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Zinc Supplementation in NFkB and Mucosal Intestinal Repair During LPS-Induced Sepsis in Rodents

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Abstract

Background: Severe sepsis increases pro-inflammatory cytokines and damage to the intestinal mucosa, causing systemic translocation to the commensal bacteria. Low zinc levels were also found in patients with sepsis.
Objective: This study aims to explain the mechanisms of sepsis improvement after zinc administration by measuring NFkB expression and mucosal intestinal repair.
Material and Methods: Samples of 40 rats were randomized into 4 group of Control, LPS, LPS-Zinc, and Zinc . Blood sampling in 2-hour after LPS or placebo administration to measure zinc level. Zinc was administered in LPS-Zinc and Zinc group, placebo was given in other groups. Blood sampling in 8, 24 and 72 hours to measure NFkB monocyte expression by sandwich-ELISA method and in 72 hours also measured the zinc content by AAS method and jejunal tissue by SEM analysis. Statistical analyzes used were one-way Anova, Kruskall Wallis, Mann-Whitney, Paired-t tests, Wilcoxon Signed Rank Test and path analysis.
Results: There were decreased in NFkB monocyte expression in the LPS-Zinc group compared to the LPS group.
Conclusion: Improvement of intestinal mucosa occured in LPS-Zinc group. The administration of zinc improves the condition of sepsis and improving intestinal mucosa (villi structure of jejunum).

Keywords: Zinc ; Sepsis ; Cytokine ; Intestinal mucosal

1. Introduction

Sepsis remains as a world health problem. Multicentre studies involving developed and developing countries found that mortality due to severe sepsis in children in hospital was still high, namely: 25%.[1] A study in Southeast Asia involving 13 public hospitals in 3 countries including Indonesia found 2% of sepsis deaths with 28% incidence of severe sepsis in children and 13% of sepsis deaths with 68% incidence of severe sepsis in adults.[2] Research at Dr. Soetomo General Hospital showed death due to sepsis was 16.7%, severe sepsis 27.08%, and septic shock 14.58%,[3] while in Cipto Mangunkusumo Hospital, from 10.3% of sepsis cases 47,8% associated with death.[4] Low plasma zinc levels were found in severe and critical sepsis patients in children and adults.[5,6,7] This actively demonstrate that low zinc levels in patients with severe and critical sepsis are still a problem.

Sepsis symptoms worsened when the host's immune response to infection is amplified and dysregulated. Resulting in transient hypotension symptoms, decreased urine production, thrombocytopenia which can result in multiple organ dysfunction.[8,9] Sepsis begins when bacteria enter the bloodstream and are recognized by the immune system through the pattern recognition receptor (PRR) which can detect pathogen-associated molecular patterns (PAMPs) from bacteria. The PRR bond with the PAMP triggers a signal cascade that activates the NFkB and AP-1 via MyD88 or TICAM-1. NFkB induces cytokines that cause symptoms of tachycardia, tachypnea, and fever. Activation of NFkB also causes injury to blood vessels. Blood vessel damage increased the vascular permeability that worsens the sepsis symptoms.[10]

Proinflammatory cytokines increased intestinal permeability and bacterial translocation occur in sepsis.[11,12] The bacterial translocation happens through two mechanisms: transcellular through enterocytes or paracellular through tight junctions. The study found that E. Coli and Proteus mirabilis in enterocytes showed a transcellular pathway through enterocytopinocytosis and bacterial invasion. Translocation through tight junctions can occur due to damage to the cytoskeleton and microtubular such as endotoxin.[13]

Sepsis handling is still not optimal, shown by high mortality.[1] Zinc administration can improve systemic immune response and repair of the intestinal mucosa. Zinc suppresses the activation of monocyte NFκB which further suppresses TNF-α.[4] Zinc reduces oxidative stress and ROS production because zinc functions as a scavenger enzyme.[15] Zinc also decreases levels of CRP, lipid peroxidase, and proinflammatory cytokines.[16] Zinc through the MAPK pathway and TGFβ-1 signaling increases intestinal permeability thereby preventing bacterial translocation.[17] The mechanism of action of zinc in sepsis through improved regulation of cytokines and intestinal mucosa in experimental animals exposed to intravenous lipopolysaccharide (LPS) as a model of sepsis is still unclear.

This study seeks to reveal the repair mechanism of sepsis after zinc administration by measuring NFkB and intestinal mucosal repair. This study used rats because it requires a sepsis model with intravenous LPS exposure, serial blood collection, and intestinal samples that can't be done in humans.

2. Methods

2.1 Preparation of animal study

This was an experimental study with a completely randomized design. Ethical clearance was given, number 700-KEP-UB. We use healthy male Sprague-Dawley rats (Rattus norvegicus) aged 10-12 weeks and excluded if there were congenital abnormalities at birth. The rats were acclimatized for two weeks at the Institute of Biosciences, Brawijaya University before being used. Feeding standard pellets and drinking water ad libitum and the husks are changed every two days. In total, 40 rats were used for our animal study then randomly divided into four group. Two group will be given a placebo, and two other as a sepsis animal model.

2.2 LPS Administration

Stimulation of LPS E. Coli serotype O111: B4 at a dose of 10 mg/kg rats body weight intravenous given to the two groups of the sepsis animal model. To facilitate intravenous administration of rats were injected with ketamine 0.1 mL intramuscular.

2.3 Serum Zinc Levels

The zinc sulfate solution was given by adding 9 mL of aquadest to 1 mL of zinc sulfate drop (10 mg / mL), the dose was 4.65 mg/kg BW for rats. A sample of rat blood was taken from the tail at the 2nd hour as much as 2mL for zinc examination and another 2,5ml blood samples at the 8th and 24th hours for ELISA and ICC tests, while at 72nd hour we collect blood samples from the rat aorta. Then divided into samples with EDTA (centrifuged at 6,000 G for 10 minutes at 25^{o}C within 30 minutes after being collected and stored in a -20^{o}C refrigerator) and without EDTA (stored in a -20^{o}C refrigerator). Measurement of zinc levels in serum using the Atomic Absorption Spectroscopy (AAS) method for its specificity, sensitivity, accuracy and less cost.[18] We begin with making a standard zinc solution of 1000 mg zinc/liter by diluting 10 mL of nitric acid to 50 mL by adding 1,000 g of zinc, diluting it again to 100 ml. As much as 1 mL of standard zinc mixed with a 5/95 to 100 mL glycerol water solution, shaken 16 times, then 1, 2, 3, 4 mL were taken into 100 mL volume flask and added with a 5/95 glycerol/water solution. Solutions (0.1, 0.2, 0.3, and 0.4 mg zinc per liter) were associated with plasma zinc concentrations of 500, 1,000, 1,500, and 2,000 g of zinc per liter. We use 1.2mL of blood with a drop of sodium citrate, then centrifuged 900 G for 20 minutes.

Take 0.5 mL of plasma and add 2 mL of de-ionized water mixed for 30 seconds, using spectrophotometry

to read the results by comparing with standard zinc solutions.[19]

Table 1. Mean Rats Body Weight in four groups

Group	n	Body Weight (g)			P
		Mean	Minimum	Maximum	
Control	5	221,40	213	229	
LPS	5	240,80	225	253	
LPS-Zinc	5	226,60	208	256	0,336
Zinc	5	232,80	202	263	

Table 2. Serum zinc levels at 2 h and 72 h after LPS E. Coli administration

Group	n	Zinc Levels (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*	

2.4 Monocytes NfKB Activities Immunocytochemical Examination

We isolated rat aortic blood monocytes in an EDTA tube. The 15 mL tube is filled with a 1: 1 ficoll solution with the volume of blood to be used and then centrifuged at a speed of 1,400 rpm, 33 minutes at room temperature. Pellets were washed using PBS (Phosphate Buffer Saline) as much as 5x the volume and centrifuged at 1,200 rpm, 10 minutes, at room temperature then added with complete RPMI (Roswell Park Memorial Institute) medium. A sterile coverslip is prepared for insertion into the well. 200 μl of the gelatin-coating solution was added and the coverslip was incubated for 10 minutes at room temperature, then the gelatin-coating solution was removed and the coverslip was dried for 15 minutes.

After isolation, monocyte cells with positive NFkB activity were stained using 4% formaldehyde for 20 minutes, washed with PBS-T (Phosphate Buffer Saline-Tween) for 2 minutes 3 times, then added 3% Hydrogen Peroxide for 10 minutes, and washed with distilled water followed by 10% addition. FBS (Fetal Bovine Serum) in PBS for 10 minutes then dried without washing. After that, NFkB primary antibody (rabbit polyclonal antirat NFkB p65 antibody) was added (1: 500) for 1 hour at 37 ° C then washed using PBS-T 3x @ 2 minutes and added 2 drops of reagent 1 solution per well and incubated for 30 minutes. Washed using PBS-T 3x @ 2 minutes followed by the addition of 2 drops of reagent and incubated for 30 minutes. Washed thrice using PBS-T for 2 minutes. Then add a solution of chromogen (1-2 drops of reagent 3B put into 1 mL of reagent 3A, vortex until well blended) as much as 2 drops per well. After that, rinsing was carried out and added with a solution of counterstained hematoxylene (50 μl in 1 mL tap water) of 100 μl per well for 1 minute, rinse for 2 minutes. Add PBS for 1 minute and rinse. After the process is complete and the slides are dry, mount the slides with glues. We used the Olympus BX53 series microscope to analyzed at 600x magnification. Microscope images are taken with a special camera (DP72 camera). Monocyte counting with NFkB activation compared to all fields of view was done with Immunoratio software.

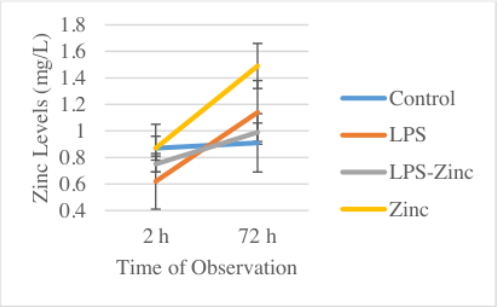

Fig 1. Comparison of zinc levels at 2 h and 72 h in 4 groups

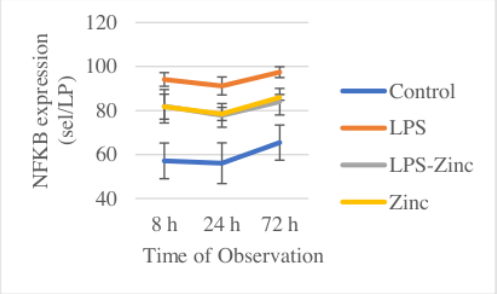

Fig 2. Comparison of monocyte NFkB expression in 4 groups

2.5 Scanning Electron Microscope (SEM) Examination on Jejunum Tissue

The jejunal tissue was collected in 72nd hour after LPS administration. jejunum segments which had been stored at -20oC, were liquefied and fixed with glutaraldehyde were cut longitudinally and placed in a box covered with paraffin wax. The mucosal surface faces upwards and is carefully washed and then cut into 5 mm. Samples were fixed for 2 hours at 4°C in 5% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.4, rinsed with cacodylate buffer, after fixation of 1% osmium tetroxide, and dehydrated with alcohol. Dehydrated samples were dried after two immersion of 1,1,1,3,3,3-hexamethyldisilazan (Aldrich), then attached to a glass object with carbon adhesive tabs or silver paint, gold-coated (Edwards Sputter Coater), scanned using a JeolS100 electron microscope with 750x magnification and a secondary detector.[20]

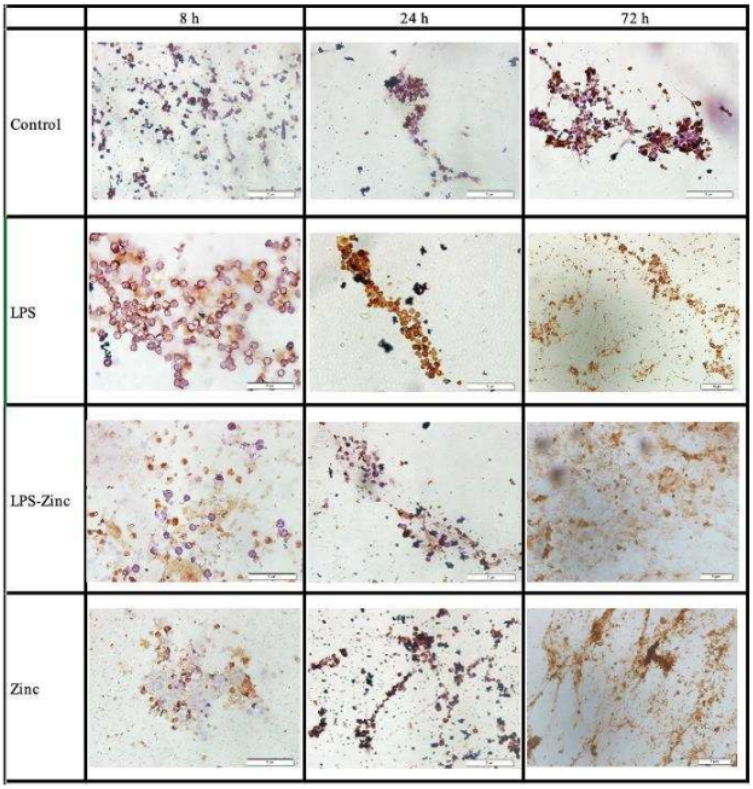

Fig 3. Microscopic image of NFkB expression on monocytes using a 600 x magnification light microscope.

2.6 Statistical Analysis

We analyzed zinc levels and intestinal mucosal damage with descriptive analysis. One-way ANOVA was used to analyze the difference between mean and standard deviation (SD) levels of zinc NFkB by first doing a normality test of all the dependent variables on all group. Comparison of group data at 8^{th}, 24^{th}, and 72^{nd} hour measurements were performed with Same Subject ANOVA analysis if the distribution was normal and Friedman's ANOVA if the distribution wasn't normal. The zinc action mechanism was analyzed using Path Analysis ($\alpha = 0.05$).

3. Result

In this study, we divided forty rats into four groups of ten each: the control group (C), the LPS group (L), the LPS-Zinc group (LZ), and the Zinc group (Z). Table 1 shows that there's no difference in body weight between groups before the treatment is given ($p > 0.05$). The LPS and LPS-Zinc groups received intravenous

administration of LPS E. Coli. The control and zinc groups with aqua dest placebo. Blood was collected (2.5mL) at the 2nd hour on five rats in each group to measure zinc levels. The aqua placebo was given to the control group and the LPS group at the 2nd, 24th, and 48th hours. We give zinc 4.65 mg/kg BW in the form of zinc sulfate solution for 10 mg/mL diluted to 1 mg/mL in the LPS-Zinc group and the zinc group at 2nd, 24th, and 48th hours. Two death occurred in the LPS group at the 8th
and 24th hours.

Table 3. NFκB Monocytes Expression in 8 h, 24 h, 72 h in 4 groups

Groups	n	Number of Monocytes Expressing NFκB			P
		8 h	24 h	72 h	
Control	5	57,12 ± 8,12[a] (44,80-67,40)	56,04 ± 9,27[a] (45,00-65,30)	65,42 ± 7,98[a] (59,20-77,95)	0,247
LPS	5	94,12 ± 3,11[c] (91,20-99,10)	91,17 ± 4,08[c] (86,55-95,80)	97,44 ± 2,46[c] (93,60-99,60)	0,077
LPS-Zinc	5	81,94 ± 7,60[b] (70,50-90,20)	77,78 ± 5,38[b] (68,90-82,40)	84,01 ± 6,04[b] (74,65-91,00)	0,092
Zinc	5	81,74 ± 5,69[b] (71,80-85,90)	78,45 ± 2,98[b] (73,20-80,40)	85,98 ± 1,31[b] (84,60-87,60)	0,041**
p		0,001*	0,001*	0,000*	

After two hours of administration of LPS E. Coli 10 mg/kg BW intravenously, we found zinc deficiency (normal zinc levels were 84 - 159 μg / dL) in LPS and LPS-Zinc groups. There were differences between the Zinc group and the other three groups (Control, LPS, and LPS-Zinc) after 72nd hours. The difference in zinc levels at the second and 72nd hours shows that three days of zinc administration can increase zinc levels (Table 2 and Figure 1). The monocytes NFkB expression analysis in the LPS group was higher than the other three groups at 8, 24, and 72 hours. Showed that zinc inhibited NFkB expression. Meanwhile, the control group was the lowest, compared to the other three groups (Table 3 and Figure 2). The monocytes' NFkB was brown in monocytes on a microscope image with 600x magnification (ocular 10x and objectively 60x), shown in Figure 3.

SEM examination of the jejunal mucosa in the experimental control group showed high jejunal villi with sufficient epithelial cell density, dense gaps between villi and regular villi arrangement (Figure 4). In the LPS group, jejunal damage was found, characterized by cell edema and wider cell gaps (Figure 5). The jejunal mucosa in the LPS-Zinc group was similar to the control group: the villi were tall and dense, a narrow gap between the villi, and the epithelial arrangement was regular (Figure 6). In the Zinc group, we found damage in the form of cell edema, loose villi spacing, and irregular epithelial arrangement (Figure 7).

4. Discussion

This study aims to reveal the action mechanism of zinc in sepsis-induced LPS E. Coli. The concept is proinflammatory cytokines induced by LPS E. Coli through activation of NFkB monocytes and damage to the jejunal mucosa as a result of sepsis. Activation of NFkB monocytes causes an increase in proinflammatory cytokines which cause tissue and intestinal mucosal damage. We use 10 mg / mL zinc sulfate solution diluted to 1 mg/mL at a dose of 2 mg/kg in humans equivalent to 4.65 mg/kg BB in mice which functions as a proinflammatory cytokine suppressant, increases anti-inflammatory cytokines, and bowel protection in sepsis. This study compared the LPS group with the control group to see the changes that occur in sepsis when compared to normal conditions. The LPS-Zinc group was compared with the LPS group to see the effect of zinc administration on sepsis. The zinc group was used to see the effect of zinc administration on normal conditions by comparing it with the control group.

Two hours after injection of intravenous LPS E. Coli, the blood zinc levels in the LPS and the LPS-Zinc group were lower than the group without injection; the control and zinc groups. The dose of LPS used in this study was 10 mg/kg body weight intravenously as used in a previous study by Utomo et al. In a study on Zinc Supplementation in Cytokine Regulation During LPS-induced Sepsis in Rodent.[21] The reduction in zinc levels 2 hours after administration of LPS in the LPS and LPS-Zinc groups in this study was not as large as the reduction in plasma zinc levels in the septic and critical conditions in the previous study (45-48 μg / dL).[5,6,7]

In this study, the average zinc level in the LPS group was 0.62 mg / L or 62 μg / dL, and in the LPS-Zinc group, the mean zinc level was 0.75 mg / L or 75 μg / dL which was included in zinc deficiency with normal values of zinc levels. 84 - 159 μg / dL,[22] whereas in the control and zinc groups normal zinc levels were found although there was no difference with the LPS and LPS-Zinc groups. This could be because in this study, the intravenous LPS E. Coli did not cause severe sepsis symptoms. Low zinc levels in sepsis were found in patients with severe and critical sepsis in children and adults which are associated with mortality.[5,6,7]

The 72-hour increase in zinc levels in the LPS group indicated a shift in intracellular to extracellular zinc and the use of zinc by monocytes as signal transduction of NFkB for proinflammatory cytokines. Zinc monocyte levels detected in serum due to centrifugation. Zinc is needed as a second messenger in monocytes so that there is an increase in intracellular zinc levels in monocytes during stimulation of the FC receptor1 .[23] In the LPS-Zinc group, the 72-hour zinc level was not different from the control group because zinc was a scavenger enzyme and anti-inflammatory. The examination of zinc levels using the AAS method reflects the total serum zinc level but does not distinguish between free and albumin-bound zinc levels. There is a decrease in total serum zinc levels, free zinc, and zinc-binding capacity by albumin in a state of sepsis. Three days of zinc administration showed a difference in plasma zinc levels in the control and the zinc group at 72 hours. The zinc levels at the second hour in the control group and the zinc group were still within normal limits. The zinc levels increases occurred at the 72nd hour in the zinc group. It is the same as other studies on giving zinc fortification to the rat's diet for 3 days can increase zinc levels in the zinc deficiency group to normal zinc levels.[24]

Significantly increased levels of NFKB were found in experimental animals that received an intravenous injection of LPS E. Coli. The administration of LPS increases the levels of NFkB through activation of the MyD88 pathway which recruits IRAK-1 and IRAK4- which phosphorylate TRAF6 resulting in activation of complex IKK and phosphorylation of IkB so that NFkB separate from IkB and go to the cell nucleus.[25] The administration of zinc in this study could significantly reduce the NFkB p65 levels in the group given LPS E. Coli was shown by a significant difference in NFkB expression between the LPS and LPS-Zinc groups: a lower NFkB expression in the LPS-Zinc group at 8, 24, and 72 hours. Other studies stated that zinc reduces NFkB activation through the activation of protein A20 which inhibits NIK (nuclear factor-kB inducible kinase) that activates IKK (I Kinase) and causes NFB activation.[26] The higher comparison of monocyte NFkB expression in the zinc group compared to the control showed the effect of zinc on monocyte cells. Giving zinc under normal conditions (in this study without giving LPS E. Coli) can increase NFkB expression in monocytes. Previous

studies also showed an increase in NFkB expression in cells exposed to zinc but in HUT-78 (Th0) cells. The upregulation mechanism of NFkB activation is through phosphorylation of IkB.[27]

The proinflammatory cytokine response to single doses of intravenous LPS E. Coli can last up to 72 hours due to damage to the intestinal mucosa resulting in systemic translocation of intestinal bacteria. The translocation of these bacteria aggravates sepsis symptoms.[11,12,28] In this study, there was damage to the jejunal mucosa in the experimental animal group injected with intravenous LPS. Zinc administration can repair the jejunal mucosal damage in the group injected with intravenous LPS. In the previous studies, zinc can improve the tight junction and barrier function of intestinal epithelial cells.[29] Giving zinc to intestinal cell cultures can induce HSp70 mRNA expression that improves the intestinal epithelial barrier.[30] We did not examine the HSP70 blood levels therefore we can not describe the effect of HSP70. Some literature said there was a protective effect of HSP70 on the intestinal barrier, and zinc administration could increase HSP70. Zinc inhibits pathways on NFkB at 8, 24, and 72 hours. The zinc inhibition pathway on NFkB activation at 72 hours is not directly but via TGF-β. Administration of zinc under normal conditions can increase the activity of NFkB and damage to the jejunal mucosa. This study's limitation is that the cytokines examined in this study cannot be determined to come from monocytes alone and can come from other sources.

Fig 4. SEM images of the jejunal mucosa of the Control group using electron microscopy with 750x magnification

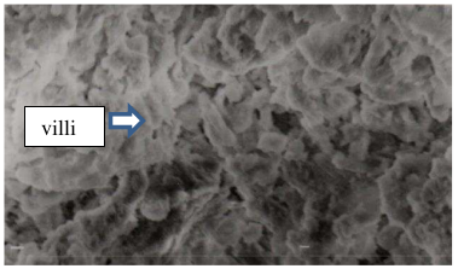

Fig 5. SEM images of the jejunal mucosa of the LPS group using electron microscopy with 750x magnification

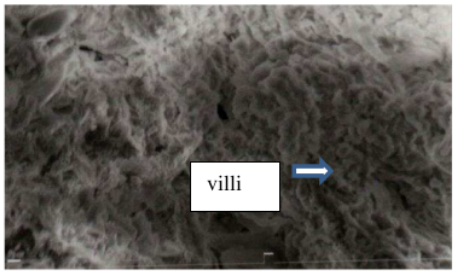

Fig 6. SEM images of the jejunal mucosa of the LPS-Zinc group using electron microscopy with 750x magnification

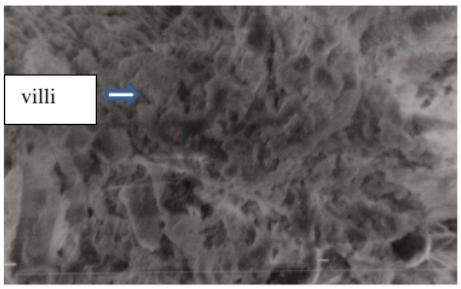

Fig 7. SEM images of the jejunal mucosa of the Zinc group using electron microscopy with 750x magnification

5. Conclusion

Oral administration of zinc decreased the expression of monocyte NFkB in an animal model of sepsis and improved the mucosal structure of the jejunum in an animal model of sepsis. The zinc mechanism in sepsis improvement is through inhibition of NFkB at 8 and 24 hours. Zinc inhibition of NFkB via the TGF-β pathway at 72 hours.

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