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Decreased Serum Cholinesterase Activity- A Reliable Diagnostic Aid for Tuberculosis

Biochemistry Section

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ABSTRACT

Introduction: Siderophore, the low molecular weight ferric iron chelator, is secreted extracellularly by *Mycobacterium tuberculosis*, an obligate aerobe. The pathogenic mycobacteria need iron as co-factor for the extracellularly secreted enzyme Superoxide Dismutase (SOD) for its pathogenicity as well as it requires iron for its metabolic functions like reduction of oxygen for synthesis of Adenosine Triphosphate (ATP), etc. The termination of impulse conduction is endorsed by the rapid hydrolysis of Acetylcholine (ACh) by Acetylcholinesterase (AChE) in the central as well as peripheral nervous system (cholinergic pathway). The inhibitors to Cholinesterase (ChE) might lead to accumulation of ACh, hyper stimulation of nicotinic and muscarinic receptors and also disruption of neurotransmission. Possibility of an inhibitor substance for AChE in Tuberculosis (TB) patients interestingly correlates with the symptom of night sweating in those subjects.

Aim: To assay the level of serum ChE in normal control, lung disease control and tubercular subjects;circumvent the serum ChE level as a diagnostic potential in TB at an early stage.

Materials and Methods: The study was conducted on total 124 subjects, and were divided into three groups: Group 1: normal control (n=31), Group 2: lung disease control (n=31) and Group 3: patients suffering from TB {3A: pulmonary TB (n=31) and 3B: extrapulmonary TB (n=31)}. Serum ChE activity for all the subjects were measured according to the method of Hestrin S. Serum ChE level was assayed for group 3 subjects after additional one month's anti-TB drug treatment and also for group 2 subjects after one month with usual treatment. The level of significance was assessed using Student's t-test.

Results: There was a significant inhibition of serum ChE activity in both pulmonary and extrapulmonary TB patients in comparison to that of in normal control as well as lung disease control subjects ($p<0.01$). With the anti-tubercular drug therapy for one month, there was significant recovery in the serum ChE activity in pulmonary as well as extrapulmonary tubercular subjects ($p<0.01$).

Conclusion: It appears that the high level of hydroxamate type of siderophores (secreted by *Mycobacterium tuberculosis* for acquiring iron) might form ACh ferric hydroxamate complex binding more strongly with serum ChE resulting in inhibition of serum ChE activity in tubercular subjects. With anti-tubercular drug therapy, there was decrease in serum ACh ferric hydroxamate complex level resulting in recovery of serum ChE activity.

Keywords: Acetylcholine ferric hydroxamate complex, Extrapulmonary tuberculosis, Pulmonary tuberculosis, Siderophore

INTRODUCTION

Worldwide, TB is one of the 10 causes of death and the leading cause from a single infectious agent {more than Human Immunodeficiency Virus, Acquired Immunodeficiency Syndrome (HIV/AIDS) cases}. TB remains the top most killer from single infectious disease claiming over 4000 lives a day globally on an average. Also, globally India has to bear the major burden (27%) of tubercular subjects [1]. At present, there does not exist a simple and rapid diagnostic test for TB; no simple and licensed serological test for diagnosing as well as monitoring the course of TB treatment. Though with the endorsement of the newer diagnostics for TB there is a noteworthy influence on the global campaign against TB, till then their full impact on the global campaign against TB is yet to be evaluated [2]. Even with the introduction of very recent diagnostics for TB, the gap between global estimates of incidence and new case notification is 4.1 million people. More accurate, rapid and cost-effective screening tests are needed to improve case detection [3].

There are a very few researches published relating the serum ChE level in TB. Nag D and Dey SC had conducted a study measuring serum ChE level in pulmonary tubercular subjects only and had reported inhibition of serum ChE activity in those subjects [4]. The other noteworthy paper published on serum ChE level showed the ratio of pleural fluid and serum ChE level for differentiating pleural transudate from exudate [5].

There are many symptoms of TB. Out of these, there are a few key signs. Night sweat that is sweating profusely during night is one of the key signs and also the degree of night sweat is an indicator of the body's level of infection. In the published literature, the excess of Tumour Necrosis Factor Alpha (TNF-α) as secreted by migratory macrophages, had been implicated as the important factor for fever, weakness, night sweating, necrosis and progressive weight loss in TB [6]. But the recent publication had foreclosed this hypothesis and abdicated any specific reason for it and declared the causation of night sweating as unanswered [7].

To unravel the cause of night sweating, the author had mulled the possible important role of siderophores, secreted by pathogenic *Mycobacterium tuberculosis*, to play with ACh and ChE. In this study, the serum ChE had been assayed separately as two groups for pulmonary tubercular as well as extrapulmonary tubercular subjects.

Mycobacterium tuberculosis has an elaborate system for acquiring iron by involving the abundant export of extra-cellular siderophores [8]. Iron is the important co-factor of the iron co-factored SOD secreted by the pathogenic mycobacteria, which has a role in the pathogenesis of TB [9]. ChE is a family of enzymes having high catalytic activity and catalysing the hydrolytic breakdown of the neurotransmitter ACh into acetic acid and choline; each molecule of ChE degrading about 25000 molecules of ACh in one second. This reaction is really necessary for allowing the cholinergic neuron to come back to its resting phase after being activated. The liberated choline is taken up by the pre-synaptic nerve and the neurotransmitter ACh is formed by the combination with Acetyl-CoA (acetyl coenzyme A) through a single step biosynthetic reaction catalysed by the enzyme choline acetyl transferase [10].

The research aimed to study the serum ChE activity in tubercular subjects, specially its activity to hydrolyse bound acetycholine ferric hydroxamate complex, if it was formed in the serum of tubercular subjects.

MATERIALS AND METHODS

The cohort study was conducted at BS Medical College and Hospital, West Bengal, India, from June, 2004 to April, 2007. Permission for the study was obtained from the Institution Ethical Committee, BS Medical College and Hospital, Bankura (vide Memo no.3/BSMC/04 dt 14-05-2004).

A total of 124 participants (aged 8-62 years) were enrolled. The study was conducted in two phases. In the first phase total 60 participants were enrolled and was conducted between June, 2004 to July, 2004. The second phase was conducted between March, 2007 and April, 2007 and the total number of subjects enrolled were 84. Before collection of blood, a verbal consent was taken from the tubercular patients as well as from the normal control and lung disease control subjects.

The following groups of subjects were taken into consideration:

1. **Normal control subjects:** (31 subjects) These were relatives of the patients with TB but without any clinical signs, symptoms or X-ray finding suggestive of TB or any sort of diseases. They were sputum negative for Acid Fast Bacilli (AFB).
2. **Lung disease control subjects:** (31 subjects) These were the patients suffering from Respiratory Tract Infection (RTI) or bronchiectasis or bronchial asthma or bronchogenic carcinoma; and certainly not suffering from TB. That they were not suffering from TB, was confirmed by clinical examination as well as investigative procedures like: (I) chest X-ray; (II) sputum for AFB; (III) ELISA technique for serodiagnosis of TB; (IV) assay of lipoarabinomannan in serum and urine. They were selected from the patients attending the Out Patient Department (OPD).
3. **Tubercular (TB) subjects:** The patients with TB (irrespective of age, sex and socio-economic status) attending the OPD, and also TB patients admitted in the Isolation Ward of the same hospital were taken into account. The TB patients were diagnosed clinically by characteristic symptoms and signs as well as by other investigative procedures like radiological investigation, sputum for AFB, Fine Needle Aspiration and Cytology (FNAC) whenever possible, ELISA technique for serodiagnosis (detection of Myco-specific serum immunoglobulin levels) [11], detection of lipoarabinomannan (the secreted antigen of mycobacteria) in the serum and urine of the subjects [12]. The tubercular subjects were divided into two groups, of 31 patients in each. Group A: included the subjects suffering from pulmonary TB; Group B: included subjects suffering from extrapulmonary TB (lymphatic TB, pleural TB, abdominal TB, disseminated TB and bone TB). In these TB subjects, the anti-TB (A-TB) drug therapy was started between 0-15 days.

In this study, besides the 62 TB patients, there were also some TB subjects enrolled for the study; but later on, they were diagnosed as Multi-Drug Resistant TB (MDR-TB) cases. Notwithstanding, those subjects were omitted from the field of result section; but the estimation of serum ChE in those MDR-TB subjects had indoctrinated a great envisage to diagnose the drug resistant (both primary and secondary) cases at an early period. In the discussion section, it had been elaborated.

In this study, all the subjects (groups 1,2 and 3) having normal liver and kidney function, normal serum glucose level were considered. To have normal Liver Function Tests (LFT) was very important in this study. It was done to exclude the possibility of having a lower serum ChE activity for deranged liver function as it had been reported by Gokani RS et al., that the serum ChE was significantly lower in liver disease patients [13].

Collection of blood: About 2 mL of blood was collected from each subject by venipuncture and the blood was left to clot; the serum so obtained was stored in the refrigerator at 2-4°C until assayed. It is to mention that the assay for serum ChE was done on the same day after preparation of the serum. Repeat blood collection was done after 30 days in lung disease control and TB subjects with usual treatment and the serum so obtained was assayed similarly for ChE. During this additional 30 days, the tubercular subjects were under Directly Observed Treatment (DOT) program under Revised National Tuberculosis Control Program (RNTCP). The lung disease control subjects were under individual treatment procedure. The serum ChE activity was measured according to the method of Hestrins [14]. The principle of the assay is based on the reaction between ACh and hydroxamate to form acetyl hydroxamate which on reacting with ferric iron (ion) in an acidic medium forms a red-purple coloured complex which is measured at 530 mμ using a spectrophotometer (Baush and Lomb Co.). ACh chloride was used as a substrate and the ChE activity in serum was expressed as units/hour/mL of serum.

STATISTICAL ANALYSIS

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

RESULTS

All the groups constituted majorly male patients. [Table/Fig-1] shows that there was a highly significant inhibition of serum ChE activity in both pulmonary and extrapulmonary TB patients, compared with normal control and lung disease control subjects. With the anti-TB drug treatment for one month, there had been a significant recovery in the serum ChE activity in both pulmonary as well as extrapulmonary tubercular subjects ($p=0.008$). On the other hand, the change in serum ChE activity in lung disease control subjects after one month's usual treatment was not significant ($p=0.1$).

With the result as depicted in [Table/Fig-1], statistical computation was looked up to engross any cut-off value of serum ChE level to diagnose TB to the earliest. The area under the normal curve between ($\bar{X}$-3SD) and ($\bar{X}$+3SD) is 99.74% of total area under the curve ($\bar{X}$ is mean and SD is standard deviation). As the serum ChE of patients with TB shows marked inhibition, the values as depicted in the right half of the normal curve have been considered for statistical computation and for that purpose three times of the SD value has been added to the mean value for pulmonary TB and extra pulmonary TB patients. Again, the serum ChE of normal control and lung disease control subjects did not show any inhibition at all. For them, the left half of the normal curve has been considered for statistical computation and three times of SD value has been subtracted from mean value for these groups of subjects.

So, if from the mean value of serum ChE level in normal control subjects is subtracted the three times of standard deviation (SD), it becomes (127.1-3×9.7)=98.0 units/hr/mL. Similarly, the said value in lung disease control subjects becomes (120.4-3×7.2)=98.8 units/hr/mL (considering the serum ChE level at the beginning). On the other hand, if with the mean value of serum ChE level at the beginning in pulmonary tubercular subjects is added the three times of SD value it becomes (24.7+3×9.6) 53.5 units/hr/mL. Similarly, Mean Value+3×SD in extrapulmonary tubercular subjects at the beginning becomes (26.5+3×8.8) 52.9 units/hr/mL. So, if in a clinically suspected TB subjects, the serum ChE level falls below 50 units/hr/mL, it might clinch to the diagnosis of TB, specially before the institution of anti-TB drug therapy.

DISCUSSION

The ACh is the neurotransmitter at the sweat gland as well as at the piloerector muscle of the sympathetic autonomic nervous

Subjects	At the baseline			After one month	p-value (Student's t-test)
	No. of male (M) and female (F) subjects	Age (years)	ChE activity in units/hr/mL	ChE activity in units/hr/mL	
1. Normal control	M-22 F-09	38.3±9.8	127.1±9.7	-	
2. Lung disease control	M-17 F-14	41.4±7.8	120.4±7.2	126.8±8.9	0.10
3. Tubercular subjects					
A. Pulmonary	M-20 F-11	40.7±12.2	24.7±9.6	64.6±4.8	0.005
B. Extrapulmonary	M-22 F-09	39.6±11.7	26.5±8.8	69.2±3.7	0.008

[Table/Fig-1]: Serum Cholinesterase (ChE) activity in normal control, lung disease control and tubercular subjects (both pulmonary and extrapulmonary) and the serum cholinesterase (ChE) activity after one month.

system. The two types of ChE are AChE and Butylcholinesterase or Butyrylcholinesterase (BChE). AChE (EC3.1.1.7) which is also known as true ChE. Red Blood Cells (RBC) ChE or erythrocyte ChE, ChE I etc., is found primarily in the blood on RBC membranes, in neuromuscular junction and neural synapses. BChE (EC3.1.1.8) also known as ChE II, pseudo ChE, plasma ChE, serum ChE etc., is synthesised in liver and is found primarily in blood plasma. The term serum ChE is generally used in the reference to a clinical test that reflects levels of both of these enzymes in the blood [15].

The major groups of siderophores include the catecholates, hydroxamates and carboxylates [16]. Siderophores produce a complex preferentially with Fe^{+3} which is a strong Lewis acid. It has preference to strong Lewis bases such as anionic or neutral oxygen atoms to co-ordinate with [17]. Again, ChE carries one or more 'peripheral' anionic sites distinct from the choline-binding pocket of the active site. The site is meant for binding ACh and other quaternary ligands acting as uncompetitive inhibitors that bind at a site clearly distinct from that occupied by the monoquaternary competitive inhibitors [18]. This site is also involved in the substrate inhibition characteristic of ChE [19]. ACh is the neurotransmitter that is responsible for hastening the rate of sweat in human. As ChE rapidly hydrolyses ACh, it is logical to note that ChE is responsible for the modulation of sweat rate. It was reported earlier that with the local application of ChE inhibitor neostigmine intradermally at the forearm with varying concentration of ACh, there was significant increase in sweat rate locally when ACh had been administered at a low to moderate concentration [20]. To examine and study for the nature of inhibition of serum ChE in tubercular subjects, Lineweaver-Burk plot was plotted and the plot had demonstrated a line parallel to the original enzyme-substrate plot, but with a higher intercept. This confirmed the uncompetitive type of inhibition [21]. As reported previously, in uncompetitive (also known as anti-competitive) type of inhibition, the enzyme inhibitor had bound only to the complex formed between the enzyme and substrate [22].

Again as reported, siderophores, being amongst the strongest binders to Fe^{+3}, the siderophore desferrioxamine has been getting greater momentum in the widespread use as chelators in iron poisoning cases and also in thalassaemia [23]. It had also been reported that the growth of *Mycobacterium tuberculosis* was impaired in mice and macrophages by the genetic disruption of siderophore expression. It demonstrates that the mycobacteria virulence is dependent on its essential role of iron acquisition by siderophores [24].

It is presumed that the Fe^{+3} present in siderophores being a strong Lewis acid does co-ordinate with anionic or neutral oxygen atom. Also, the peripheral anionic site present in ChE is involved in substrate inhibition of uncompetitive nature. It appears that in the serum of tubercular subjects, ACh ferric hydroxamate complex might be formed, which binds more strongly with serum ChE and remains unhydrolysed and as a result inhibition of ChE was the result in tubercular subjects. It is evident therefore that high level of siderophores excreted extracellularly for acquiring iron by pathogenic *Mycobacterium tuberculosis* might be correlated with the formation of high concentration of serum ACh ferric hydroxamate complex which remains bound to serum ChE. Anti-tubercular drugs cause a decrease in serum siderophore level resulting in diminution of serum hydroxamate level in tubercular subjects and therefore a decrease in ACh ferric hydroxamate complex level. So, the ChE enzyme activity in the serum of tubercular subjects increases with treatment with anti-TB drugs.

As reported earlier, the enzymatic inhibition in uncompetitive type could not be reversed by increasing the substrate concentration. Also, the same research reported uncompetitive type of inhibition of AChE by tertiary amines (R_3N) [25]. In another study published by the index authors, it was reported that with zinc supplementation (50 milligram of elemental zinc orally daily) for one month along with the usual anti-TB drug treatment, this recovery of serum ChE activity was more significant in both pulmonary and extrapulmonary TB subjects [26]. Zinc, being an antioxidant element inhibits the formation of superoxide in the host tissue and also hastens the process of normal compartmentalisation of iron in the tissues. So, oral supplementation of zinc destabilises the decompartmentalisation of iron and hastens the process of compartmentalisation of iron. Thus, it helps to prevent *Mycobacterium tuberculosis* from getting iron which is very much needed as co-factor for the iron co-factored SOD secreted extracellularly by the pathogenic mycobacteria to obtain the soluble oxygen by dismutation reaction, which is very much needed for this obligate aerobe. Anti-tubercular drugs along with zinc when instituted, there was qualitative and quantitative recovery of serum ChE activity in tubercular patients, possibly due to the replacement of Fe^{+3} ions from ACh complex by zinc causing a qualitative and quantitative recovery of serum ChE activity in tubercular subjects. The tubercular subjects later diagnosed as MDR-TB were omitted from the field of study, but for them the serial measurement for serum ChE level was continued even after 30 days. Some of those patients had shown no noteworthy recovery of serum ChE activity from the very beginning. These subjects might be diagnosed as primary drug resistant. On the other hand, the rest of the subjects with anti-TB drugs had an initial appreciable recovery of serum ChE activity but later on showed a fall in serum ChE activity. Those subjects might be diagnosed as secondary drug resistant tubercular subjects.

It is noteworthy to mention that the enzyme ChE, which has been assayed in this study, in the serum of tubercular subjects is of host origin. The author has two recent publications elaborating the two biomarkers; Glutamine Synthetase (GS) and SOD for early diagnosis of TB [27,28]. These two enzymes are secreted as leaderless proteins extracellularly by pathogenic *Mycobacterium tuberculosis*. GS, an essential enzyme for the survival and growth of *Mycobacterium tuberculosis* had been demonstrated to be present in the serum of subjects suffering from pulmonary as well as extrapulmonary TB; whereas normal control and lung disease control subjects did not have detectable serum GS activity at all.

The GS activity as detectable in the serum of tubercular subjects was of *Mycobacterium tuberculosis* origin as the serum GS activity of those subjects was reduced while incubating the serum with L-methionine-S, R-sulfoximine, a selective inhibitor of *Mycobacterium tuberculosis* GS [27]. Again, there was an elevated serum level of SOD in tubercular subjects (both pulmonary and extrapulmonary), which had been resistant to sodium Cyanide (NaCN). This iron co-factored SOD was *Mycobacterium tuberculosis* origin, which had been secreted extracellularly by the pathogenic mycobacteria [28]. Elaboration of two *Mycobacterium tuberculosis* origin biomarkers (SOD and GS) in an enhanced concentration and the inhibited host origin serum ChE concentration might increase the specificity for diagnosing TB at a very early stage by assaying serum SOD, GS and ChE simultaneously.

Limitation(s)

The activity of both AChE and butylcholinesterase has been measured together in this study. As reported by Macabeo APG, et al., globospiramine, the bisindole alkaloid, and deoxyvobtusine identified from *Voacanga globosa* had exhibited potent anti-TB and BChE activity, respectively. The rapport of deoxyvobtusine had been mentioned with BChE form of ChE [29]. So, assay of the two forms of ChE separately might unravel some facts regarding the pathogenesis of *Mycobacterium tuberculosis.*

CONCLUSION(S)

The assay of ChE activity in serum for diagnosing TB is a simple, rapid, not too expensive method. Nevertheless, this assay will be helpful to diagnose pulmonary as well as extrapulmonary TB cases with confidence. Also, the assay is very praiseworthy to monitor the effectiveness of treatment and also to diagnose the primary and secondary drug resistant cases effectively as mentioned already. This simple and not too expensive assay might be an effective weapon to break the transmission of TB by instituting early and prompt treatment.

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