Original Article

Correlation of Plasma Zinc with Neutralising Antioxidant Enzymes and Cellular Immune Responses in Healthy Nigerian Adults

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ABSTRACT

Background: Considerable information is available on the role of micronutrient Zinc in many aspects of immune function and protection of cell membranes from oxidative damage in diseased states. To our knowledge, there are no reports relating Zinc levels with different stages neutralizing antioxidant enzymes and cellular immune processes (leucocyte migration, engulfment and intracellular killing of phagocytosis) among healthy Nigerians.

Methodology: In 50 healthy Nigerians, cellular phagocytic mechanism [percentage leucocyte migration (%LM) and intracellular killing (%NBT)] were determined by microscopy; inflammatory cytokines [Plasma interleukin 6 (IL-6) and 8 (IL-8)] were determined using ELISA; respiratory burst indices [plasma catalase (CAT), superoxide dismutase (SOD), myeloperoxidase (MPO), hydrogen peroxide (H_2O_2) and nitric oxide (NO)] were determined by spectrophotometry. Zinc (Zn) was determined using AAS. Phagocytic indices, cytokines and respiratory burst indices were correlated with plasma Zn levels using Spearmans Correlation analysis at $\alpha_{0.05}$.

***Results*:** The result of the study shows that plasma IL-8 level was negatively correlated with Zn level while catalase was positively correlated with Zn level in healthy Nigerians.

Conclusion: *T*aken together, these findings suggest that immuno-potential effect of Zn may include production of protective antioxidant neutralizing enzyme (catalase) and reduction of IL-8 inflammatory cytokine.

Recommendation: These findings raise the possibility that Zn supplementation or Zn containing diets may be beneficial to individuals with intracellular infection or inflammatory diseases.

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INTRODUCTION

The World Health Organization recommends a daily zinc intake of 9.4–10 mg and 6.5–7.1 mg for men and women, respectively[1] so as to meet zinc's daily requirement. In health, human body contains 2–4 grams of zinc[2] as located in the skeletal muscle, bone, liver and the skin, and in other tissues[3]. Internal zinc balance is regulated by activities of two metal transporter protein families (Slc39a4 and Slc39a5). Most labile zinc is absorbed via intestinal epithelial cells into the plasma by Slc39a4[4] while excess zinc is excreted using kidneys[5] and the intestine[6] by Slc39a5. Zinc levels affect number and function of immune cells (macrophages, neutrophils, dendritic cells, mast cells, T cells and B cells)[7, 8]. Zinc also play essential roles in the signaling and inflammatory output of monocytes and macrophages, including activation of mitogen-activated protein kinase and nuclear factor kappa-

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light-chain-enhancer of activated B cells (NF-κB)[9], reduction of lysosomes integrity[10], activation of cryopyrin[11], induction of interleukin-1 beta (IL-*1β)* secretion by macrophages[12], reduction of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-*α)* in human monocytes[13].

Leucocytes are involved in host defense responses including phagocytosis, antigen presentation and immunomodulation[14], cytokine production and other immune system processes[15]. Activation of the immune system results in increased generation of reactive oxygen species (ROS) excess of which damages immune cells and this is neutralized by Zn[16]. Zn inbalance is detrimental to health because Zn deficiency increases susceptibility to infection[17, 18]. Zinc supplementation also decreased oxidative stress biomarkers and decreased inflammatory cytokines. Studies on the experimental model of Zn deficiency in humans showed that zinc deficiency increased the generation of IL-1β and its mRNA in human mononuclear cells following LPS stimulation. Zinc supplementation upregulated A20, a zinc transcription factor resulting in decreased generation of inflammatory cytokines[16, 17, 18, 23].

Apart from Zn, other micronutrients are known to boost immunity. Micronutrients most needed to sustain immunocompetence include vitamins A, C, D, E, B2, B6 and B12, folic acid, beta carotene, iron, selenium, and zinc[19]. Iron deficiency favours M1 macrophages and the development of a Th1 immune. Moreover, iron as a co-factor of myeloperoxidase is required for the generation of reactive oxygen species (ROS) in the respiratory burst[20]. Antioxidative function makes selenium an essential element for the immune system by protecting immune cells like phagocytes from oxidative stress caused by the respiratory burst. Se deficiency caused a reduced secretion of the Th2-stimulating cytokine IL-10 by dendritic cells (DCs) while that of the Th1-stimulating cytokines (IL-12p40 and IFN-γ) increased[21].

Vitamin A regenerates skin and mucosa thereby maintaining external barrier against invading pathogens, directly modulates proliferation and differentiation of immune cells, regulates DC differentiation into specific subsets that present antigens to $CD4^+$ Th cells and induces inflammatory Th17 cells that secrete IL-17[22]. Folate deficiency was associated with reduced maturation of DCs, lower secretion of IL-12, TNF-α, IL-6 and IL-1β by DCs and impaired differentiation of $CD4^+$ T lymphocytes[37]. Vitamin C deficiency leads to impaired phagocytosis and respiratory burst. Vitamin C has also been shown to promote the proliferation, differentiation and maturation of T-lymphocytes[38]. Immune tolerance is particularly promoted by vitamin D acting through dendritic cells to stimulate the differentiation of regulatory T cells. In addition, vitamin D also has a stimulatory effect on Th2 cells, thus contributing to the humoral defence borne by B lymphocytes[39]. Vitamin E is a major nutritive antioxidant which plays an important role during immune reactions by protecting cells and functional components from damage by reactive oxygen and nitrogen species released against pathogens during the respiratory burst. Additionally, vitamin E also exerts a direct effect on T cells by restoring the recruitment of signalling molecules after the formation of an immune synapse between an antigen presenting cells and a naïve T cell[40]

Above literatures suggest that Zn regulates leucocyte phagocytic functions and inflammation in a variety of ways. Also, oxidative stress and chronic inflammation which are important contributing factors to several chronic diseases are ameliorated by Zn.

MATERIALS AND METHODS

Subject population

This is a longitudinal study using convenient sampling method. The participants comprised of 50 healthy adult staff and students in University College Hospital, Ibadan, Nigeria. The participants did not have hypertension, diabetes mellitus, cardiovascular disease, cerebrovascular disease,

cancer, communicable diseases, chronic renal disease or inflammatory conditions. Also excluded were those that drink alcoholic beverages or cigarette smokers. Each participant was given 20mg Zn sulphate daily for 3 months which was orally taken in the presence of the investigators. Blood sample was collected a day after the last dose of Zn sulphate. Written, informed consent was obtained from all participants and the research was conducted in compliance with the Helsinki Declaration.

Plasma Isolation

Whole blood was collected in a covered test tube with lithium heparin anticoagulant and carefully mixed. Plasma was removed after centrifuging at 1500 ×g for 10 minutes in a centrifuge, the liquid component (plasma) was immediately transferred into a clean polypropylene tube using a Pasteur pipette and stored at 2–8°C.

Percentage Leucocyte Migration

Percentage leucocyte migration (%LM) was determined as previously described[24]. Leukocytes were isolated from whole blood using 6% dextran. After separation of plasma by centrifugation, 6% dextran was mixed with cells sediment (1:1) and incubated for 45 minutes at 37°C. Leukocyte-rich supernatant was obtained and washed 3 times in Kreb-Ringers solution, filled into capillary tubes, and anchored into a migration chamber filled with either Kreb-Ringers solution or antigen (BCG) and Kreb-Ringers solution (1:50). This was incubated for 18 hours at 37°C and the area of LM in the chamber containing antigen was compared with the area of migration in the chamber without antigen. The %LM was calculated as follows:

%LM = (area of migration in antigen solution/area of migration in medium alone) x 100.

Percentage Nitroblue Tetrazolium Dye Reduction.

Percentage nitrobluetetrazolium (%NBT) dye reduction was based on a previously described method[24]. For stimulated NBT procedure, 50 µL of NBT solution (0.2% NBT), 25 µL heparinized blood, and 25 µL of stimulant solution (nonviable bacterial extract) were mixed gently, incubated at 37°C for 10 minutes, and then incubated at 25°C for 10 minutes. A thick smear of the mixture was prepared and allowed to air dry. Air-dried smear was treated with Wright stain for 15 seconds and flooded with distilled water for 30 seconds before rinsing in water and air-drying. Two hundred leukocytes were counted under oil immersion objective and leukocytes showing dark formazan deposit were recorded as positive. The percentage of bacterially stimulated NBT was calculated as:

%NBT = [leucocyte with dark formazan deposit (positive)/total leukocytes counted] x 100.

Micronutrient analysis

Plasma concentration of Zn was determined using Atomic Absorption Spectrophotometry (Buck Scientific, 210, Atomic Absorption Spectrophotometer, Connecticut, USA) as previously described[25].

Cytokine analysis

Plasma concentrations of cytokines interleukin-6 (IL-6) and IL-8 were determined by enzyme linked immunosorbent assay (ELISA) as previously carried out[26]. Assay protocol was as specified by ELISA kit manufacturer (Life Technologies Corp, USA).

Superoxide Dismutase (SOD) activity determination

The SOD activity was determined using the method of Misra and Fridovich (1972) as previously carried out[27]. This method is based on the principle that SOD inhibits the autoxidation of epinephrine at pH 10.2.

Catalase (CAT) activity determination

Catalase activity was determined as previously carried out[27]. This method is based on the principle that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the

formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured at 570 nm.

Myeloperoxidase (MPO) activity determination

MPO activity was determined as previously described[27]. The rate of decomposition of H_2O_2 by peroxidase, with guaiacol as hydrogen donor, produced tetraguaiacol which was measured at 436 nm.

Hydrogen peroxide determination

Hydrogen peroxide concentration was determined as described by Wolff (1994) and previously carried out[27]. The assay is based on peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange to form Fe^{3+}-xylenol orange complex with an absorbance maximum of 560 nm. Plasma H_2O_2 was determined by comparing absorbance with standard solutions of H_2O_2.

Nitric oxide (NO) determination

Plasma nitric oxide concentration was determined using Griess reagent (Sulpanilamide and N-1-napthyethylene-diamine dihydrochloride) as previously described[27]. The assay is based on a reaction that utilizes sulpanilamide and N-1-napthyethylene-diamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Nitrite forms coloured chromophore with reagent, with an absorbance maximum at 540nm.

Statistical Analysis

Data obtained were presented as mean ± S.D for Zn, %NBT, %LM, IL 6 and IL 8 while SOD, MPO, CAT, NO and H_2O_2 were presented as mean (Interquartile Range). Spearman Rank Correlation was used to establish correlation between %NBT, %LM, IL 6, IL 8, SOD, MPO, CAT, NO and H_2O_2 with Zn levels. Values were considered significant at $p<0.05$.

RESULTS

The values of cellular %NBT, %LM, plasma Zn, IL-6, IL-8, SOD, MPO, CAT, NO and H_2O_2 were presented in Table 1. The values are within normal ranges. In Table 2, Spearman's Rank Correlation analysis showed that IL-8 was negatively correlated with Zn level while catalase was positively correlated with Zn level in healthy Nigerians ($p< 0.05$). All values of the variable falls within the normal ranges, as follows %NBT ($\geq$60%), %LM ($\leq$ 80%), plasma Zn *(60-130µg/dl)*, IL-6, IL-8, SOD, MPO, CAT (0.01-0.08*U/mg protein)*, NO *(12.2-69.4µmol/l)* and H_2O_2 *(11-59µmol/l)*

Table 1: Plasma Zn levels and Mean Phagocytic Indices in Apparently Healthy Nigerians

Variables	participants (n=50)
Zn*(µg/dl)*	89.53±17.89
NBT*(%)*	83.33±7.58
%LM*(%)*	58.00±2.0
IL-6*(pg/ml)*	8.01±3.92
IL-8*(pg/ml)*	80.04±15.46
SOD*(U/ml)*	0.19(0.14-0.26)
CAT *(U/mg protein)*	0.03(0.02-0.05)
MPO*(U/ml)*	8.27(7.23-9.59)
H_2O_2*(µmol/l)*	311.0(228.5-336.0)
NO*(µmol/l)*	12.75(9.47-16.08)

Table 2: Correlation of phagocytic indices with plasma Zn in healthy Nigerians

		Zn
%NBT	r	0.018
	P	0.773
%LM	r	-0.066
	P	0.290
IL-8	r	-0.146
	P	0.020*
IL-6	r	-0.036
	P	0.568
SOD	R	-0.085
	P	0.172
MPO	R	0.087
	P	0.160
CAT	R	0.127
	P	0.041*
H_2O_2	r,	0.004
	P	0.955
NO	R	0.014
	P	0.821

*Significant at $p<0.05$

DISCUSSION

The present study showed that zinc-mediation of phagocytic mechanism is likely to be at multiple levels. It is clear from this study that not all aspects of phagocytosis are affected equally by Zn intake. For example, IL-8 which is a leucocyte chemoattactant had negative correlation with Zn level while catalase which is a mediator of leucocyte intracellular killing was positively correlated with Zn level. Phagocytosis, a hallmark of innate cellular immune defenses that plays important role in protection against microbes was altered by Zn[28], but different aspects of phagocytosis were not specifically studied. Phagocytosis can be divided into phases, which include leucocyte migration to the infected foci, engulfment and intracellular killing. These phases employ various mechanisms that are controlled by a combination of factors to ensure clearance of foreign body.

Superoxide (O_2-) produced by NADPH oxidase activity is converted to hydrogen peroxide (H_2O_2) through dismutation within the phagosome[29]. H_2O_2 which is the first effector molecule that mediates microbicidal effect of phagocytes[30] can further react with O_2- to generate other reactive oxygen species (ROS) having ability to kill the intra-phagosomal pathogens[31]. Catalase (CAT) is a major scavenger of H_2O_2 which protects host cell from oxidative damage by excessive H_2O_2[32]. Positive correlation of catalase activity with Zn level seen in this present study could be the ability of host cell to control tissue damage resulting from the actions of excessive free radical production. This study posits that increased host plasma CAT activity might have been induced by Zn intake, this however needs further clarification. Iron is known to form complex with catalase whose activity is also iron-dependent[41]. It is likely that iron and zinc may contribute to catalase activities via completely separate pathways. This corroborates conclusions that supplementation with a combination of iron and zinc is effective in reducing iron deficiency anemia[42] and higher hemoglobin concentrations[43].

Hydrogen peroxide is a primary chemoattractant of immune cells to wounds during injury[33]. In the present study, Zn level was positively correlated with catalase activity. This implied that catalase activity increased with Zn level therefore catalase breakdown of H_2O_2 was increased and leucocyte chemoattractant effect of H_2O_2 was also decreased which caused reduced inflammation during Zn intake. IL-8 is primarily responsible for the recruitment of monocytes and neutrophils through a chemotactic gradient to attract, retain and activate cells to site of inflammation[34]. Also like H_2O_2, IL-8 was reported to stimulate oxidative burst activity[35]. Therefore, negative correlations between plasma IL-8 level with plasma Zn meant a reduction of inflammation as Zn level increases.

Foods high in zinc include oysters, beef, chicken, tofu, pork, nuts, seeds, lentils, yogurt, oatmeal, and mushrooms with daily value for Zinc as 11mg. Plant foods like nuts and seeds are good sources of zinc. High zinc fruits include avocados, blackberries, pomegranates, raspberries, guavas, cantaloupes, apricots, peaches, kiwifruit, and blueberries. These fruits provide 2-12% of the daily value per cup. Nuts and seeds high in zinc include squash seeds, pumpkin seeds, pine nuts, cashews, sunflower seeds, pecans, chia seeds, flax seeds, brazil nuts, and almonds. Zinc found in plant foods like fruits is not as bioavailable as zinc in animal foods[36].

CONCLUSIONS AND RECOMMENDATION

The vital role that the micronutrient zinc plays in maintaining health and reducing diseases have been known for many years. The present study suggests that immuno-potential effect of Zn may include production of protective antioxidant neutralizing enzyme (catalase) and reduction of IL-8 inflammatory cytokine. Thus, Zn supplementation or Zn containing diets is recommended for subjects experiencing infections in which phagocytosis is central to resistance and conditions involving apoptosis, damaging effects of oxygen radicals and inflammation.

Study Limitation: Non-determination of the levels of zinc, cellular activity including inflammatory mediators and neutralizing enzymes (SOD, catalase, MPO etc.) before zinc supplementation.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

Authors' Contributions: Both authors contributed equally to this work.

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