Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Evaluation of serum zinc level and IL-18 mRNA expression in children with pneumonia

Shixiong Yang, Jin Shi*

Department of Paediatrics, Edong Healthcare Group, Huangshi Maternity and Children's Health Hospital, Huangshi, Hubei, 435000, China

*Correspondence to: shijin20210309@163.com

Received July 24, 2021; **Accepted** September 27, 2021; **Published** November 22, 2021

Doi: http://dx.doi.org/10.14715/cmb/2021.67.3.26

Abstract: Pneumonia is currently one of the leading causes of death in children. Increased susceptibility to pneumonia may be due to their decreased immunity. One of the reasons for the decrease in immunity is zinc deficiency. In children with pneumonia, on the other hand, some cytokines are secreted, resulting in inflammation that spreads, persists, and makes treatment difficult for specialists. In this study, we investigated the serum zinc level in children with pneumonia and healthy children. Also, we tried to find its relationship with IL-18 mRNA expression as an inflammatory cytokine. For this purpose, serum zinc levels and IL-18 mRNA expression were evaluated in 120 children aged 3-60 months with pneumonia and 120 healthy children. After taking 2ml of blood from children and measuring serum zinc level, the level of the IL-18 mRNA was measured by real-time PCR. Total RNA was extracted by bioZOL™-G RNA Isolation Reagent kit. The primary cDNA was amplified by the extracted RNA, and in the next step, 2µl of cDNA were amplified by specific primers to measure IL-18 mRNA. The Beta-actin gene was also used as internal control and housekeeping gene. Results showed that the level of zinc in the patient group was 412.625±28.87µM and in the control group was 514.40±49.67µM. This difference was statistically significant (P=0.0053). Also, the expression of the IL-18 gene was increased in children with pneumonia, significantly (P=0.0015). Therefore, from the results, it can be deduced that children with zinc deficiency were at higher risk for a lung infection. Inflammatory cytokines such as IL-18 also were increased in these children. Hence, it can be concluded that zinc levels can reduce the expression of IL-18 mRNA and play an important role in the prevention and treatment of children with pneumonia.

Key words: IL-18; Inflammatory cytokines; Pediatric pneumonia; Serum zinc level.

Introduction

Lower respiratory infection (pneumonia) is one of the leading causes of child mortality in developing countries (1). The susceptibility to infection is higher in malnourished children due to decreased cellular immunity (2). Zinc deficiency may be one of the reasons for reduced immunity in children with malnutrition (3). Zinc is an important chemical element with different functions in humans. Zinc has been shown to play a role in neurotransmission, immune activity, development, the activity of certain hormones, olfactory function, and wound healing (4). The essential role of zinc in the synthesis of protein and nucleic acid has also been proven (5). Also, the role of zinc in shortening the fever period and speeding up recovery in pediatric respiratory infections is well known. Even some studies have found that routine zinc intake in children 6 months to 3 years old is effective in reducing the incidence of pneumonia (6). This may be due to an improvement in the immune system, especially an increase in CD4 (5). Numerous studies have shown that serum zinc levels in children with pneumonia have a significant reduction relative to the control group (7, 8). Zinc has also been shown to improve gastrointestinal infections (diarrhea) in recent years (9, 10).

Pneumonia, or acute infection of the lung parenchyma, is most often caused by bacterial or viral agents, but the causative agent is often undetectable (11). In this disease, infection and tissue damage invoke cells involved in inflammation, such as macrophages, to the site of injury (11). Inflammation and tissue damage induces the secretion of cytokines from these cells, leading to an acute phase response. These include inflammatory cytokines, interleukin-6 (IL-6) and IL-18 in humans and in mice, which are produced by macrophages (12). A study in rats infected with *Streptococcus pneumoniae* reported a sharp rise in IL-6 and IL-18 (13). These cytokines activate the JAK-STAT pathway and after STAT phosphorylation by JAK, STAT binds to the hepcidin promoter and increases transcription of the hepcidin gene (14, 15). Other inflammatory cytokines, such as tumor necrosis factor-alpha (α-TNF) and microbial products may also activate hepcidin gene transcription (15).

IL-18 was first detected in the liver cells of mice exposed to the lipopolysaccharide of the bacterium *Propionibacterium acnes* (16). IL-18 is similar in structure and function to the IL-1β family. It is biologically synthesized as a 24 kDa protein precursor. The conversion of this protein to the active form of 18 kDa is performed by the converting enzyme IL-1β (caspase-1), which is essential for the biological activity of IL-18. IL-18 plays a major role in defense against pathogens by stimulating the innate immune response and increasing the TH1 response. This cytokine causes the cytotoxicity of NK cells and the production of IFN-γ from the cells (17).

Because timely diagnosis and effective treatment of this disease are very important in children, so the pres-

Table 1. The sequence of primers and probes.

Gene		Sequence
IL-18	Forward	5′- GACCAAGGAAATCGGCCTCTA -3′
	Reverse	5′- CCATACCTCTAGGCTGGCTATCTT -3′
	Probe	FAM-ATTCTGACTGTAGAGATAATGCACCCCGGAC-TAMRA
β-actin	Forward	5′- AGCCTCGCCTTTGCCGA -3′
	Reverse	5′- CTGGTGCCTGGGGCG -3′
	Probe	FAM-CCGCCGCCCGTCCACACCCGCC-TAMRA

ent study was performed to investigate the serum level of zinc and IL-18 mRNA expression in children with pneumonia and compare it with healthy children to find out the effect of zinc on IL-18 mRNA expression.

Materials and Methods

Patients

In this case-control study, all children who were admitted to the pediatric ward of the hospital with a diagnosis of pneumonia were studied in the absence of the following; age less than 3 months and more than 60 months, malnutrition, diarrhea, localized infection except pneumonia, underlying disease, dehydration, hemolysis, antibiotics and zinc, seizures, and more than 12 hours of incontinence. A total of 120 children with pneumonia were eligible for the study.

Because severe cases of pneumonia (tachypnea with severe retraction of intercostal muscles, cyanosis, loss of consciousness, toxicity) are admitted to the ICU of children, they were not included in the study. Children in good general condition (who could be fed orally at the beginning or most after 12 hours) were included in the study. The mean time of hospitalization in these patients was 5 ± 1.5 days.

120 healthy children of the same age as the case group and of appropriate weight and height who had been referred for monthly visits or vaccinations were included in the study as a control group after obtaining parental consent.

Measurement of serum zinc level

Two milliliters of venous blood were taken from all these children. The blood was then poured in acid-washed polypropylene tubes and immediately transferred to a laboratory under sterile conditions for centrifugation and serum separation. After centrifugation of the blood and separation of the serum from the globules, the serum was transferred to another sterile tube and stored in a freezer at -80°C. After obtaining the calculated number of samples and serum level, it was measured by atomic absorption spectrophotometry.

RNA extraction and cDNA preparation

Total RNA was extracted using a solution Biozol (Bioflux-Japan) according to the manufacturer's protocol, and the amount and purity of the extracted RNA were determined using a nanodropspectrometer (USA). Optical Density RNA was measured at 260/280 nm. Samples with the OD 260/280 nm ratios ranging from 1.8 to 2.2 were used for cDNA synthesis. In this study, 2µg of RNA was converted to single-stranded cDNA using Fermentas Revert Primer (Random Hexamer) AidTM First Strand cDNA synthesis Kit, according to the manufacturer's protocol. There were two stages of incubation: the first 5 minutes at 65°C and the second incubation for 10 minutes at 25°C and then 60 minutes at 42 °C. Finally, the reaction was completed with 10 minutes of heating at 72°C. The synthesized cDNA was stored at -70°C for later use.

The reaction of Real-Time PCR (TaqmanProb)

In this study, the IL-18 gene was studied as the target gene and the β-actin gene was used as the housekeeping gene. The primers were designed by GENERUNNER software, PERLPRIMER and NCBI database (www.ncbi.nlm.nih.gov/BLAST). The sequence of primers is shown in Table 1.

All Real-Time PCR reactions were performed on a Corbett Rotor Gene TM 3000. The time-heating schedule of the device was set in three stages. The first stage was 95°C for 10 minutes, which led to the denaturation of cDNA molecules. The second stage was 45 cycles, which included 95 °C for 15 seconds for denaturation, 60°C for 60 seconds for annealing and extension. These reactions were performed at a final volume of 25µl in 0.1 ml microtubes. Compounds of each reaction consisted of 12µl of TaqMan Universal PCR Master Mix (2X-rotor gene-Germany), 0.4µl of 10 pM primers and 0.2µl of 10 pM probes, 7µl DNase free water, 2µl of template cDNA, then the sample was placed in the analyzer and 45 cycles were amplified. For data analysis, the ΔCt gene in each sample was calculated from the differentiation of the corresponding Ct gene, and the Ct β-actin gene was calculated as the reference gene.

Statistical analysis

The collected data were analyzed using SPSS 16, Mann Whitney U test for gene expression in two different groups, and t-test. GraphPad Prism 5 Demo software was used to prepare the relevant figures.

Results

Mean serum zinc levels

A total of 240 children were studied in two groups of 120 children. The mean age of the subjects was 13.7±6.31 months. In both groups, 62.9% were boys and 37.1% were girls. The level of zinc in the patient group was 412.625±28.87µM and in the control group was 514.40±49.67µM. This difference was statistically significant (P=0.0053). This means that serum zinc levels in children with pneumonia were significantly reduced. In both patient and control groups, 73.7% of cases (177 children) were in the age range of 3-12 months and 63 children were in the age range of 1-5 years. The mean serum level of zinc in the two groups with pneumonia and control in general and by age group under one year and above one year is shown in Table 2.

Table 2. Comparison of mean serum zinc levels (µM) in pneumonia and control groups by age.

	Pneumonia Group (µM)	Control Group (µM)
<1 year old	465.82 ± 25.03	532.59 ± 46.13
≥1 year old	359.43 ± 32.71	496.21 ± 53.21
Total	412.625 ± 28.87	514.40 ± 49.67

IL-18 mRNA expression

Statistical analysis showed that there was a significant difference between IL-18 mRNA expression in children with pneumonia and children in the control group (Figure 1). The expression of the IL-18 gene was increased in children with pneumonia, significantly (P=0.0015).

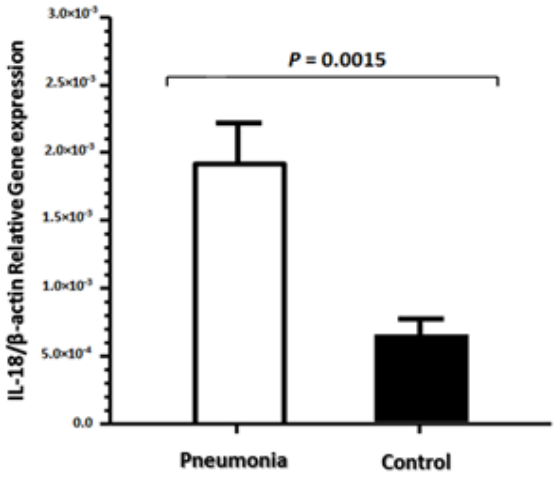

Figure 1. IL18 mRNA expression in children in pneumonia group and children in the control group.

Discussion

Zinc is an element that plays an important role in human development and immune function. In recent years, the role of zinc in many diseases has been discussed. Zinc levels in children with fever and seizures have been studied and the deficiency of this element has been stabilized in these cases (18). The deficiency of this element has a role in causing neural tube defects and prevention and reduction of these defects by consuming this element has been proven. The role of this element in wound healing due to surgery has also been shown (19). The most controversial issue in recent years about zinc is its role in infectious diseases and reducing the mortality of these diseases by consuming this element. The deficiency of this element in children with diarrhea has been shown in numerous studies (20). In one study, zinc consumption during diarrhea was shown to reduce the severity and duration of diarrhea (21).

Other infections that are associated with zinc deficiency are considered to be pulmonary infections (pneumonia). A 2004 study by Mahalanabis *et al*. (22) found that zinc consumption during pneumonia reduced fever and improved infection time. Another study conducted in 2004 by Brooks *et al*. (7) also confirmed the result. In a study similar to the present study conducted on children with pneumonia, serum zinc levels in children with pneumonia were significantly lower than in the control group. Thus, the zinc level was 176±98 u/dl in the control group and 90±51 u/dl in children with pneumonia (10). In the present study, the deficiency of this element was shown in children with pneumonia compared to healthy children. Serum zinc level was 514.40 ± 49.67µM in the control group and 412.625 ± 28.87µM in the patient group. This difference was statistically significant.

IL18 is a multifunctional immune system-regulating cytokine that is elevated in the human body in autoimmune and infectious diseases such as rheumatoid arthritis, pneumonia, and leprosy tuberculosis (23, 24). This suggests that pneumonia is involved in increasing IL-18. Unlike many cytokines, IL-18 mRNA may have a long half-life (25). In fact, untranslated IL-18 region 3′ lacks the AUUUA sequence, which is an unstable sequence. The high level of IL-18 transcripts in infectious and non-infectious tissues indicates that the biological activity of this cytokine may be controlled after translation (26-28).

IL-18 requires a post-translational enzymatic process to be biologically activated. Analysis of pneumonia shows the presence of an adult isoform of 18kDa IL-18 in the positive and negative cases of the disease. However, previous studies on intestinal mucus in patients with colitis show 18kDa IL-18 expression. But the 24kDa precursor IL-18 has also been found in the intestinal mucus of healthy individuals. Quantitative techniques such as ELISA to detect IL-18 cannot differentiate between mature IL-18 and IL-18 precursors (28). In rheumatoid arthritis, in which high levels of IL-18 protein were demonstrated using Western blotting, evaluation of complete IL-18 protein in ELISA biopsy specimens showed IL-18 depletion (29).

In general, according to the results of this study, children with zinc deficiency are at higher risk for a lung infection. Inflammatory cytokines such as IL-18 should also be increased in these children. Therefore, it can be concluded that zinc levels can reduce the expression of IL-18 mRNA and play an important role in the prevention and treatment of children with pneumonia.

In this study, the amount of zinc and IL-18 mRNA expression in children with pneumonia were evaluated and compared with healthy children. The results showed that the amount of zinc in children with pneumonia was significantly lower than in healthy children, and the level of IL-18 gene expression in these children was significantly increased compared to healthy children. Therefore, according to the results of this study, children with zinc deficiency are at higher risk for a lung infection. Inflammatory cytokines such as IL-18 should also be increased in these children. Therefore, it can be concluded that zinc levels can reduce the expression of IL-18 mRNA and play an important role in the prevention and treatment of children with pneumonia.

References

1. Nathan AM, Teh CSJ, Jabar KA et al. Bacterial pneumonia and

its associated factors in children from a developing country: A prospective cohort study. PloS one 2020; 15(2): e0228056.
2. Chisti MJ, Tebruegge M, La Vincente S, Graham SM, Duke T. Pneumonia in severely malnourished children in developing countries–mortality risk, aetiology and validity of WHO clinical signs: a systematic review. Trop Med Int Health 2009; 14(10): 1173-1189.
3. Rolles B, Maywald M, Rink L. Intracellular zinc during cell activation and zinc deficiency. J Trace Elem Med Biol 2021: 126864.
4. Wessels I, Rolles B, Slusarenko AJ, Rink L. Zinc deficiency as a possible risk factor for increased susceptibility and severe progression of COVID-19. Br J Nut 2021: 1-42.
5. Brion LP, Heyne R, Lair CS. Role of zinc in neonatal growth and brain growth: review and scoping review. Pediatr Res 2021; 89(7): 1627-1640.
6. Vickram S, Rohini K, Srinivasan S et al. Role of Zinc (Zn) in Human Reproduction: A Journey from Initial Spermatogenesis to Childbirth. Int J Mol Sci 2021; 22(4): 2188.
7. Brooks WA, Yunus M, Santosham M et al. Zinc for severe pneumonia in very young children: double-blind placebo-controlled trial. Lancet 2004; 363(9422): 1683-1688.
8. Shakur MS, Malek M, Bano N, Islam K. Zinc status in well nourished Bangladeshi children suffering from acute lower respiratory infection. Age (mo) 2004; 32(6.52): 30-36.64.
9. Baqui AH, Black RE, Walker CLF et al. Zinc supplementation and serum zinc during diarrhea. Indian J Pediatr 2006; 73(6): 493-497.
10. Strand TA, Adhikari RK, Chandyo RK, Sharma PR, Sommerfelt H. Predictors of plasma zinc concentrations in children with acute diarrhea. Am J Clin Nutr 2004; 79(3): 451-456.
11. Chen H-H, Shaw DM, Petty LE et al. Host genetic effects in pneumonia. Am J Hum Genet 2021; 108(1): 194-201.
12. Harun-Or-Roshid M, Ali MB, Mollah MNH. Statistical meta-analysis to investigate the association between the Interleukin-6 (IL-6) gene polymorphisms and cancer risk. PloS one 2021; 16(3): e0247055.
13. Delogu G, Famularo G, Tellan G et al. Lymphocyte apoptosis, caspase activation and inflammatory response in septic shock. Infection 2008; 36(5): 485-487.
14. Damsky W, Thakral D, McGeary MK, Leventhal J, Galan A, King B. Janus kinase inhibition induces disease remission in cutaneous sarcoidosis and granuloma annulare. J Am Acad Dermatol 2020; 82(3): 612-621.
15. Vaddi K. The Role of the JAK/STAT Signalling Pathway in Immunoregulation of Gastrointestinal Cancers. *Immunotherapy for Gastrointestinal Cancer*: Springer; 2017: 147-186.
16. Okamura H, Nagata K, Komatsu T et al. A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxic shock. Infect Immun 1995; 63(10): 3966-3972.
17. Ghayur T, Banerjee S, Hugunin M et al. Caspase-1 processes IFN-γ-inducing factor and regulates LPS-induced IFN-γ production. Nature 1997; 386(6625): 619-623.
18. Wessels I, Maywald M, Rink L. Zinc as a gatekeeper of immune function. Nutrients 2017; 9(12): 1286.
19. Maggini S, Maldonado P, Cardim P, Fernandez Newball C, Sota Latino E. Vitamins C, D and zinc: synergistic roles in immune function and infections. Vitam Miner 2017; 6(167): 2376-1318.1000167.
20. de Almeida Brasiel PG. The key role of zinc in elderly immunity: A possible approach in the COVID-19 crisis. Clin Nutr ESPEN 2020; 38: 65-66.
21. Black RE. Progress in the use of ORS and zinc for the treatment of childhood diarrhea. J Glob Health 2019; 9(1).
22. Mahalanabis D, Lahiri M, Paul D et al. Randomized, double-blind, placebo-controlled clinical trial of the efficacy of treatment with zinc or vitamin A in infants and young children with severe acute lower respiratory infection. Am J Clin Nutr 2004; 79(3): 430-436.
23. Kaplanski G. Interleukin-18: Biological properties and role in disease pathogenesis. Immunol Rev 2018; 281(1): 138-153.
24. Dinarello C, Novick D, Kim S, Kaplanski G. Interleukin-18 and IL-18 binding protein. Front immunol 2013; 4: 289.
25. Tone M, Thompson S, Tone Y, Fairchild PJ, Waldmann H. Regulation of IL-18 (IFN-gamma-inducing factor) gene expression. J Immunol 1997; 159(12): 6156-6163.
26. Hida N, Shimoyama T, Neville P, Dixon M, Axon A, Crabtree J. Increased expression of IL-10 and IL-12 (p40) mRNA in Helicobacter pylori infected gastric mucosa: relation to bacterial cag status and peptic ulceration. J Clin Pathol 1999; 52(9): 658-664.
27. Bilal I, Xie S, Elburki M, Aziziaram Z, Ahmed S, Jalal Balaky S. Cytotoxic effect of diferuloylmethane, a derivative of turmeric on different human glioblastoma cell lines. Cell Mol Biomed Rep 2021; 1(1): 14-22.
28. Azeez, S., Jafar, S., Aziziaram, Z., Fang, L., Mawlood, A., Ercisli, M. Insulin-producing cells from bone marrow stem cells versus injectable insulin for the treatment of rats with type I diabetes. Cell Mol Biomed Rep 2021; 1(1): 42-51.
29. Wawrocki S, Seweryn M, Kielnierowski G, Rudnicka W, Druszczynska M. IL-18 and related function proteins associated with tuberculosis severity and screening for active TB among patients with non-mycobacterial community-acquired pneumonia (CAP). Saudi J Biol Sci 2020; 27(11): 3035-3045.
30. Nozaki Y, Ri J, Sakai K et al. Inhibition of the IL-18 receptor signaling pathway ameliorates disease in a murine model of rheumatoid arthritis. Cells 2020; 9(1): 11.