ORIGINAL RESEARCH ARTICLE

Beneficiary Effect of Zinc Supplementation in Tuberculosis as Reflected by Serum Level of Diagnostic Biomolecules

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Abstract

Aim and objective: This author had indoctrinated a higher *Mycobacterium tuberculosis* (*Mtb*) origin serum superoxide dismutase (SOD), detectable *Mtb* origin serum glutamine synthetase (GS) and inhibited host origin serum cholinesterase (ChE) as diagnostics for tuberculosis (TB). *Mycobacterium tuberculosis* by secreting abundant siderophores, the Fe^{+3} chelators, scavenges iron (Fe) from transferrin, lactoferrin, etc.; and thus, major decompartmentalized state of Fe takes place in host tissues, generation of superoxides is accentuated, which are used up by *Mtb* for dismutation reaction to evolve soluble oxygen for survival of this obligatory aerobe. Zinc (Zn), a redox inert metal, accelerates reversion to normal compartmentalized state of Fe by replacing Fe from thiol group binding site. Zn, by decreasing generation of reactive oxygen species renders an onslaught on *Mtb*. In this study, the author had mulled the effect of Zn supplementation (25 mgm of elemental Zn daily orally for 1 month) on the serum level of TB diagnostics as mentioned.

Materials and methods: Serum SOD, GS, and ChE were assayed for TB patients at baseline and also after 1 month with anti-TB drugs as two groups; one without and other with Zn supplementation. Same parameters were also measured for normal control and lung disease control subjects at baseline.

Result: Significant decrease in serum SOD ($p = 0.01$) and GS ($p = 0.01$) in TB patients with Zn supplementation for 1 month had been recorded in comparison to those without Zn supplementation. Also, recovery of serum ChE activity with Zn supplementation was significant ($p = 0.002$).

Conclusion: With the assertive and veritable improvement by instituting Zn supplementation in TB patients as reflected by serum level of diagnostic parameters, it is a great solemnity to promulgate that oral Zn supplementation might be added to anti-TB (A-TB) drug regime for early onslaught on *Mtb* and also for an effective weapon preventing development of primary drug resistance.

Keywords: Cholinesterase, Extrapulmonary tuberculosis, Glutamine synthetase, Metallothioneins, Pulmonary tuberculosis, Superoxide dismutase, Zinc transporters, Zrt-Irt-related proteins.

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Introduction

It is important to mention that tuberculosis (TB) is the 13th leading cause of death and stands second leading infectious killer after COVID-19 (exceeding that of HIV/AIDS). Again, the current dogma as perceived, there are about a quarter of world's population latently infected with *Mycobacterium tuberculosis* (*Mtb)*. This fearful number of latent TB infection (LTBI) indicates a great reservoir for individuals at risk to develop active TB. So, the need of hour is to have utmost attention to screen out LTBI and to undertake measures to block the progression to active TB and thus to end TB strategy for 2050 to be achieved.[1]

This author had indoctrinated in his previous papers, the assay of three enzymes namely superoxide dismutase (SOD), glutamine synthetase (GS), and cholinesterase (ChE) as early diagnostics for both pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB). Chattopadhyay DK, had demonstrated detection of L-methionine-S,R-sulfoximine (MSO) sensitive serum GS as a reliable diagnostic for TB.[2] The index author had recorded highly elevated sodium cyanide (NaCN) resistant SOD, being *Mtb* origin, as plausible diagnostic for TB.[3] The same author had also demonstrated inhibited serum ChE as a praiseworthy diagnostic for TB.[4] Chattopadhyay had also put forward measurement of the ratio of serum SOD and whole blood glutathione peroxidase (GPx) as TB diagnostic and assay of serum iron (Fe) as well as total iron binding capacity (TIBC) as a preliminary study for survey shortlisting suspected TB patients (pts).[5,6]

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Zinc (Zn), a divalent metal belonging to group (Gp) 12 of periodic table, is actually redox-inert owing to filled *d*-shell. On the other hand, Zn^{+2} is functional as Lewis acid and functions by integrating in a tetrahedral array with amino acid side chains like cysteine, histidine, aspartic, and glutamic acid.[7] The binding takes place with its four ligands. Zn, truly the ubiquitous metal, is essential for not less than 2800 macromolecules and even more than 300 enzymes to develop their structure and function.[8] In adult human body, total Zn content is about 2–3 gm. Only 0.1% of body Zn is present in serum of which 80% loosely bound to albumin and 20% tightly bound to α2-macroglobulin.[9]

For maintaining Zn homeostasis, ten Zn transporters (ZnTs) and fourteen Zrt-, Irt-related proteins (ZIP) are present in human

cells.[10] ZnTs promote Zn efflux from cells into intracellular vesicles and hence reduce intracellular Zn availability. On the other hand, ZIP transporters by promoting extracellular Zn uptake and releasing vesicular Zn into the cytoplasm, increase intracellular Zn availability. Both these ZnT and ZIP transporters by differential responsiveness to excess or less dietary Zn-intake, the noteworthy tissue-specific expressions, do maintain normal homeostasis of Zn in body to physiological stimuli through hormones and cytokines.[11] Again, it was reported that 20% of intracellular Zn did bind with ubiquitous cysteine-rich and low-molecular-weight metallothioneins (MTs). Metallothioneins by playing a significant role in metal uptake, distribution, storage, and release; act as a cellular Zn buffer.[12]

Remarkable changes in Zn homeostasis had been observed in inflammatory reactions. In acute phase response (APR), the serum Zn concentration decreases owing to re-distribution of Zn from plasma into organs, predominantly liver.[13] This fall in serum Zn level might be an endeavor to deprive the invading pathogens of Zn. On the other hand, macrophages simultaneously have the role of increasing the concentration of Zn to intoxicate phagocytosed microorganisms.[14] Interestingly, in bacteria, a Zn^{+2} pump had been demonstrated to catalyze active Zn^{+2}-transport utilizing p-type ATPase.[15] It had been reported that an increase in intracellular Zn accomplishes neutralization of reactive nitrogen and oxygen species, plays a vital role in energy metabolism, stimulates synthesis of proteins in general and specifically acute phase proteins in liver.[16] In inflammatory pathways, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling pathway plays the main role regulating the apoptosis controlling genes and also the genes controlling cell adhesion, proliferation, innate and adaptive immune responses, expression of proinflammatory cytokines, acute phase proteins, matrix metalloproteinases (MMPs), etc.[17] Von Bulow et al. had reported that Zn suppressed LPS-induced activation of I kappa B kinase beta (IKKβ) and ultimately TNF-α production in human monocytes.[18]

This author had categorically reported that Zn supplementation might replace Fe from binding with thiol groups and thus reverts the decompartmentalized state of iron to normal compartmentalized state of iron.[19,20] With Zn supplementation, the extracellular secretion of Fe-cofactor SOD (*sod* A) by *Mtb* in abundance having no leader peptide (SOD in other bacteria is strictly intracellular), responsible for its pathogenicity [to generate soluble oxygen by dismutation reaction with superoxide ($O_2^{\cdot}$)] gets a jolt by normal compartmentalization of iron in host tissues; and thus bactericidal action is hastened resulting in a decrease in mycobacterial load.[21] It had also been reported by the index author that anti-TB (A-TB) drugs with simultaneous Zn supplementation might replace Fe^{+3} from acetylcholine (Ach)-ferric hydroxamate complex binding more strongly with serum ChE and thus hastens the recovery of serum ChE activity.[4,22] The present author also had demonstrated significant serum GS decrease with Zn supplementation along with A-TB drug therapy.[23]

Zinc has prominent role in reducing oxidative stress by scavenging and decreasing production of reactive oxygen species (ROS). But, high Zn^{+2} level impairs mitochondrial function and activates mitochondrial lipoamide dehydrogenase (LADH) oxidase and thus abundant ROS production occurs.[24] So, in this study, a moderate dose of Zn containing 25 milligram of elemental Zn orally daily for 1 month in the form of zinc sulfate had been instituted for TB pts along with A-TB drugs to mull its effectiveness as reflected by the changes in serum level of diagnostic biomolecules for TB. Notwithstanding, half of the TB pts kept under A-TB drug therapy for 1 month were administered oral Zn supplementation and the other half of TB pts were kept only under A-TB drug therapy for 1 month but without any Zn supplementation. The diagnostic TB parameters had been assayed for TB pts at baseline and after 1 month with A-TB drug therapy with or without Zn supplementation. A comparison study of these two groups (Gps) had been made.

Materials and Methods

The cohort study was conducted at BS Medical College and Hospital, Bankura, West Bengal, India, from June 2004 to May 2009. A total of 224 participants (aged 8–64 years), including normal control (NC), lung disease control (LDC), PTB, and EPTB subjects were enrolled for the study. The purpose of study was explained to all participants and before collection of blood, informed verbal consent was obtained from each of the subjects. For under-aged participants, verbal consent was sought and obtained from their legal guardians before collection of blood. The study was conducted in four phases with 56 participants being enrolled in each phase. The subjects were divided into three Gps.

1. Group I: (n = 56) NC – They were healthy relatives of TB pts having no clinical symptom, sign or any finding suggestive of TB or any sort of disease. They were sputum-negative for acid-fast bacilli (AFB). Collection of blood was made from each subject for estimating aforesaid parameters at baseline.
2. Group II: (n = 56) lung disease control (LDC) – These patients were suffering from respiratory tract infection (n = 21) or chronic obstructive pulmonary disease (n = 14) or bronchial asthma (n = 11) or bronchiectasis (n = 8) or bronchogenic carcinoma (n = 2). They were selected from pts attending out-patient-department (OPD) of BS Medical College and Hospital, Bankura. For each subject of this group, blood sample was collected at baseline only.
3. Group III: TB pts – Patients suffering from TB (irrespective of age, sex, and socioeconomic status attending OPD of BS Medical College and Hospital, Bankura, and also TB pts admitted in TB-ward of the same hospital were taken into account. Diagnosis of TB was made clinically by characterized symptoms and signs as well as by other investigative procedures like radiological investigation, sputum for AFB, fine needle aspiration and cytology (FNAC) whenever possible. The ELISA technique for serodiagnosis (detection of mycospecific serum immunoglobulins level) was also done as an added procedure.[25,26] Multidrug resistant TB pts were not considered for this study. It was an exclusion criterion regarding TB pts concerned for this study. All the subjects in this study had normal liver function tests (LFT) and that was an inclusion criterion for the study as transferrin, a major Fe-binding protein in human, is synthesized predominantly in liver. Based on prior diagnosis, TB pts were divided into two subgroups as follows:
   a. Subgroup IIIA (n = 56): It had included pts suffering from PTB.
   b. Subgroup IIIB (n = 56): It had included pts suffering from extra-PTB (EPTB); e.g., TB lymphadenitis (n = 12), TB pleural effusion (n = 23), TB meningitis (n = 9), lupus vulgaris (n = 1), and also spinal (n = 1), hip joint (n = 6), intestinal (n = 1), urinary bladder (n = 1), and miliary (n = 2) TB.

For all those TB pts, A-TB drug therapy was started between 0 and 15 days. Collection of blood was done for all these TB pts at baseline. All TB pts were kept under directly observed treatment (DOT) program as recommended by Revised National Tuberculosis Control Program (RNTCP). Repeat blood collection was done after 30 days' treatment. During this additional 30 days' treatment, half of TB pts under sub-Gps A and B of group III were administered oral supplementation of Zn daily in the form of zinc sulfate containing 25 mgm of elemental Zn.

Collection of blood: Morning fasting blood samples were collected by venipuncture. Serum, so obtained was transferred in clean and sterile Eppendorf tube and kept in a refrigerator at 2–4°C until assayed on the same day of sample collection.

Procedure Methodology

- Estimation of serum GS: The highly specific method of transfer reaction was used for assaying GS activity.[27] Gamma-glutamyl hydroxamate so formed in the reaction; L-glutamine + Hydroxylamine → γ-glutamyl hydroxamate + NH_3; in presence of sodium arsenate and ADP; was made to react with ferric chloride to form orange-brown colored γ-glutamyl hydroxamate-Fe^{+3} complex. The OD of the product was measured at 540 nm. The serum GS activity in milli units (mu)/mL was determined from standard curve drawn with a pure sample of γ-glutamyl hydroxamate. One unit of enzyme is equivalent to the amount of GS that can produce one micro-mole of γ-glutamyl hydroxamate per minute at 37°C.
- Estimation of serum SOD: Serum SOD activity was assayed using the reagent kits of Randox Laboratories Ltd as per the manufacturer's instruction. The absorbance at 505 nm was measured with the help of spectrophotometer. The degree of inhibition of the reaction was used as a measure of SOD and expressed as units/mL (U/mL). One unit of SOD equals to the amount of SOD causing 50% inhibition of reduction rate per minute.[28]
- Estimation of serum ChE: The principle of the assay (method of Hestrin S) is based on reaction between ACh and hydroxamate to form acetyl-hydroxamate which on reacting with ferric ion (Fe^{+3}) forms a red-purple colored complex in an acidic medium, which is measured at 530 nm using a spectrophotometer.[29] Acetyl choline chloride was used as substrate and serum ChE activity was expressed as units/hour/mL of serum.

Note: In this study, consolidated standards of reporting trials (CONSORT) was adopted. This evidence-based study is for reporting randomized control trials (RCT). It is meant to facilitate quality assessment by reporting in a full and clear manner.

Statistical Analysis

Statistical analysis for the results was made using the Statistical Software for Social Sciences (SPSS Version 21.0). The level of significance was assessed using an independent Student's *t*-test. The $p < 0.05$ was taken to be statistically significant.

Results

Serum GS, the TB diagnostic had decreased significantly after A-TB drugs treatment but without Zn supplementation ($p = 0.005$). With A-TB drugs along with Zn supplementation, ($n = 28$); the decrease in serum GS in TB pts was very significant when compared with those of without Zn supplementation ($n = 28$); ($p = 0.001$) (Tables 1 and 2). Another TB diagnostic marker, serum SOD had shown significant decrease ($p = 0.03$) with A-TB drug treatment for 1 month but without Zn supplementation. On the other hand, a more significant decrease ($p = 0.01$) of serum SOD in TB pts had been recorded with A-TB drug treatment with Zn supplementation ($n = 28$), when compared with those kept under A-TB drugs alone ($n = 28$), (Vide Table 2). On the other hand, there was significant recovery of serum ChE activity in TB pts with A-TB drugs for 1 month, without Zn supplementation ($p = 0.005$). But, with Zn supplementation along with A-TB drugs for 1 month, ($n = 28$); the recovery of serum ChE was more significant ($p = 0.002$) when compared with those without any Zn supplementation ($n = 28$) (Table 2). Serum levels of the said parameters for NC and LDC subjects at baseline had been depicted in Table 1.

To evaluate the outcome of Zn supplementation in TB pts along with A-TB drugs, the receiver-operating characteristic (ROC) analysis was adopted. The corresponding empirical ROC curve was drawn by the non-parametric method. The curve as well as corresponding area under the curve (AUC) had established that Zn supplementation along with A-TB drugs had a predictive and

Table 1: Serum GS, SOD, and ChE of normal control and lung disease control subjects at baseline

Parameters	*Normal control subjects at baseline (n = 56)*	*Lung disease control subjects at baseline (n = 56)*
Serum GS (milliunits/mL)	Undetectable	Undetectable
Serum SOD (units/mL)	128 ± 31	144 ± 33
Serum ChE (units/hr/mL)	134.6 ± 10.4	119.7 ± 7.4

Table 2: Serum GS, SOD, and ChE of tuberculosis patients at baseline and after 1 month with A-TB drugs and with or without zinc supplementation

	Pulmonary TB				*Extrapulmonary TB*			
		After 1 month with A-TB drugs				*After 1 month with A-TB drugs*		
Parameters	*At baseline n = 56*	*Without Zn supplementation n = 28*	*With Zn supplementation n = 28*	*p-value*	*At baseline n = 56*	*Without Zn supplementation*	*With Zn supplementation*	*p-value*
Serum GS (milliunits/mL)	17.86 ± 5.4	4.78 ± 2.3	0.98 ± 0.34	0.001	17.20 ± 5.1	4.61 ± 2.7	0.94 ± 0.27	0.001
Serum SOD (units/mL)	1438 ± 102	986 ± 88	742 ± 47	0.01	1386 ± 81	972 ± 59	723 ± 32	0.01
Serum ChE (units/h/mL)	34.4 ± 10.6	62.7 ± 8.6	86.4 ± 6.8	0.002	36.8 ± 9.7	66.4 ± 7.8	88.8 ± 7.2	0.002

substantial ability to hasten rapid bacterial killing and well-being for TB pts so far the level of serum TB diagnostics concerned.

Discussion

Mycobacterium tuberculosis requires iron (Fe) for its growth and survival in host tissues. Iron is the cofactor of SOD (*sod* A) secreted extracellularly in abundance having no leader sequence. On the other hand, SOD in other bacteria is strictly intracellular. The intracellular (RBC) mammalian SOD which is copper (Cu)-zinc (Zn) cofactor and does contain leader peptide had revealed lesser degree increase than serum SOD (mycobacterial origin) in TB patients.[21] Fe plays a pivotal and important role for pathogenesis and virulence of mycobacteria. *Mycobacterium tuberculosis* while using β-oxidation pathway does survive nutrient pathway inside macrophages, the phagosomes with the help of membrane-bound NADPH-oxidase system reduces O_2 to superoxide ($O_2^{\cdot}$) initiating oxidative burst which interestingly is very well used up by *Mtb* by dismuting it with its abundant extracellular secretion of Fe-cofactor SOD to generate soluble $O_2^{\cdot}$ required badly for survival of this obligate aerobe. Not only that, this Fe-cofactor SOD released extracellularly in the vicinity of the organism neutralizes toxic $O_2^{\cdot}$ before they might reach the outer wall of the organism.[5] For acquiring Fe, *Mtb* secretes out extracellularly mycobactin and carboxymycobactin, the lower molecular weight (MW) Fe^{+3}-specific siderophores having high affinity for Fe and thus scavenging Fe not only from host insoluble and host protein-bound iron (e.g., transferrin, lactoferrin, and ferritin) but also from iron in mineral phases like oxide and hydroxides of Fe.[20] Through mycobacterial membrane proteins (Mmps) and also with associated transport proteins, *Mtb* secretes out desferricarboxymycobactin which does chelate Fe^{+3} and is converted to ferricarboxymycobactin. Then by using Irt A/Irt B transporters present on cytoplasmic membrane and with the help of FAD-mediated reductase enzyme of Irt A protein, *Mtb* reductively removes Fe (Fe^{+3} to Fe^{+2}) and mediates plausible internalization of Fe.[30] With presence of abundant siderophores, the Fe^{+3} chelators and stronger binding sites for iron and also diffusible one, remarkable decompartmentalized state of iron sets in host tissues. This decompartmentalized state of Fe in host tissues is augmented by the veritable decrease in serum transferrin level in TB pts as a result of infection as well as protein–energy malnutrition.[6] Also, reduction of pH of vesicles by hydrogen-proton pump (H^+ATPase) is required for Fe-bound transferrin to release its iron. But, *Mtb* by preventing phagosome acidification and lysosome fusion decreases Fe-dissociation from transferrin and is able to acquire Fe from endosomal holotransferrin.[20] In that scenario, Fe^{+3} by complexing with high-affinity siderophores, the diffusible one, and also by decreasing and damaging normal Fe-binding sites and altering the barriers; introduces a state of major decompartmentalized state of iron in host tissues in TB pts as siderophores having more affinity toward Fe^{+3} become major transporters of Fe instead of transferrin.[20] In prevailing major decompartmentalized state of Fe, increased generation of $O_2^{\cdot}$ takes place owing to Fe-catalyzed oxidation of thiol gps. Interestingly, it is insinuated that generated $O_2^{\cdot}$ is used up by *Mtb* for dismutation reaction.[19,20] Superoxide dismutase by acting as $O_2^{\cdot}$ Scavenger curtails generation of reactive nitrogen intermediates (RNI), the formation of which strongly correlates with antimycobacterial activity of macrophages with L-arginine dependent induction. So, *Mtb* with the liberation of SOD scavenges $O_2^{\cdot}$ and inhibits evolution of RNI and renders a basal level of nitric oxide (NO) and saves themselves from onslaught.[5] Interestingly, *Mtb* escapes killing by hydrogen peroxide (H_2O_2) so formed in dismutation reaction by breaking down H_2O_2 mediated by mycobacterial catalase-peroxidase protein and alkyl hydroperoxide reductase protein encoded, respectively, by *kat*G and *ahp*C.[31]

Out of the unique family of protein exporters, WXG-100 secretion system (ESX/WSS) is secreted by mycobacterial cell. They consist of ESX-1 to ESX-5. ESX-3 promotes siderophore-mediated Fe and Zn acquisition for *Mtb*.[32] Zinc uptake regulator (ZUR), the Zn-sensing transcriptional regulator factor of ferric uptake regulator (FUR) superfamily of proteins, is abundant in bacterial species and is a regulator of Zn import and Zn homeostasis. Zn is essential but deleterious in excess for the pathogen. The pathogen always endeavors to get access to the host Zn-reservoir. Moayeri et al. had reported that host adopts to defend by either limiting Zn availability or intoxicating the invading pathogen with Zn excess as higher concentration of Zn is toxic to pathogen cell.[33] Zn supplementation leads to increased polymorphonuclear (PMN) cell chemotaxis and increased phagocytosis of invading pathogens. Zinc plays a crucial role by orchestrating adaptive immunity through several mechanisms. Zinc is involved in recognition of major histocompatibility complex (MHC) class 1 by natural killer (NK) cells; hastens adhesion of monocytes to endothelial cells by augmenting the synthesis of proinflammatory cytokines like interleukins (IL); IL-1β, IL-6, and TNF-α and also by subsequent T-cell lymphocytosis and increased B-cell and subsequent antibody production; plays a crucial role for the balance between different T-cell subsets.[34] Also, as mentioned already in Introduction Section, Zn has the role of suppressing IKKβ and NF-Kb in infection and Zn^{+2} by being imported by ZIP8 into macrophages, monocytes and lung epithelium ultimately results in inhibition of TNF-α production.[18]

Iron is accessible to two oxidative forms namely ferrous (Fe^{+2}) and ferric (Fe^{+3}) in aqueous media and thus readily taking part in lots of diverse electron transport reactions like molecular oxygen and nitrogen activation, oxygen binding with hemoglobin (Hb), myoglobin, etc. To combat kinetic barrier redox reaction, release of Fe as water soluble Fe^{+2} is essential.[20] As far as the redox equilibrium of Fe^{+2}–Fe^{+3} couple, free radicals are involved in the process. Therefore, Fe overload and Fe toxicity in biological system are manifested by increased free radical intermediates. Vital sites, liable to Fe-catalyzed oxidation, are kept in hydrophobic milieu or by binding with catabolically inert Zn-ion kept well protected from Fe-interaction.[20] Zn, being redox neutral (unlike Fe and Cu) and more electropositive than Fe, might replace Fe from binding with critical thiol group and curtailing $O_2^{\cdot}$ production.

With Zn supplementation, there is a jolt in formation of soluble $O_2^{\cdot}$ accelerating *Mtb* killing.[19,20] Zn^{+2} also prevents generation of ROS by competing with redox-active metal ions, protecting sulfhydryl (-SH) gp in proteins, increasing the anti-oxidant Cu-Zn cofactor SOD activity and also by induction of metallothionins (MTs). Metallothionins function as Zn-chaperons regulating gene expression and activity of metalloproteins and metal-dependent transcription factors. MTs are in fact a link between Zn and cell redox status acting against oxidative stress and participating in immune response.[12] In addition to antioxidant role of Zn, the pro-oxidant role of Zn had been reported with aberrant increase in Zn level. As mentioned already in Introduction Section, the aberrant Zn released from organelles like mitochondria and lysosomes and/or

from binding sites like MT might cause secondary oxidative stress mediated by different oxidant-producing enzymes through the inhibition of metabolism and mitochondrial functions. Also, Zn in hypoxia can activate NADPH-oxidase through phosphorylation modulation, thus initiating ROS accumulation.

The moderate dose of Zn (25 mgm elemental Zn daily orally for 1 month) for TB pts was below the lowest observed adverse effect level (LOAEL) based on SOD activity in erythrocytes with Zn intake.[35] This dose of Zn supplementation was without any toxic symptoms and signs. This author previously had undertaken a study to assay serum Zn level at baseline for TB pts and also after 1 month with daily oral Zn supplementation along with A-TB drugs. At baseline, while lymph gland TB pts had shown significant decrease in serum Zn level, the PTB pts had recorded significantly increased value. With Zn supplementation for 1 month along with A-TB drug treatment, the hyperzincemia in PTB and hypozincemia in lymph gland TB pts were restored to almost normal serum Zn levels.[36] The putative observation for almost normalization of serum Zn level with Zn supplementation might be due to orchestration of the roles played by Zn-transporters (ZnTs), reducing intracellular Zn availability; the intracellular Zn-importer protein ZIPs and intracellular cysteine-rich, metal-binding protein MT as elaborated in Introduction. Different stimuli like cell stress, etc., leads to an increase in reactive oxygen and nitrogen species which subsequently release free Zn from Zn pool specially MT. Zn stimulates an increase of MT, the major Zn-binding protein. In a cell-free system, the Zn-transfer process takes place by the direct interaction between apo-zinc binding peptides and MT. *Mycobacterium tuberculosis* has the important role to protect cells from the onslaught of electrophiles and exposure to oxidants by protecting the sulfhydryl gps (-SH). *Mycobacterium tuberculosis* has a unique role to play to regulate Zn level and its distribution in intracellular spaces.[37] With Zn supplementation in TB pts, there was an egress of Fe out of cells under the hastening process of reversion to normal compartmentalization state of Fe in host tissues and the egressed Fe was used up for hemoglobin synthesis as reported by Chattopadhyay DK.[20] Now, let the present researcher interpret and discuss the serum level of the TB diagnostics parameters as obtained after 1 month's oral Zn supplementation along with A-TB drug treatment and its perspectives.

A significant decrease in serum SOD had been reported in TB pts with Zn supplementation for 1 month along with A-TB drugs when compared with those of with TB pts with A-TB drug treatment for 1 month but without Zn supplementation. The immune function and the antioxidant role of Zn have already been elaborated in this study. Zinc by decreasing generation of $O_2^{\cdot}$ creates a setback for *Mtb* with lesser production of soluble $O_2^{\cdot}$ by dismutation reaction. Also, Zn^{+2} as a Lewis acid and integrating in a tetrahedral array with amino acid side-chain (like cysteine, histidine, etc.); might replace Fe from cofactor site of SOD and thus decreases virulence of *Mtb* and hastens *Mtb* killing.

Glutamine synthetase is an abundant and omnipresent biomarker in the serum of TB pts. Glutamine synthetase, the dodecamer of identical 53 Kda, not only has the pivotal role in cell nitrogen and hence essential for synthesis of cell wall component poly-L-glutamate/glutamine present exclusively in pathogenic mycobacterium; but also helps the bacteria to prevent phagosome lysosome fusion by altering ammonia level and hence the pH. Though four *Gln* A genes do encode for *Mtb*, only *Gln* A1 is highly expressed and thus essential for *Mtb* homeostasis.[38] The present research work has elaborated a significant decrease in serum GS in TB pts with oral Zn supplementation for 1 month along with A-TB drugs when compared with that of those with A-TB drugs only. The veritable cause behind the beneficial effect of Zn supplementation in bringing down serum GS level significantly in TB pts may be explained by the praiseworthy decrease in dismutation reaction and hence generation of soluble $O_2^{\cdot}$ required for survival of *Mtb*. With enhanced bacterial killing, the extracellular GS secretion by *Mtb* is decreased and hence significant decrease in serum GS. In this connection, while entrenching assay of serum GS as reliable and fastidious as diagnostic marker for TB, it is to mention that Tumani et al. had reported endogenous very low level of serum GS in normal subjects and with some of the diseases. Using sandwich enzyme immunoassay and immunoblotting, serum GS in normal control subjects ($n = 35$) was 36 ± 27 pg/mL, in pts with amyotrophic lateral sclerosis ($n = 8$) it was 116 ± 6.2 pg/mL, in Alzheimer's disease ($n = 9$) 111 ± 53 pg/mL, 72 ± 59 pg/mL with vascular dementia ($n = 15$), 77 ± 32 pg/mL with Parkinson disease ($n = 5$) and 74 ± 32 pg/mL with schizophrenia ($n = 4$).[39] These very low level of serum GS is, in fact, undetectable by transfer reaction. So, assay of serum GS by transfer reaction will not at all be interfered by the very low level of endogenous serum GS level in normal control subjects or with the ailments as mentioned above. Two metal ions bind with bacterial GS, one with high and the other with low affinity. As per the hard-soft-acid-base (HSAB) theory, for GS cation active binding site, Mn^{+2}, Mg^{+2}, Ca^{+2}, and Co^{+2} (i.e., the hard metal ions) are preferably chosen than the borderline metals like Cd^{+2} and Zn^{+2}. It is interesting to note that lower affinity metals do stabilize the active binding site of GS with hard metals.[40] These observations along with knowledge about the binding sites of GS with substrate as well as nucleotide binding sites might be the important research subjects to posit and confirm the effect of Zn supplementation on the binding sites of GS. Also, with meticulous experimental study on the nucleotide binding sites, it might have suave observations to use for targeting drug design.

Besides these two TB biomarkers, this researcher had demonstrated inhibition of serum ChE as a diagnostic for TB.[4] As reported by index author, siderophores bind strongly with Fe^{+3}, a strong Lewis acid having preference to strong Lewis bases like anionic binding site. Acetylcholine is the neurotransmitter responsible for modulation of sweat rate. ChE possesses peripheral anionic sites which are meant for binding with ACh and other quaternary ligands acting as uncompetitive inhibitors.[4] So, higher concentration of Fe^{+3}- scavenger siderophores can be correlated with the formation of higher concentration of serum ACh-ferric hydroxamate complex binding more strongly with serum ChE and remains unhydrolyzed.[4,22] So, significant inhibition of serum ChE activity had been recorded in TB pts at baseline. With the inhibitory effect of ChE, the substrate ACh is not hydrolyzed rapidly and there is hastening of rate of sweating in TB pts, the night sweating, one of the cardinal signs for TB.[4] With A-TB drug therapy, there was recovery of the activity of serum ChE; but with Zn supplementation along with A-TB drug therapy, the recovery of serum ChE activity was much significant when compared with those under A-TB drug therapy alone. Zn, besides its role as anti-oxidant and also as an agent hastening normal compartmentalization of Fe in host tissues; might also replace Fe^{+3} from ACh-ferric hydroxamate complex resulting in qualitative and quantitative recovery of serum ChE activity.[4,22]

Conclusion

Zinc supplementation along with A-TB drugs in TB, hastens decompartmentalized state of iron to revert back to normal compartmentalized state of iron. Zinc, by replacing Fe from thiol binding site and inhibiting generation of $O_2^{\cdot}$ mongers the very pathogenicity of *Mtb*. The dose of Zn as used in this study (25 mgm elemental Zn daily orally) may be added in the regime of A-TB drugs under RNTCP. The dose of Zn was below LOAEL and without any pro-oxidant effect. Also, with robust attack on metabolic process and pathogenicity of *Mtb* from the very beginning of drug treatment, incidence of primary drug resistance may be curtailed.

Clinical Significance

As discussed in this study, intracellular Zn-homeostasis is assiduously regulated by ZIP, ZnT and also a family of intracellular, thiol-rich protein, the MTs which have the role of exchanger of Zn with other proteins in the cell as both Zn-acceptor and Zn-donor. With reversible dissociation of Zn^{+2} of MT and with oxidation of sulfur donors contribute toward intracellular Zn signaling. Bouron et al. had reported mobilization of Zn^{+2} through cellular membrane by some membrane transporter proteins like voltage-gated calcium channels, transient receptor proteins (TRP) and also by glutamate and ACh receptors.[41] These fledgling, suave, and meticulous observations like the role played by MTs in Zn-homeostasis; and also mobilization of Zn through glutamate and ACh receptors would encourage the researchers to conduct elegant research to unravel the relationship among Zn-homeostasis, MTs, membrane transporter proteins. This author has elaborated the relevant information from published literatures and also from his own published research works in detail in Discussion Section, which hopefully would throw more focus on the beneficial effects of Zn supplementation in TB. Those investigative works might enlighten other beneficiary effects of Zn supplementation and also on the line of drug target-ultimately enabling global population to become free of menace of TB ravaging human civilization badly.

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