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RESEARCH ARTICLE

STUDY OF COPPER, ZINC AND CERRULOPLASMIN ALBUMIN RATIO IN PULMONARY TUBERCULOSIS PATIENTS ON DIFFERENT DRUG REGIMES

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ABSTRACT

Introduction: Pulmonary Tuberculosis (PTB) is a severe bacterial infection that continues to be the main cause of morbidity and mortality. Compromised immune status due to micronutrient deficiency is one of the key factors predisposing to PTB infection. The aim of the present study was to assess the trace elements Copper and Zinc status in PTB patients and to evaluate the diagnostic role of Cerruloplasmin (Cp) and Cerruloplasmin Albumin ratio (Cp/Alb).

Method: 120 Adult PTB patients (60 newly diagnosed and 60 relapse) attending the Institute of Respiratory Disease, SMS Medical College, Jaipur and 60 healthy non family members of patients / Controls were enrolled for the study. After informed consent, sputum examination and Routine Biochemical analysis along with Serum Copper (Cu), Zinc (Zn) and Cerruloplasmin (Cp) were estimated in fasting sample for all subjects.

Result and Discussion: Serum Protein, Albumin and Zinc were significantly reduced in PTB group as compared to controls due to increased catabolism, anorexia, pre-existing undernutrition and Acute Phase Response associated with PTB. Serum Cu and Cp were significantly high which may be due to increased hepatic synthesis of acute phase proteins. Cu correlated strongly only to Cu/Zn ratio but the later was found to have good correlation with all parameters estimated. Similarly, Cp/Alb ratio correlates well with Cp as well as Cu and Zinc.

Conclusion: Trace elements alteration in PTB is significant and can be used in nutritional monitoring of patients. Early estimation of micronutrient status can help identify at risk individuals and prevent occurrence of relapse. This would also enhance survival and early recovery of patients. Cp/Alb ratio can be used as additional tool to assist diagnosis and Prognosis of PTB.

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INTRODUCTION

Many essential elements present in our body in minute quantity perform functions indespinsable for growth and maintenance of tissue. The trace elements are examined critically as potential key factors in various diseases. Two important trace elements Copper (Cu) and Zinc (Zn) play role in pathophysiology of pulmonary tuberculosis due to their immunomodulatory functions. Poor Micronutrient status adversely affect immune competence that not only enhances susceptibility to infection but also influence morbidity and mortality of disease. This works involves study of trace elements Cu and Zn along with Cerruloplasmin (Cp) to detect deficiency at early stage so that supplementation could be planned in highly malnourished patients. Such Nutritional monitoring will help in early recovery, prevention of relapse and increase survival. Zn is a micromineral and a component of more than 200 different enzyme systems. It is a cofactor for Superoxide Dismutase (SOD) with role in carbohydrate and Protein metabolism, influence immune strength and wound healing. Its deficiency causes impaired Cell mediated immunity (CMI), decrease circulating T- Cell count thereby reducing Tuberculin reactivity, decreases phagocytosis and compromise neutrophil function, thereby increase susceptibility to infection (Ramakrishnan *et al*., 2008). Cu is an essential trace element

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and cofactor of several enzymes such as Cytochrome Oxidase of Mitochondrial ETC and Cytosolic SOD. 95% of total Serum Cu is bounded to Cp, which is an extracellular antioxidant synthesized by hepatocytes and 5 % is bounded nonspecifically and loosely to Albumin (Alb) (Robert *et al*., 2003). Alb bound fraction is exchangeable with tissue. Albumin (69Kda) is the most abundant globular plasma protein (60%). It is synthesized by liver and is a nonspecific carrier of various substances and metal ions. Beside its transport function, it helps to maintain plasma colloidal osmotic pressure and provide antioxidant protection by functioning as serum peroxidase in the presence of reduced Glutathione (Cernat *et al*., 2011). Epidemiological data consistently show that reduced Albumin level is associated with increased mortality (Cernat *et al*., 2011). Cerruloplasmin (160Kda) is an alpha 2 globulin containing 95% Cu, giving it a blue colour (6-8 atoms of Cu are present per molecule of Cp). It acts as an extracellular antioxidant and possess ferroxidase activity that catalyzes oxidation of ferrous to ferric iron with production of H_2O_2 and O_2^-. Macrophages and Lymphocytes also produce small amount of Cp (Anuradha *et al*., 2014). Recent review shows that trace element deficiency have adverse effect on immune status (Choi *et al*., 2015). Apart from dietary factors, host pathophysiology also affect trace element absorption, utilization and serum concentration with altered level seen in various disease including TB. They have impact on clinical outcome and are thus related to disease control (Choi *et al*., 2015). This work is undertaken to assess if changes in micronutrient factors might assist diagnosis and prognosis of TB leading to decrease morbidity and mortality by early treatment and proper supplementation. Their estimation will help evaluate therapeutic effect and monitor nutritional regime. Cerruloplasmin / Albumin (Cp/Alb) ratio and Cu/Zn ratio were also calculated and correlation among all parameters was studied. Micronutrient status do affect immune strength and susceptibility to MTB infection. Knowledge of trace element is essential to plan nutritional supplememtation which is equally significant to chemotherapy in recovery of patient and prevention of re-activation.

MATERIALS AND METHODS

The study was conducted in Department of Biochemistry, SMS Medical College, Jaipur in collaboration with Institute of Respiratory Diseases (IRD) Jaipur. 60 Newly diagnosed PTB patients (Group A), 60 relapse cases (Group B) and 60 healthy controls (Group C) were enrolled for the study. Controls were tested free of MTB, without any previous or present symptoms of TB or any other pulmonary disease and non family members of patients. Patients with drug resistant TB (MDR), extrapulmonary TB, those with significant renal, cardiac, neoplasm or respiratory disease (other than PTB like lung cancer) etc., diabetes, endocrine or genetic disorder were excluded from the study. HIV positive cases, Pregnant or lactating women and those on oral nutritional supplements were also excluded. All subjects gave their written consent to participate in the study that was approved by institutional Ethic committee.

Sample collection and Bacteriological examination

Routine clinical history along with personal details were noted for all subjects. Two consecutive sputum sample of each patient were collected and subjected to Acid fast Staining. In order to determine Smear Positivity Extent (SPE), number of Acid Fast Bacilli (AFB) were counted microscopically and analyzed as follows:1. No AFB in 100 fields-negative; 1-9 AFB in 100 fields-scanty ; 10-99 AFB in 100 fields +1 ; 1-10 AFB per field +2 and more than 10 AFB per field +3.

After an overnight fast (12 hrs), venous blood was drawn from each subject under aseptic technique in plain vial and serum separated. Glucose (GOD-POD method), Serum Urea (Urease U V kinetic), Creatinine (Jaffe Reaction, End point method), Total protein (Biuret), Albumin (BCG dye binding), Copper (Chromogen Di-Br-PAESA, end point), Zinc (5-Br-PAPS, end point) and Cerruloplasmin (Immunoturbidimetric method) were estimated in all samples on fully autoanalyzer Imola 3, Randox. Proposed standard protocol was followed for all tests.

Statistical Analysis

Quantitative data were expressed as Mean ± SD. Comparison was made using ANNOVA- with Post hoc tukey -t test (independent sample t-test). $P < 0.05$ was considered significant. Pearson Correlation was used to assess correlation among various parameters.

RESULTS AND DISCUSSION

PTB is a potentially hazardous infectious and highly prevalent disease caused by Mycobacterium Tuberculosis (MTB) accounting for 4 lakhs deaths in India alone (Muthuraj *et al*., 2010). Emergence of Multidrug resistant MTB strains and reactivation of latent TB are on increase due to MTB induced immune suppression and is responsible for its worldwide prevalence and high mortality and morbidity (Sharma *et al*., 2015). Among various factors, Micronutrient status is an important determinant of immune strength since certain trace elements play essential role in macrophage phagocytosis, intracellular killing and eventual bacterial clearance (Edem *et al*., 2015). As seen in table 1, no significant difference was seen among all groups with regard to age, gender distribution and routine parameters of study population. Serum Protein and Albumin level were significantly reduced in PTB patients than controls as well established in various studies ($p<0.001$) (Akiibinu *et al*., 2007). Albumin is a component of plasma antioxidant activity and negative APP that decreases in any inflammatory condition, injury or stress as a result of increased metabolic need for tissue repair and free radical utilization (Akiibinu *et al*., 2007). Further leakage via vascular endothelium, reduced hepatic synthesis, increased catabolism and anorexia in TB and pre-exsisting malnutrition clearly explain low protein and Albumin level in patients than controls ($p<0.001$) (Table 1). Low level in relapse may also be due to hepatotoxicity of Anti-tubercular Therapy (ATT). Our study demonstrated significant decrease in Zn in fresh TB cases with slight increase in relapse. Both were significantly less than controls (Table 2). Mean Cu and Cu/Zn ratio in newly diagnosed PTB patients were 172.0± 64.2 ug/dl & 1.76± 0.81 and in relapse cases was 168.4± 46.8 ug/dl & 1.44± 0.70 respectively. It was significantly higher than controls (127.6± 30.1 ug/dl & 1.25± 0.42) (Table 2), and in line with many previous reports. Elevated Cu/Zn ratio was seen in different pathological condition and neoplasm with ratio exceeding 2 indicates severity of infection. Reduction in Cu and Cu/Zn ratio after therapy (151.9 ug/dl and 1.58) was seen as compared to before treatment levels i.e. 187.9 ug/dl and 2.4 respectively along with increase in Zn level i.e. from 87.3 ug/dl to 118.3 ug/dl following ATT (Kassu *et al*., 2006).

Table 1. General Characterstics and Routine parameters of study population

S. No.	General Characterstics	Newly Diagnosed (Group A)	Relapse cases (Group B)	Controls (Group C)	Significance (p value)
1.	No. of cases (N)	60	60	60	
2.	No. males	42 (70)	35 (58.3)	50 (83.4)	0.041 N S
3.	No. females	18 (30)	25 (41.7)	10 (16.6)	
4.	Average Age (in years)	52.53 ± 19.6	59.87± 15.1	40.23 ± 10.2	0.526 NS
5.	Sputum Status N(%)				-
	Negative	22 (36.66)	25 (41.66)	60 (100)	-
	+1	25 (41.66)	24 (40.0)	0 (0)	-
	+2	8 (13.33)	8 (13.33)	0 (0)	-
	+3	5 (8.3)	3 (5.00)	0 (0)	-
6.	Blood Sugar (mg/dl)	87.57±25.66	88.43±32.09	81.12±17.88	NS
7.	Urea (mg/dl)	32.5±10.6	28.54±7.6	31.66±8.43	NS
8.	S. Creatinine (mg/dl)	1.07±0.54	1.03± 0.65	0.92 ± 0.43	NS
9.	S. Protein (g/dl)	6.40 ± 0.53	7.04 ± 0.23	7.88 ± 1.16	<0.001 S
10.	S. Albumin (g/dl)	3.07 ± 0.69	3.86 ± 1.00	4.52 ± 0.81	<0.05 S

Values are in terms of Mean ± Standard deviation. Values are in no.& percentage in given in parenthesis.

Table 2. Trace Element status and Cerruloplasmin Level in study population

S. No.	Biochemical Parameters	Newly Diagnosed (Group A)	Relapse cases (Group B)	Controls (Group C)	Significance (p value)
1	S. Copper (ug/dl)	172.0 ± 64.27	168.4 ± 46.8	127.6 ± 30.1	<0.001
2.	S. Zinc (ug/dl)	109.23 ±45.0	124.52 ± 18.89	112.7 ± 43.7	<0.05
3.	Cu/ Zn Ratio	1.76 ± 0.81	1.44 ±0.07	1.25 ± 0.42	<0.001
4.	S. Cerruloplasmin (mg/dl)	61.6 ± 18.7	65.24 ± 14.8	37.8 ± 10.5	<0.001
5.	Cerruloplasmin Albumin ratio (Cp/Alb)	20.80 ± 11.6	18.57 ± 10.0	9.8 ± 7.3	<0.001

Values are in terms of Mean ± Standard deviation

In another study, involving untreated newly diagnosed PTB patients, Cu/Zn ratio was 1.35± 0.46 and in controls was 0.69± 0.19 ($p<0.001$), thus supporting our finding and speculating the role of Cu/Zn ratio in early diagnosis and prognosis of PTB (Yerrajwala *et al*., 2016). Imbalance in Cu and Zn level causes defective functioning of immune mechanism and is found to be associated with pathophysiology of PTB. Similarly, children with PTB exhibits elevated Cu and reduce Zn level that tends to normalize after 4 months of ATT, however most studies proposes lowering in Zn more pronounced than elevation in Cu (Yerrajwala *et al*., 2016; Reza *et al*., 2007). Alteration in dynamics of trace elements and association of Cu/Zn ratio with acute phase of disease has been documented in other studies as well (Moraes *et al*., 2011). In accordance to our study, some workers found significant improvement in Zn level during course of ATT. Zn level in Newly diagnosed PTB patients was 59.88± 7.82 ug/dl, after two months of ATT was (74.70± 6.50 ug/dl) and after completion of ATT was (99.82± 14.23 ug/dl) (Bhandari *et al*., 2013). A highly significant ($p<0.001$) reduction in serum Zn in PTB patients of all age groups (53.4±2.96 ug/dl) as compared to controls (85.2± 2.16) with gradual improvement with ATT provide strong evidence that basic cause of Zn deficiency is underlying disease. Further in the above study, Zn correlated with the stage of tubercular lesion showing maximum deficiency in patients with far advanced lesion (Gulam *et al*., 2009). In other words, it showed that Zn supplementation improves effect of tubercular medication, affects disease outcome and be a mandatory constituent of treatment protocol (Gulam *et al*., 2009). In line to the above study, another finding reported that 93 % of PTB subjects had serum Zn level below cut off (11 mmol/L) and only 7 % had Zn level within the reference range (11-19 mmol/L) (Uttra *et al*., 2011). Rise in Cu and fall in Zn level was considered to be a sensitive index of tuberculosis infection. Patients with demonstrable AFB (Acid fast bacilli) in smear had significantly higher Cu level and lower Zn (175.4±19.3 & 54.6±16.5 ug%) compared to those who did not had AFB in their sputum smear (139.8± 39.4 & 71.7± 22.5 ug%). Such alteration in Cu & Zn also correlated with disease severity which may be direct for sputum containing AFB or indirect such as nature and extent of pulmonary radiographic lesion (Khanna *et al*., 1982). Increase in serum Zn (93.70±10.38 to 109.31±26.62 ug/dl) and decrease in Cu level (from 132.9± 19.76 to 122.5± 21.87 ug/dl) with concomitant decrease in Cu/Zn ratio after 6 months of ATT was found in another study (Porfallah *et al*., 2011). Inspite small variations, all the above findings and ours confirmed high Cu level and Cu/Zn ratio in PTB and reversal of picture with improvement in clinical status of patients. Various factors are attributed the observed pattern of trace element in PTB. Metalothionein, a Zn carrier alpha-2 macroglobulin is induced by Acute phase Response (APR) in PTB and functions in transport of Zn from plasma to liver and other tissue.

IL-1 and other cytokines increases its hepatic synthesis (Porfallah *et al*., 2011). Such redistribution of Zinc, impaired absorption and utilization by MTB, anorexia and hepatotoxic effect of ATT in relapse cases with pre-exsisting undernutrition are factors causing reduced Zinc level in patients group in our study. Rise in Cu level is due to non specific increase in Cu binding Acute phase protein (APP) Cerruloplasmin as found in various infection diseases including PTB. Cu and Zn changes in opposite direction causing significant raised Cu/Zn ratio in Newly diagnosed and relapse cases i.e. 1.76±0.81 and 1.44±0.07 respectively than controls (Table 2). Further Cu/Zn ratio correlated strongly to Cu, Zn and Cp level as well as to Cp/Alb ratio indicating its association with pathophysiology of PTB (Table 3). Serum Cu and Cu/Zn ratio increases progressively with sputum positivity as shown in table 4, with mean value of Cu in 72 Sputum positive patients was 171.35± 42.2 ug/dl and in 48 sputum negative cases was 166.0 ± 42.56 ug/dl. Cu/Zn ratio in Sputum positive and negative cases was 1.698±0.78 and 1.52± 0.80 respectively. The above findings suggest that Cu/Zn ratio can be valuable laboratory tool to enhance sensitivity of the exsisting diagnostic methods. Association of trace elements and Cu/Zn ratio with sputum

Table 3. Correlation between various parameters in 120 PTB patients: Values are correlation coefficient (r)

S. No	Biochemical parameters	Copper	Zinc	Cu/Zn ratio	Cerruloplasmin	Cp/Alb ratio
1	Copper	1.000	-0.2933	+ 0.7106	+0.5934	+0.5152
2	Zinc	-0.307	1.000	- 0.7508	-0.3696	-0.3618
3	Cu/Zn ratio	+ 0.722	-0.7508	1.000	+0.5450	+0.5100
4	Cerruloplasmin	+ 0.5143	-0.3620	+0.5410	1.000	+0.8312
5	Cp/Alb ratio	+ 0.6071	-0.3618	+ 0.5088	+0.8312	1.0000

Table 4. Mean value of trace elements and Cerruloplsmin level in relation to sputum positivity in 120 PTB patients

S. No.	Biochemical Parameters	Negative (N= 48)	+ 1 (N= 49)	+2 (N= 15)	+ 3 (N= 8)
1	S. Copper (ug/dl)	165.9± 42.56	169.17 ± 42.21	165.00 ± 41.16	194.14 ±46.95
2.	S. Zinc (ug/dl)	116.02±32.7	115.5 ± 32.3	107.2 ± 32.38	120.21 ± 35.8
3.	Cu/ Zn Ratio	1.527 ± 0.86	1.609 ± 0.79	1.94 ±0.757	1.82 ±0.90
4.	S. Cerruloplasmin (mg/dl)	56.66 ± 18.59	57.17 ± 18.26	51.74 ± 17.36	63.65 ± 17.5
5.	Cerruloplasmin Albumin ratio (Cp/Alb)	17.25 ± 7.13	18.40± 7.08	18.77 ± 7.014	18.96 ± 6.87.

Values are in terms of Mean ± Standard deviation.

positivity suggests their role in assessing effectiveness of ongoing antitubercular therapy.

Cp/Alb ratio in newly diagnosed PTB was 20.8±11.6, in relapse cases was 18.57± 10.0 and that in controls was 9.8± 7.3($p<0.001$) (Table 1). Significant difference was seen in Cp level and Cp/Alb ratio among all groups with mean value of Cp and Cp/Alb ratio in sputum positive cases was 56.58±18.26 and 18.44±7.09 and in sputum negatives cases was 56.66±18.59 and 17.16± 7.13 respectively. The values were significantly higher than controls ($p<0.001$). Studies have shown significantly high Cp in newly diagnosed TB cases (1603.8± 222.7 IU/L), than after 6 months of ATT (1001.8 ± 201.6 IU/L) and controls (864.4± 106.4 IU/L) with similar pattern observed in Cp/Alb ratio (Batra *et al*., 2007). Another study found significantly high Cp/Alb ratio in newly diagnosed PTB patients (9.321± 2.25) than relapse PTB cases (7.465± 1.32) (Makadia and Jain, 2014) suggesting that Cp/Alb ratio can be used to study treatment response in PTB patients. Progressive decrease in Cp and Cp/Alb ratio in PTB patients was seen during course of ATT. Fresh PTB patients had Cp and Cp/Alb ratio 61.3±5.74 mg/dl and 0.025±0.017 respectively that decreases to 50.40±5.05 mg/dl and 0.015±0.003 after 2 months of onset of ATT. It further dips to 33.92± 6.70 and 0.008±0.002 after completion of treatment (Ramesh *et al*., 2012). It explains the utility of these parameters in assisting diagnosis and follow up of PTB. Cp/Alb ratio was observed as 0.028±0.004 in Newly diagnosed PTB, 0.010± 0.002 in patients after completetion of treatment and 0.009±0.001 in healthy controls (Gamit *et al*., 2016). A significantly high Cp/Alb ratio was again seen in newly diagnosed PTB patients (21.76) as compared to on treatment group (19.0± 4.4), cured Group (13.5± 3.5) and control (9.6±1.9) even when the study population consist of 25 subjects in each of the above groups (Begum *et al*., 2016). Cp/Alb ratio showed good correlation with serum Cu(r=0.6071), Cu/Zn ratio (r=0.5411) and to Cp level (r=0.8312) as compared to correlation of Cp alone with other parameters (Table 3). It is well known that infection induces reduction in plasma albumin and increase in Cp level in human and experimental animals (Ali *et al*., 2013). Cp is associated with generation of oxidant products H_2O_2 and O_2^- causing peroxidative damage induced by cytokines such as interleukin- 6 and Tumor necrosis factor- alpha that also causes a dramatic shift in the plasma concentration of essential micronutrients. All the above discussion suggest role of Cp/Alb ratio is assisting diagnosis and prognosis. Inspite of advanced nuclear techniques, MTB culture still remains gold standard method for diagnosis of TB. It takes about 7 days for bacterial growth (Rapid Diagnostic test for Tuberculosis, 1997), hence there is requirement of cost effective Biochemical parameters to assist early diagnosis.

Conclusion

Active PTB patients showed reduced Zinc and raised Cu and Cp level reflecting the ongoing inflammatory process. And their association to tuberculo-toxemia. From our finding and discussion of previous reports, it seems likely that Cu/Zn ratio and Cp/Alb ratio may serve as sensitive diagnostic tool and helps to assess beneficial effects of ATT as compared to Cerruloplasmin alone. However, large scale research is required to validate the role of these parameters in diagnosis and prognosis of tuberculosis.

Compliance with Ethical Standards

This study received no funding from any source. The authors declare that they have no conflict of interest.

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