

RESEARCH ARTICLE

Zinc Administration Affects Bronchial Mucosal NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β of Zinc-deficient Rats

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Abstract

BACKGROUND: Risk of acute respiratory infections in children less than 5 years of age is up to 95%. Zinc deficiency is one of the main risk factors. This study aimed to explore the effect of zinc on the bronchial mucosae inflammatory status expressed by nuclear factor (NF)- κ B p105/p50, NF- κ B p65, interleukin (IL)-8, and IL-1 β .

METHODS: Twenty-four Wistar rats were divided into 4 groups: normal zinc diet group without zinc supplementation (Z1), normal zinc diet group with zinc supplementation (Z2), zinc deficient diet group without zinc supplementation (Z3), and zinc deficient diet group with zinc supplementation (Z4). NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β were measured by immunohistochemical staining.

RESULTS: The inflammatory status of bronchial mucosae between Z1 and Z2 groups showed no difference ($p=0.055$). However, the inflammatory status of bronchial mucosae between Z3 and Z4 groups showed significant difference ($p<0.01$). Multivariate factorial design showed that zinc supplementation was beneficial when given to zinc deficient diet group with regard to decrease p-NF- κ B p65, IL-8 and IL-1 β levels ($p<0.001$) and increase dendritic cell ($p=0.022$).

CONCLUSION: Zinc administration under conditions of zinc deficiency affects the inflammatory status, as shown by the decrease of p-NF- κ B p65, IL-8 and IL-1 β and the increase of NF- κ B p105/p50.

KEYWORDS: zinc, NF- κ B, p105/p50, p65, IL-8, IL-1 β , rat

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Introduction

Child mortality due to acute respiratory infection (ARI) reached 1.9 million in 2000, with the highest levels seen in developing countries, particularly in children under 5 years old. In the developing countries, this age group has the highest risk of death from ARI, reaching as high as 95%. (1) The Basic Health Research conducted in Indonesia states that the national prevalence of overall ARI reached 25.50%. While in the group of infants and children younger than 5 years, as many as 35% suffered from ARI. (2) Zinc

deficiency is one of the main risk factors for acute respiratory infections. (3)

Zinc is an essential component of several enzymes and cofactors in signaling pathways. Controlling inflammatory signaling pathway through correlated underlying factors such as interleukin (IL)-1 β (4-6) and IL-8 (7), and also transcription factors, such as NF- κ B (6-9), is required for normal development (10,11), apoptotic induction of tumor/cancer cell (12-14), and cellular function. (15)

Zinc plays a role in the inflammatory system by a variety of mechanisms such as protecting the mucociliary cytoplasmic apparatus (tubulin and basal bodies), and

inhibiting Nuclear Factor (NF)- κ B translocation into the nucleus, which prevents the subsequent expression of pro-inflammatory cytokines. Zinc deficiency will provoke pro-inflammatory cytokines, which could damage the mucous membrane of respiratory tract, leading to respiratory tract infections.(15-17) Zinc deficiency affects the function of cells belonging to innate and acquired immunity and can cause complications such as secondary infections and cell damage.(18) Zinc deficiency increased the levels of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-8 cytokines.(19,20)

Unfortunately, effects of zinc supplementation on the inflammatory system of bronchial mucosa were not extensively studied. There was one study conducted in septic mice (21), but not in healthy rats. Therefore, current research was conducted to investigate the effect of zinc administration on the inflammatory bronchial mucosal status by analysing NF- κ B p105/p50, phosphorylated-NF- κ B (p-NF- κ B) p65, IL-8, and IL-1 β of normal and zinc deficiency healthy rats.

Methods

Animal Treatment and Sample Collection

Twenty-four male Wistar rats, aged 5 weeks, 60-100 g were divided into 4 groups: normal zinc diet group without zinc supplementation (Z1), normal zinc diet group with 60 ppm/day zinc supplementation (Z2), zinc-deficient diet group without zinc supplementation (Z3), and zinc-deficient diet group with 120 ppm/day zinc supplementation (Z4). All treatments were performed until 3 weeks. After that, rats were sacrificed and bronchial mucosae were collected. The mucosae were fixed and processed for making paraffin blocks. The study was conducted in the Biochemistry Laboratory, Faculty of Medicine, Universitas Airlangga/ Dr. Soetomo General Hospital, Surabaya. The research protocol was approved by the Research Ethics Committee for Animal Care and Use, Faculty of Veterinary Medicine, Universitas Airlangga (No. 055-KE).

Immunohistochemistry

Bronchial mucosal paraffin blocks were sliced in 4 μ m, de-paraffinized and antigen retrieved. After washing with phosphate buffered saline (PBS), the tissue sections were incubated with 3% hydrogen peroxide and incubated with 2% bovine serum albumin. Then each of the following primary antibodies was applied. For NF- κ B p105/p50 detection,

a mouse monoclonal anti-NF- κ B p105/p50 antibody (Cat# NB100-56583, Novus Biologicals, Centennial, CO, USA) was applied. For p-NF- κ B p65 detection, a mouse monoclonal anti-phospho-NF- κ B p65 (Ser536) antibody (Cat# sc-136548, Santa Cruz Biotechnology, Dallas, TX, USA) was applied. For IL-8 detection, a rabbit polyclonal anti-IL-8 antibody (Cat# orb229133, Biobyte, St. Louis, MO, USA) was applied. For IL-1 β detection, a rabbit polyclonal anti-IL-1 β antibody (Cat# AAR15G, Bio-Rad, Hercules, CA, USA) was applied. After the first antibody, N-Histofine High Stain HRP (MULTI) (Nichirei Biosciences, Tokyo Japan) kit was used. The peroxidase activity was visualized by immersing tissue sections in N-Histofine DAB-2V (Nichirei Biosciences), resulting in a brown reaction product. Tissue sections were finally counterstained with hematoxylin and mounted.

Immunohistochemical Evaluation

Cells with overexpressions of NF- κ B p105/p50, p-NF- κ B p65, IL-8 and IL-1 β were examined, documented and counted. Five fields/slide/rat were selected and documented under a light microscope with 400x magnification, then counted by two trained examiners.

Statistical Analysis

Counted cells were statistically analyzed with SPSS Statistics, version 17.0 (SPSS Inc., Chicago, IL, USA). Student's t-test was used to analyse differences of number of cells expressing NF- κ B p105/p50, p-NF- κ B p65, IL-8 and IL-1 β in each group. The multivariate analysis of variance (MANOVA) factorial design was used to determine the effect between zinc status and zinc supplementation on immun-expression levels of NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β . Data were analysed with 95% confidence level. The p -value<0.05 was considered significant.

Results

Immuno-expressions of NF- κ B p105/p50 (Figure 1), p-NF- κ B p65 (Figure 2), IL-8 (Figure 3) and IL-1 β (Figure 4) were seen clearly in bronchial mucosae of all Z1-Z4 groups. Immuno-expression levels of NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β in the bronchial mucosae of Z1 and Z2 groups were not significantly different (Table 1). Immuno-expression levels of NF- κ B p105/p50, IL-8, and IL-1 β in the bronchial mucosae of Z1 group were merely slightly higher than the ones in Z2 group.

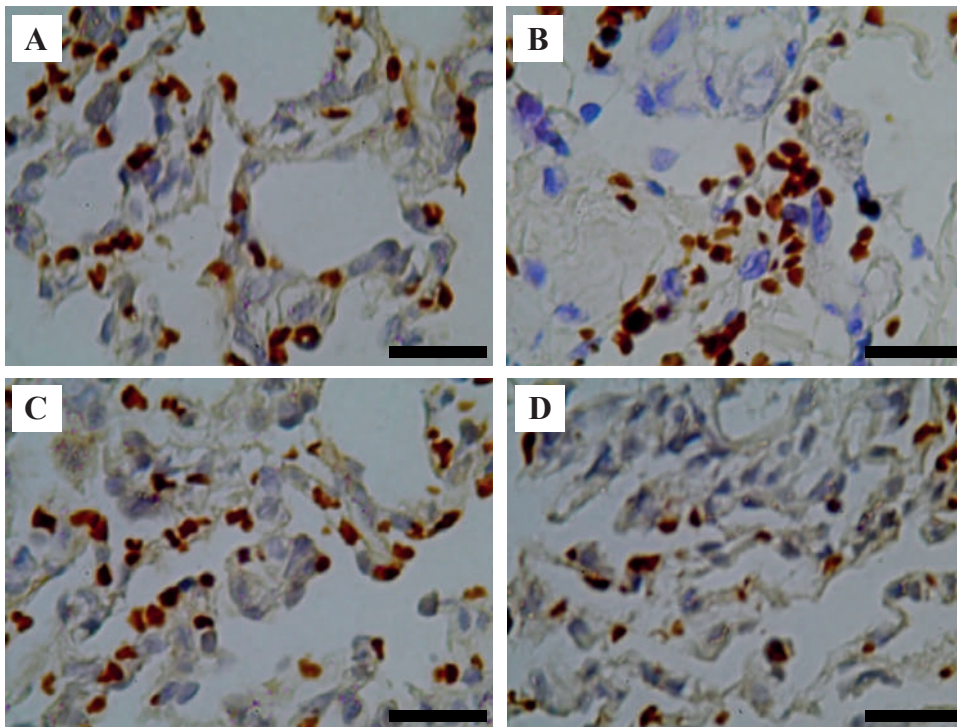


Figure 1. Immuno-expression of NF- κ B p105/p50 in bronchial mucosae. A: Z1 group, B: Z2 group, C: Z3 group, D: Z4 group. Black bar: 10 μ m.

Meanwhile immuno-expression levels of NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β in the bronchial mucosae of Z3 and Z4 groups were significantly different (Table 2). Immuno-expression levels of, p-NF- κ B p65, IL-8, and IL-1 β in the bronchial mucosae of Z4 group were significantly lower than the ones in Z3 group. Interestingly, immuno-expression level of NF- κ B p105/p50 in the bronchial mucosae of Z4 group was significantly higher than

the one in Z3 group. The MANOVA factorial design showed significant results of zinc status with zinc supplementation on the immuno-expression levels of NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β (Table 3). In addition, individual zinc status and individual zinc supplementation were shown to significantly affect immuno-expression levels of p-NF- κ B p65, IL-8, and IL-1 β but not the one of NF- κ B p105/p50.

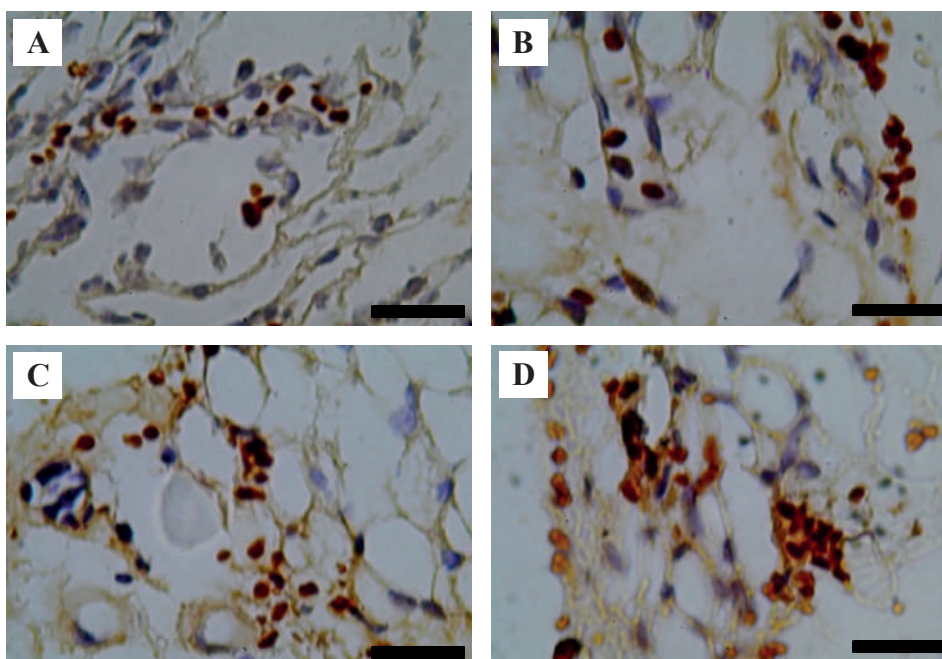


Figure 2. Immuno-expression of p-NF- κ B p65 in bronchial mucosae. A: Z1 group, B: Z2 group, C: Z3 group, D: Z4 group. Black bar: 10 μ m.

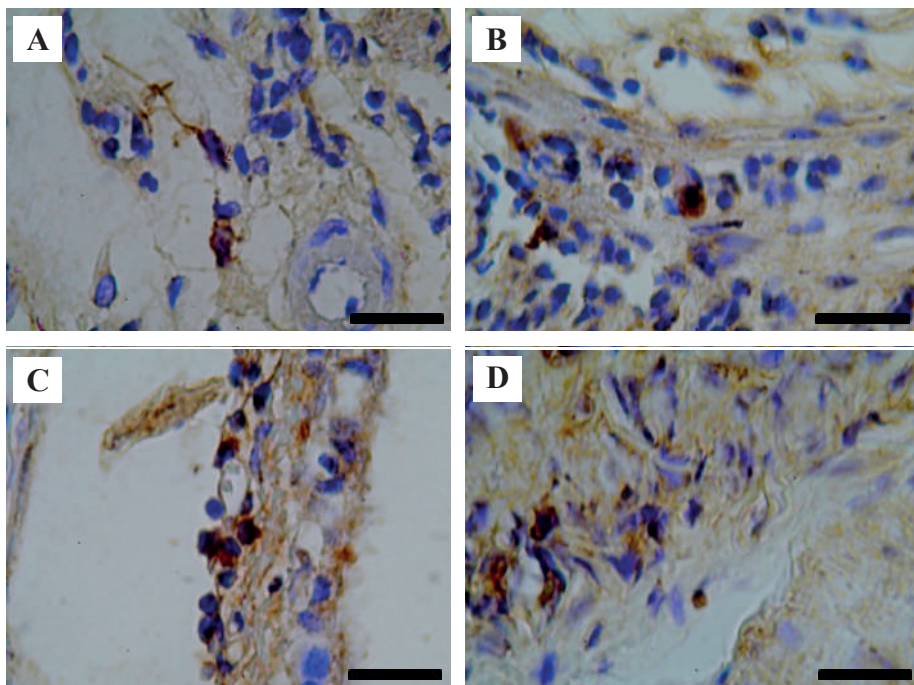


Figure 3. Immuno-expression of IL-8 in bronchial mucosae. A: Z1 group, B: Z2 group, C: Z3 group, D: Z4 group. Black bar: 10 μ m.

Discussion

Present study showed that zinc supplementation under normal conditions had no effect on the inflammatory status of bronchial mucosae, as shown by immuno-expression levels of NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β . Previous study reported that zinc supplementation on respiratory

tract infections of children with cystic fibrosis showing a not significant difference of IL-8 and IL-1 β serum levels compared to placebo.(22) Zinc supplementation would not be beneficial to individuals with normal zinc level, but zinc-deficient individuals responded to zinc supplementation. (23) Present results also showed that zinc administration had an effect on bronchial mucosal inflammatory status in deficient condition.

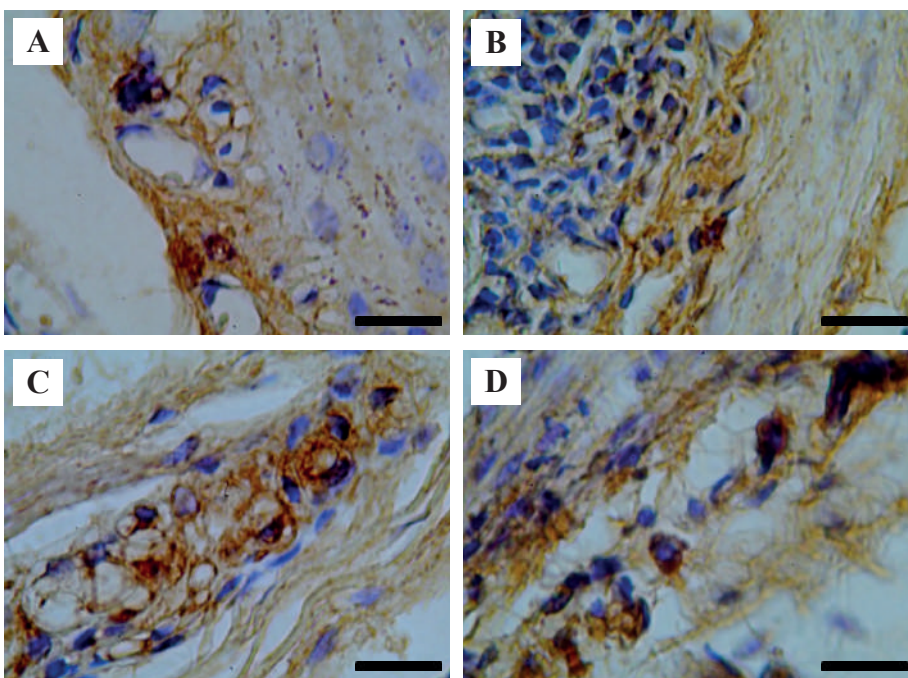


Figure 4. Immuno-expression of IL-1 β in bronchial mucosae. A: Z1 group, B: Z2 group, C: Z3 group, D: Z4 group. Black bar: 10 μ m.

Table 1. Immuno-expression levels of NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β in the bronchial mucosae of normal zinc diet group. Z1 group: normal zinc diet group without zinc supplementation, Z2 group: normal zinc diet group with zinc supplementation.

Variable	Normal Zinc Diet Group		<i>p</i> [*] (univariate)	<i>p</i> ^{**} (multivariate)
	Z1 Group (Mean \pm SD)	Z2 Group (Mean \pm SD)		
NF- κ B p105/p50	12.40 \pm 3.21	20.20 \pm 0.84	0.001	0.055
p-NF- κ B p65	2.40 \pm 1.14	2.00 \pm 0.71	0.524	
IL-8	3.00 \pm 1.23	3.80 \pm 1.48	0.380	
IL-1 β	3.00 \pm 1.58	3.60 \pm 1.52	0.557	

*Independent t-test; **Hotelling's T2 test.

Table 2. Immuno-expression levels of NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β in the bronchial mucosae of zinc deficient diet group. Z3 group: zinc deficient diet group without zinc supplementation, Z4 group: zinc deficient diet group with zinc supplementation.

Variable	Zinc Deficient Diet Group		<i>p</i> [*] (univariate)	<i>p</i> ^{**} (multivariate)
	Z3 Group (Mean \pm SD)	Z4 Group (Mean \pm SD)		
NF- κ B p105/p50	5.50 \pm 1.38	12.60 \pm 2.70	<0.001	<0.001
p-NF- κ B p65	19.30 \pm 1.97	7.80 \pm 2.86	<0.001	
IL-8	20.30 \pm 1.63	11.20 \pm 1.30	<0.001	
IL-1 β	22.17 \pm 3.97	10.40 \pm 1.14	<0.001	

*Independent t-test; **Hotelling's T2 test.

In present study, p-NF- κ B p65, IL-8 and IL-1 β were increased in zinc-deficient rats without zinc supplementation while NF- κ B p105/p50 was not influenced by zinc supplementation. Theoretically zinc deficiency might cause epithelial damage, which stimulate dendritic cells to secrete IL-8 and IL-1 β in the inflammatory process of the bronchial mucosal epithelium. Then the signal transduction will

occur, leading to increment of NF- κ B p65 phosphorylation, then p-NF- κ B p65 translocate into the nucleus. As for the inactive form of NF- κ B will decrease, including NF- κ B p105/p50, which is remained in the cytoplasm.

Zinc administration will increase zinc levels in the intracellular system and inhibit the production of proinflammatory cytokines through several channels. In

Table 3. MANOVA factorial design of zinc status and zinc supplementation on immuno-expression levels of NF- κ B p105/p50, p-NF- κ B p65, IL-8, and IL-1 β in the bronchial mucosae of Wistar rats.

Main Effects and Interaction Effects	Variable	Mean square	<i>p</i> [*]	<i>p</i> ^{**}
Effects of zinc status (normal/ zinc deficiency)	NF- κ B p105/p50	322.73	<0.001	<0.001
	p-NF- κ B p65	668.48	<0.001	
	IL-8	784.1	<0.001	
	IL-1 β	850.85	<0.001	
Effects of zinc supplementation (yes/no)	NF- κ B p105/p50	253.8	<0.001	<0.001
	p-NF- κ B p 65	192.61	<0.001	
	IL-8	112.4	<0.001	
	IL-1 β	154.93	<0.001	
Interaction effects of zinc status (normal/deficiency) with zinc supplementation (yes/no)	NF- κ B p105/p50	0.013	0.721	<0.001
	p-NF- κ B p65	148.44	<0.001	
	IL-8	100.74	<0.001	
	IL-1 β	200	<0.001	

*Tests of Between-Subjects Effects; **Wilks' Lambda.

addition, zinc induces zinc finger protein and inhibits activation of the NF- κ B pathway through Tumor Necrosis Factor Receptor (TNF-R)-associated Factor (TRAF).(20,23) Zinc administration in HL-60 cells was shown to decrease gene expression and the production of inflammatory cytokines, as well as decreasing markers of oxidative stress. (20) Therefore, in present study, by supplementation of zinc, the zinc deficiency-caused inflammatory signaling pathway was inhibited, resulting down-regulation/decrease of p-NF- κ B p65 and upregulation/increase of NF- κ B p105/p50.

Conclusion

Zinc administration has an effect on bronchial mucosal inflammatory status, which is expressed by the decrease of p-NF- κ B p65, IL-8 and IL-1 β levels and the increase of NF- κ B p105/p50 level in rats with zinc deficiency.

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