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Original Article

Biochemistry Section

Superoxide Dismutase: A Biomarker for Early Diagnosis of Tuberculosis

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ABSTRACT

Introduction: Tuberculosis (TB) is a major public health concern in developing countries. Traditional diagnostic methods such as microscopic detection of organism and histopathological examination are limited in their capacity to provide information on prognosis and treatment efficiency. Abundantly secreted extracellular Superoxide Dismutase (SOD) protects the *Mycobacterium tuberculosis (MTB)*, and elevated levels of the biomolecule are found in tissues and fluids of tubercular patients.

Aim: To estimate the levels of serum SOD in lung control disease and tubercular patients; assess the diagnostic potential of SOD assay for detection of tuberculosis at an early onset stage and quantify the serum SOD activity to monitor the course of infection and determine the effectiveness of the anti-Tubercular (anti-TB) drug treatment.

Materials and Methods: The present observational study was conducted on 180 participants at B.S. Medical College and Hospital, Bankura, West Bengal, India. The participants were divided into three groups: Group 1: Normal Control (n=30), Group 2: Disease Control/Lung-Disease Control (n=27) and Group 3: Tubercular Subjects (3A- Pulmonary TB and 3B- Extra-pulmonary TB) (n=76). Serum SOD levels of the participants were measured spectrophotometrically. The serum SOD levels of the patients were re-measured after one-month of A-TB drug treatment. In addition, to increase the specificity of the test, the serum of tubercular subjects was incubated with different concentrations of Sodium Cyanide (NaCN) and then assayed for SOD activity. The level of significance was assessed using Student's t-test.

Results: The serum SOD level in tubercular subjects (both pulmonary and extra-pulmonary; with treatment started between 0-15 days) were significantly (p-value <0.01) elevated as compared to the control and lung-disease subjects. Significant decrease in serum SOD levels was observed after one-month of A-TB drug treatment signifying a decrease in mycobacterial load in host tissues. The iron co-factored SOD secreted by *M.tuberculosisis* was found to be resistant to NaCN whereas Copper-Zinc (Cu-Zn) co-factored SOD was inhibited by NaCN.

Conclusion: The serum SOD assay, used in the present study, differentiated between human and mycobacterial origin-SOD, on incubation with NaCN, and diagnosed pulmonary as well as extra-pulmonary TB cases with confidence. Thus, it can be used as a simple, rapid, inexpensive, yet highly sensitive and specific- assay for detection of both human and bovine tuberculosis and primary and secondary drug-resistant cases.

Keywords: Anti-tuberculosis treatment, Diagnostic marker, Extra-pulmonary tuberculosis, Pulmonary tuberculosis, Sodium-cyanide

INTRODUCTION

Tuberculosis (TB) is a bacterial disease caused by a complex of *Mycobacterium tuberculosis* (MTB) [1]. TB is a worldwide pandemic, with ~10.0 million new cases and 16 million deaths in 2017. In 2016, India alone accounted for about a quarter of global TB burden, with an estimated incidence of 2.79 million cases and 0.48 million death. About 40% of the infected Indian population has latent TB rather than active TB disease [2]. A highly infectious person can transmit the disease to 10-15 persons per year. Therefore, an accurate early diagnosis and prompt initiation of treatment are required for both timely management of symptomatic cases and prevention of further transmission [3].

Presently, the diagnosis of TB is mainly centred on the detection of causative agent in the clinical biological specimens. Smear microscopy is the most common and cheapest method of diagnosis; however it is limited by requirement of high concentration of bacilli in the sample (5000-10,000/mL) and inability to differentiate between drug-resistant or drug-sensitive mycobacterial species [4]. The available radiographic methods are non-specific and require additional examinations. The known bacterial culturing procedures can identify MTB in over 80% cases, but they require highly trained personnel, specialised equipments and have longer wait time (~2-6 weeks) due to slow growth of MTB. The nucleic acid amplification diagnostic test is rapid, highly specific (98-100%) and fairly sensitive (>95%), yet their use is limited to developing countries, due to the associated complexity and cost. Both tuberculin and cytokine assay have marginal role in diagnosis of TB since they cannot differentiate between active and latent infection in an individual [2-4].

Considering all the limitations associated with the currently available diagnostic methods, the need of the hour is to develop a rapid, inexpensive yet highly specific and sensitive test for detection of tuberculosis and to monitor the course of infection.

Various studies report the role of SOD in survival of MTB. SODs are extracellular metalloenzymes, produced in the initial stages of growth of bacteriae that catalyzes the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen and thus protect the *M. tuberculosis* against the oxidative stress in the host [5,6]. *M. tuberculosis* produces two types of SOD enzyme, SOD-A which encodes an iron-co-factored enzyme and SOD-C which encodes for a copper-zinc co-factored enzyme [7-9]. Elevated production and export of SOD by *M. tuberculosis* prevent the elimination of the tubercle bacilli by the innate immunity and therefore plays a cardinal role in the pathogenesis of tuberculosis [10-12].

Based on the extensive literature research, the present study was conducted to estimate the levels of serum SOD in normal, lung control disease and tubercular patients, and to assess the diagnostic potential of SOD assay for detection of tuberculosis at an early onset stage. In addition, the serum SOD activity was quantified to monitor the course of infection and determine the effectiveness of the anti-TB drug treatment.

MATERIALS AND METHODS

This observational study was conducted at B.S. Medical College and Hospital, Bankura, West Bengal, India, from June 2004 to April 2007. The study protocol was approved by the Institutional Ethical Committee (vide Memo No 3/BSMC/04dt 14-05-2004). A total of 133 participants (aged 8-62 years) were enrolled for the study.

The study was conducted in two phases at an interval of one-month.

The subjects were divided into three groups:

Group 1: Control subjects (n=30): The healthy relatives of the tubercular patients, without any clinical signs, symptoms or X-ray finding suggestive of tuberculosis or any sort of diseases were recruited. Their sputum was negative for acid fast bacilli.

Group 2: Disease-control subjects (Lung disease control (n=27): Included OPD patients suffering from Respiratory Tract Infection (RTI) or bronchiectasis or bronchial asthma or bronchogenic carcinoma. None of them had any clinical signs or symptoms of tuberculosis. The subjects were under individual treatment procedure (not certainly under A-TB drug regime).

Group 3: Tubercular subjects (n=76): The Tubercular (TB) patients (irrespective of age, sex and socioeconomic status) admitted in the Isolation Ward and patients attending the Outpatient Department of B.S. Medical College and Hospital were taken into account. Based upon the prior diagnosis, the patients were categorised as Subgroup A: Pulmonary (n=42) and Subgroup B: Extra- Pulmonary (n=34). In all these tubercular subjects the anti-TB drug (A-TB) therapy was started between 0-15 days. Multi Drug Resistant (MDR) TB cases were not considered in this study. The tubercular subjects were under Directly Observed Treatment (DOT) programme under Revised National Tuberculosis Control Programme (RNTCP).

Nota Bene (NB): All the subjects under study (Groups 1,2, 3A and 3B) had normal liver and kidney functions and normal serum glucose level.

The detailed purpose of the study was explained to all the participants and informed verbal consent was obtained, before collection of blood samples. About 2 mL of morning blood sample was collected by venipuncture in plain vacutainer. The samples were allowed to clot for ~2 hours and the serum was obtained by centrifugation at 2200 rpm for 15 minutes. After the centrifugation, the serum was transferred to clean and sterile eppendorf tubes and was stored at 2-4°C until further analysis. The serum samples of lung disease control and tubercular subjects were recollected after 30 days of usual treatment. The serum SOD levels were assayed on the same day of collection.

Serum SOD activity was assayed using Ransod Kit {Randox Laboratories Ltd., Cat No. SD125} as per the manufacturer's instruction. The absorbance was measured at 505 nm using a spectrophotometer (Baush and Lomb Co.). SOD was measured from the degree of inhibition of the reaction and expressed as unit/mL (U/mL). One unit of SOD is equal to the amount of SOD required to cause 50% inhibition of reduction rate (per minute), i,e., the change of absorbance per minute [13].

For determination of the sensitivity of SOD enzyme activity towards NaCN, the sera of tubercular subjects (both pulmonary and extra-pulmonary) were incubated with 40 µm, 400 µm and 4000 µm of NaCN for two hours at 37°C. For control measures, purified *M.tuberculosis* iron-cofactored SOD enzyme and also human erythrocyte Cu-Zn co-factored SOD were incubated with the same concentration of NaCN. The activity of SOD enzyme for all the samples was measured using Ransod kit (Randox Laboratories Ltd.,).

For determination of percentage of inhibition of SOD activity, SOD levels prior to incubation with NaCN were taken as baseline 100% value and changes in the SOD activity on incubation with varying concentration of NaCN (40,400 and 4000 µm) were assayed.

STATISTICAL ANALYSIS

The results were analysed using Statistical Software for Social Sciences (SPSS, version 21.0). The level of significance was assessed using independent Student t-test. $p<0.05$ was considered statistically significant.

RESULTS

The serum SOD levels of both pulmonary and extra-pulmonary tubercular subjects (anti-TB drugs started between 0-15 days) were significantly higher than normal control subjects ($p<0.01$) and disease control subjects ($p<0.01$). After an additional treatment with A-TB drug therapy for one month, the serum SOD level fell dramatically ($0.05>p>0.02$) [Table/Fig-1].

Group	Subjects	Before treatment		After one month treatment		p-value
		n	Serum SOD (U/mL)	n	Serum SOD (U/mL)	
1	Normal control	30	126±37	_	_	
2	Disease control (Lung disease control)	27	142±31	27	131±23	0.1
3	**Tubercular subjects**					
3A	Pulmonary	42	1413±103*	42	978±72*	0.03*
3B	Extra-pulmonary	34	1226±76*	34	869±57*	0.04*

[Table/Fig-1]: Serum SOD levels before and after one month treatment.
*Tubercular Subjects: Treatment started between 0-15 days. Independent Student's test. *p<0.05 statistically significant.

Upon incubation with NaCN, no significant inhibition was observed in the serum SOD activity in the test sera of tubercular subjects. Very low degree of inhibition in the activity of SOD was observed in *M.tuberculosis* Fe-co-factored SOD and human erythrocyte showed significant inhibition of Cu-Zn SOD activity when incubated with NaCN. Significant inhibition was also observed in serum SOD activity of normal control and lung disease control subjects [Table/Fig-2]. This suggests that the SOD activity in tubercular subjects was of Mycobacterial origin rather than the human (host) origin.

DISCUSSION

Superoxide dismutase is a ubiquitous metalloenzyme that maintains an optimal redox environment in the bacterial cytoplasm and prevents DNA cross-linking, lipid peroxidation and cysteine-cysteine bonding of essential enzymes [14]. *M. tuberculosis* secretes large amount of

Concentration of NaCN (µm)	Serum SOD Activity					
	Normal controls (n=24)	Lung disease control (n=22)	Pulmonary tubercular subjects (n=29)	Extra-pulmonary tubercular subjects (n=23)	M.tuberculosis Fe-cofactored SOD	Human Erythrocyte Cu-Zn cofactored SOD
0	100	100	100	100	100	100
40	21±1.7	23±2.1	83±4.7*	81±4.1*	98	19
400	15±1.1	18±1.8	78±3.8*	77±4.3*	94	12
4000	11±0.8	13±1.2	73±5.3*	71±3.3*	91	09

[Table/Fig-2]: Serum SOD activity (%) on incubated with different concentrations of sodium cyanide.
Independent Student's test. *p<0.05 statistically significant.

leader peptide-independent iron-co-factored SOD (FeSOD or SOD-A) in the extracellular medium during initial stages of growth [7,15,16]. Fe-SOD scavenges ROIs and contributes to the pathogenicity by inhibiting NF- κB activation and mononuclear cell apoptosis [17,18].

In the first phase of the present study, the serum SOD activity was measured in tubercular subjects on A-TB drugs started between 0-15 days and then after one-month of A-TB treatment. Significantly elevated serum SOD levels were observed in both pulmonary and extra-pulmonary tubercular subjects, which can be used as diagnostic marker for early detection of tuberculosis infection. The serum SOD levels fell by an average of 31% in pulmonary TB subjects and 29% in patients with extra-pulmonary TB after one month of A-TB drug treatment. These findings were similar to Nag D et al, study where SOD levels of tubercular subjects decreased after one-month of A-TB treatment and zinc supplementation [19]. The supplemented zinc binds to thiol group of the free iron necessary for bacterial growth and thus reduces the level of SOD [19-21]. These variations in enzymatic activity and the degree of abundance can be used as a framework for the development of a simple assay to diagnose tuberculosis.

In the second phase of the study, an increase in the degree of inhibition of serum SOD activity was observed in normal and lung disease control subjects on incubation with increasing concentration of NaCN. Cyanide and its derivatives are metabolic inhibitors that cause oxidative stress by inhibition of antioxidant enzymes. Mammalian SOD enzymes (SOD-A and SOD-C) are co-factored with Copper (Cu) and Zinc (Zn) whereas *M. tuberculosis* SOD enzyme (SOD-A) is complexed to iron (Fe) [9,22]. Human erythrocyte Cu-Zn co-factored SOD showed enhanced inhibition towards NaCN, whereas Fe-co-factored SOD was found to be relatively resistant to NaCN. These varied responses of host and bacterial SOD activity towards NaCN, adds up to the specificity of the assay. These findings also suggest the presence of Fe co-factored bacterial SOD in the serum and thus justifies the elevated SOD levels in tubercular subjects.

In some patients, the SOD levels remained constant at the beginning of the A-TB drug treatment which indicated the primary drug resistance, whilst some patients initially responsive to treatment developed secondary drug resistance and their SOD levels rebounded. Therefore, the patients on A-TB drug treatment should be regularly screened to determine the effectiveness of the ongoing treatment.

LIMITATION

The sample size of the study was small, further studies on larger sample size are required. The study was very fundamental, there is scope to include *E.coli* Fe-co-factored SOD in determining the degree of inhibition by NaCN and the exact degree of resistance of Fe-co-factored *M.tuberculosis* SOD towards NaCN can also be estimated.

CONCLUSION

The SOD assay serves as an effective technique for early diagnosis of tuberculosis to prevent the transmission by timely institution of suitable treatment (A-TB drugs). The assay also helps in monitoring course of treatment, detection of primary and secondary drug resistance and diagnosis of both pulmonary and extra-pulmonary, as well as human and bovine tuberculosis cases. Incubation with NaCN, increases the sensitivity of the assay and help in differentiation of human and myco-bacterial origin SOD. Minimal serum requirement and rapid processing time, the assay readily may be used in developing countries with limited resources.

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