank test)

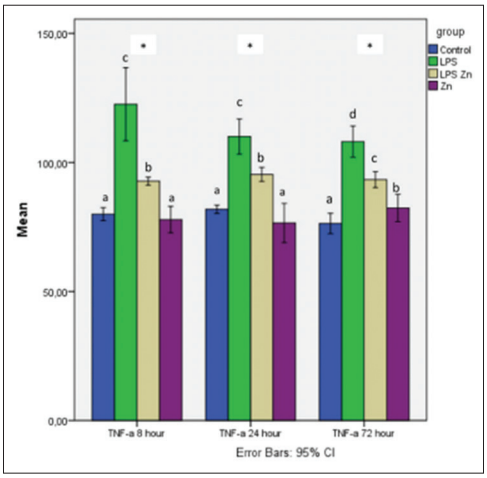


Figure 3: Tumor necrosis factor-alpha levels in treatments after 8, 24, and 72 h. *Significant at α = 0,05; [a,b,c,d]Same letters in the same hour revealed no difference between groups (multiple comparisons Games–Howell/Mann–Whitney)

DISCUSSION

Zinc supplementation for 3 days can increase zinc levels in rats previously given a diet that is deficient in zinc.[4] Increased plasma zinc levels were also seen in other studies but with a longer duration of administration of 2–4 months.[3,18-20] ZnT lowers intracellular zinc by efflux from cells or influx to intracellular vesicles so that extracellular or plasma levels increase, while Zrt-and Irt-like protein (ZIP) promotes zinc transport from extracellular fluid or intracellular vesicles to the cytoplasm so that cytoplasmic levels increase. ZnT1 messenger RNA (mRNA) levels in leukocytes increased significantly after Zn supplementation, resulting in an increase in serum zinc because ZnT promotes cell efflux from cells to extracellular or to blood circulation.[21] Zinc can stimulate monocytes directly to secrete TNF-α.[9] Zinc exposure of >100 μM in monocyte cells can increase levels of TNF-α.[21]

Pro-inflammatory cytokine levels of TNF-α in the zinc group were higher than the control group at 72 h while IL-6 cytokines in the zinc group were higher than controls at 8th and 24th h, whereas at 72nd h there were no differences between controls and zinc group for IL-6 levels. TNF-α levels and IL-6 zinc groups were lower than the LPS and LPS-zinc groups.

In this study, zinc administration under normal conditions could increase the levels of proinflammatory cytokines TNF-α and IL-6. TNF-α has an essential role in inflammation and administration of rh-TNF in experimental animals can cause symptoms of hypotension, metabolic acidosis, massive pulmonary bleeding, acute tubular necrosis in the kidneys, and gastrointestinal bleeding lesions.[14,15] Zinc supplementation can increase TNF-α levels. In a study conducted by Chu *et al*. in type 2 diabetes mellitus patients, it was found that TNF-α mRNA increased.[24] The same thing was also found in the study conducted by Meksawan *et al*., i.e., an increase in TNF-α in monocyte and lymphocyte transmembrane, which was

Table 2: Plasma zinc levels at 2 h and 72 h after *Escherichia coli* lipopolysaccharide administration

Group	*n*	Plasma zinc (mg/L)		*P*
		2 h	72 h	
Control	5	0.87±0.09 (0.75–0.97)	0.91±0.22[a] (0.57–1.19)	0.670
LPS	5	0.62±0.21 (0.42–0.97)	1.14±0.24[a] (0.94–1.54)	0.039**
LPS-Zinc	5	0.75±0.06 (0.71–0.86)	0.99±0.07[a] (0.94–1.11)	0.043**
Zinc	5	0.87±0.18 (0.62–1.12)	1.49±0.17[b] (1.33–1.74)	0.001**
P		0.073	0.001*	

*Significant at a=0.05, **Significant at a=0.05 (paired *t*-test/Wilcoxon Signed-rank test), [a,b]Same superscript in one column showed no difference between groups (multiple comparisons LSD). LPS: Lipopolysaccharide, LSD: Least significant difference

Table 3: Tumor necrosis factor-α plasma concentration at 8, 24, and 72 h

Group	*n*	TNF-a plasma concentration (pg/mL)			*P*
		8 h	24 h	72 h	
Control	5	79.95±2.04[a] (76.55–81.55)	81.55±1.30[a] (80.55–84.05)	76.35±3.19[a] (71.55–80.55)	0.029**
LPS	5	122.55±11.40[c] (111.55–136.55)	110.05±5.48[c] (106.55–119.05)	108.05±4.87[d] (101.55–114.05)	0.034**
LPS-Zinc	5	92.75±1.25[b] (91.55–94.55)	95.35±2.17[b] (91.55–96.55)	93.35±2.49[c] (91.55–96.55)	0.211
Zinc	5	77.85±4.15[a] (71.55–81.55)	76.55±6.12[a] (71.55–86.55)	82.35±4.27[b] (76.55–86.55)	0.101
P		0.000*	0.001*	0.001*	

*Significant at a=0.05 (Brown–Forsythe/Kruskal–Wallis), **Significant at a=0.05 (same-subject ANOVA/ANOVA Friedman), [a,b,c,d]Same superscript in a column revealed no difference between groups (multiple comparisons Games–Howell/Mann–Whitney). TNF: Tumor necrosis factor, LPS: Lipopolysaccharide

Table 4: Interleukin-6 plasma concentration at 8, 24, and 72 h

Group	*n*	IL-6 plasma concentration (pg/mL)			*P*
		8 h	24 h	72 h	
Control	5	1.58±0.09[a] (1.42–1.64)	1.39±0.11[a] (1.31–1.55)	1.57±0.05[a] (1.53–1.63)	0.091
LPS	5	4.91±0.40[c] (4.25–5.21)	5.57±0.16[d] (5.54–5.93)	3.39±0.38[c] (3.09–4.05)	0.000**
LPS-Zinc	5	2.15±0.09[b] (2.06–2.25)	3.83±0.19[c] (3.64–4.11)	2.51±0.15[b] (2.34–2.74)	0.000**
Zinc	5	2.10±0.17[b] (1.91–2.27)	2.21±0.44[b] (1.81–2.72)	1.84±0.26[a] (1.55–2.14)	0.304
P		0.000*	0.000*	0.001*	

*Significant at a=0.05 (Brown–Forsythe/Kruskal–Wallis), **Significant at a=0.05 (Same subject ANOVA), [a,b,c,d]Same superscript in a column revealed no difference between groups (multiple comparisons Games–Howell/Mann–Whitney). IL: Interleukin, LPS: Lipopolysaccharide

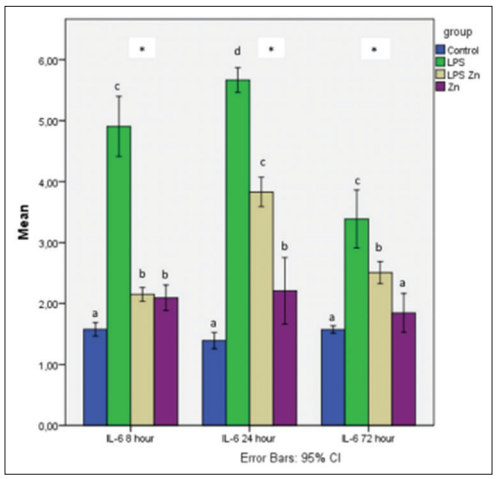


Figure 4: Interleukin-6 levels in treatments at 8, 24, and 72 h. *Significant at α = 0.05; [a,b,c,d]Same letters in the same hour revealed no difference between groups (multiple comparisons Games-Howell/Mann–Whitney)

useful as an immune response to cancer, but plasma TNF-α did not increase.[25] Zinc supplementation can increase NFkb activation through tyrosine phosphorylation and induction of protein kinase C. Tyrosine phosphorylation is used for signal induction of TLR-4 by LPSs. Tyrosine phosphorylation is performed by PTK and is degraded by PTP, and zinc is a potent inhibitor of PTP.[9] Activation of NFkB after administration of zinc then downstream inflammatory mediators TNF-α, IL-6, IL-1b, and IL-8.[25]

The increase in the IL-6 levels of the zinc group in this study occurred at the 72nd h. Increased IL-6 can determine SIRS due to infection and is associated with mortality.[26,27] IL-6 increases also occur in severe sepsis and cause an increase in the capillary leak and decrease in intestinal contractions.[12,28] In this study, there was an increase in IL-6 in the zinc group compared to controls, but it was still lower than the LPS and LPS-zinc groups so that severe symptoms also did not appear. This is by previous studies that showed an increase in IL-6 in monocyte cell groups exposed to 100 µM zinc content and above, whereas 30 µM zinc exposure did not increase IL-6.[22]

Low zinc levels and increased IL-6, IL-8, IL-1g, and TNF-α cytokines occur in sepsis compared to healthy individuals.[29] Low plasma zinc levels in sepsis are caused by the influence of pro-inflammatory cytokines including IL-1g and IL-6, which activate STAT-mediated signals and upregulation of ZIP14 and ZIP6, which trigger plasma zinc influx into the intracellular.[21]

LPS enters the system and then is recognized by TLR4 of monocytes and is transmitted through signal transduction, which activates nuclear factor kappa B (NFκB).[30] Activated NFκB induces the expression of cytokines IL-6,[31] IL-8,[32] and TNF-α.[33] LPS is bound by LPS-binding protein which is captured by TLR-4 on cell membranes than through signal MyD88, IRAKs, and recruitment of adaptors TRAF6 then recruits IKK complex lead to phosphorylation and degradation of IkB inhibitors. NFkB is active and translocated to the nucleus to activate the pro-inflammatory cytokine gene.[34]

In a review conducted by Foster and Samman of several studies, it was found that zinc supplementation in normal adult conditions for 8 weeks increased IL-2, TNF-α, and IFN-g whereas in elderly it was found that zinc supplementation for 48 days increased plasma IL-6 levels.[17]

CONCLUSION

Zinc supplementation under normal conditions increases proinflammatory cytokines TNF-α and IL-6, so it needs caution and needs to monitor clinically for zinc supplementation under normal circumstances. From this study, we suggested that zinc supplementation in normal condition should be used by cautions because it can increase the pro-inflammatory cytokine.

ACKNOWLEDGMENT

We thank our colleagues from Bioscience Institute Brawijaya University who provided insight and expertise that greatly assisted the research, although they may not agree with all of the interpretations/ conclusions of this paper.

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Source of support: Nil; Conflict of interest: None Declared

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Proofs corrections

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AQ1	**name of corresponding author is right, email: is right, and add: mrmartono73@gmail.com**
AQ2	**MOF = Multiple Organ Failure (page 1)** **DIC = Disseminated Intravascular Coagulation (page 1)** **TMB= Tetramethylbenzidine (page 2)** **OD = Optical Density (page 3)** **ZnT = Zn Transporter (page 4)**
AQ3	**These groups are comparable, as Table 1 and Figure 1 shows that there were no differences in the body weight of the rats in the four groups. (page 3, column right, paragraph 1 in RESULTS)** **From Table 4 and Figure 4 shows IL-6 levels in the zinc group were higher than the control group at 8th and 24th h, but lower than the LPS and LPS-zinc groups (page 4, column left, paragraph 2)**
AQ4	**It's already right**
AQ5	**SPSS v.21 (IBM, New York, United States)**
AQ6	**it's written in *extra if you need***
AQ7	**It's already right (type 2 diabetes mellitus)**
AQ8	**From Table 4 and Figure 4 shows IL-6 levels in the zinc group were higher than the control group at 8th and 24th h, but**

	lower than the LPS and LPS-zinc groups (page 4, column left, paragraph 2)
AQ9	**ABSTRACT** **Background** **Zinc supplementation in young adults has been found to increase dose-dependent pro-inflammatory cytokines. Increased proinflammatory cytokine has been found in severe sepsis and shock conditions. To determine the effect of zinc administration on normal conditions on pro-inflammatory cytokines compared to sepsis and normal conditions.** **Methods** **A total sample of 40 rats was randomized into four control groups, lipopolysaccharide (LPS), LPS-zinc, and zinc. Placebo normal saline was given intravenously to the control and zinc groups, whereas in the LPS and LPS-zinc groups, intravenous Escherichia coli LPS was given. Blood collection was carried out at the 2nd h after administration to measure zinc levels, and an oral distilled water placebo was given to the control group and LPS, while the LPS-zinc and zinc groups were given zinc supplementation orally for 3 days. At 8, 24, and 72 h, blood was collected to measure tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels. At the 72nd h, blood was also taken to measure zinc levels. Examination of TNF-α and IL-6 levels used the enzymelinked immunosorbent assay Sandwich technique, while zinc levels used atomic absorption spectroscopy.** **Results** **The TNF-α level in the zinc group at 72nd h was higher than the control group, while the zinc group's IL-6 level was higher than the control group at 8th and 24th h. The levels of TNF-α and IL-6 in the zinc group were lower than LPS and LPS-zinc groups.** **Conclusions** **Zinc supplementation under normal conditions increases the cytokines of TNF-α and IL-6. KEY WORDS: Interleukin-6, Normal conditions, Tumor necrosis factor-alpha, Zinc**

Extra if you need (AQ6)

Page number	Column	Paragraph number from top	Line number from top of paragraph	Add and correct text with (correction)
1	Right	1	12	2) were seen.[5,6]
2	Right	3	13	Zinc content was examined by atomic absorption spectrophotometry (AAS) by Smith et al. (18)

4	Right	2	7	Zinc supplementation for 3 days can increase zinc levels in rats previously given a diet that is deficient in zinc.[19]
4	Right	2	8-10	Increased plasma zinc levels were also seen in other studies but with a longer duration of administration of 2–4 months.[3,18-20]
4	Right	2	20	blood circulation.[22]
4	Right	2	22	μM in monocyte cells can increase levels of TNF-α.[23]
5	Left	1	10	mediators TNF-α, IL-6, IL-1b, and IL-8.[26]
5	Left	2	14	with mortality.[27,28]
5	Right	1	1	and decrease in intestinal contractions.[12,13]
5	Right	1	7	30 μM zinc exposure did not increase IL-6.[23]
5	Right	2	16	intracellular.[22]
6	Left and right	3	All references	1. Black RE. Zinc deficiency, infectious disease and mortality in the developing world. J Nutr. 2003;133(5, 1):1485S–1489S. 2. Fischer Walker CL, Ezzati M, Black RE, Walker CLF, Ezzati M, Black RE. Global and regional child mortality and burden of disease attributable to zinc deficiency. Eur J Clin Nutr. 2009;63(5):591–7. 3. Ganatra HA, Varisco BM, Harmon K, Lahni P, Opoka A, Wong HR. Zinc supplementation leads to immune modulation and improved survival in a juvenile model of murine sepsis. Innate Immun. 2017;23(1):67–76. 4. As'ad S, Yusuf I. The effects of zinc supplementation on the TNF-a profile and diarrhea in severely malnourished children of low income family. Med J Indones. 2003;12(4):247–51. 5. Bao B, Prasad AS, Beck FWJ, Snell D, Suneja A, Sarkar FH, et al. Zinc supplementation decreases oxidative stress, incidence of infection, and generation of inflammatory cytokines in sickle cell disease patients. Transl Res. 2008;152(2):67–80. 6. Prasad AS, Beck FWJ, Bao B, Fitzgerald JT, Snell DC, Steinberg JD, et al. Zinc supplementation decreases incidence of infections in the elderly: Effect of zinc on generation of cytokines and oxidative stress. Am J Clin Nutr. 2007;85(3):837–44. 7. Kahmann L, Uciechowski P, Warmuth S, Plümäkers B, Gressner AM, Malavolta M, et al. Zinc Supplementation in the Elderly Reduces Spontaneous Inflammatory Cytokine

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Acceptance letter From JPR solutions

(Drug Invention Today : Online ISSN NO: 0975-7619) Journal url: http://jprsolutions.info/view_journal_new.php?journal_id=2	http://jprsolutions.info
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To,

Martono T Utomo
Department of Child Health, Faculty of Medicine, Airlangga University, Surabaya, East Java, Indonesia

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Authors: Martono T Utomo, Subijanto M Sudarmo, Ketut Sudiana

Affiliation Department of Child Health, Faculty of Medicine, Airlangga University, Surabaya, East Java, Indonesia have recommended by editorial Board (JPR Solutions) for publication in Drug Invention Today.

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