ORIGINAL RESEARCH ARTICLE

Ratio of Serum Superoxide Dismutase and Whole Blood Glutathione Peroxidase: A Noteworthy Parameter for Tuberculosis Diagnosis

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Abstract

Aim and background: *Mycobacterium tuberculosis* secretes extracellularly abundant amount of two proteins superoxide dismutase (SOD) and glutamine synthetase (GS) having no leader sequences. The pathogenesis of *M. tuberculosis* is contributed by the secretion of iron-cofactored SOD which scavenges reactive oxygen intermediates (ROI) by dismutation reaction and also blocks activation of NF-kB and mononuclear cell apoptosis. The obligatory aerobe catalase-positive *M. tuberculosis* also secretes selenoenzyme glutathione peroxidase (GPx) catalyzing reduction of peroxides produced by dismutation reaction at the expense of reduced glutathione. In this study, the author has computed the statistical ratio of serum SOD and whole blood GPx level and mulled to use that as a diagnostic marker for tuberculosis (TB) and to monitor the effectiveness of drug therapy.

Materials and methods: The participants were divided into three groups: Normal control; 2-Lung disease control and 3-TB patients (3A-pulmonary and 3B-extrapulmonary). The serum SOD and whole blood GPx activity were measured spectrophotometrically for all participants initially. Both of these parameters were assayed again after 1 month's usual additional treatment for groups II and III.

Results: The ratio as calculated in TB patients is >9 and 8 times, respectively, than those of normal and lung disease control subjects. With anti-TB drug therapy for 1 month, there was a significant decrease in the ratio.

Conclusion: The higher magnitude of the ratio might be well utilized to diagnose TB, the serial measurement of the said ratio during the course of A-TB drug treatment might confer effectiveness of drug therapy and diagnose drug-resistant cases.

Keywords: Extrapulmonary tuberculosis, Hydrogen peroxide, Myeloperoxidase, Oxidative burst, Pulmonary tuberculosis, Siderophores, Superoxide, Thiol cofactor, Transferrin.

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Introduction

Though there is a considerable increase in detecting tuberculosis (TB) cases, there is still a gap globally between the new cases reported in 2019 (about 7.1 million) and the estimated incident cases in 2019 (about 10.0 million).[1] This large gap is stated to be due to underreporting and under-diagnosing of TB cases.[1] Research study had been performed to make the assay for glutamine synthetase (GS) and superoxide dismutase (SOD) in the serum of TB patients, both pulmonary TB (PTB) and extrapulmonary TB (EPTB). These two leaderless proteins are released extracellularly by *Mycobacterium tuberculosis* (*M. tuberculosis*). In most other bacteria, these two enzymes are strictly intracellular.[2] The high GS level is essential for efficient regulation of nitrogen metabolism and enhanced growth under restrictive conditions *in vivo*.[3] This GS activity in the serum of TB subjects was *M. tuberculosis* origin as the serum GS activity was reduced while being incubated with L-methionine-S,R sulfoximine (MSO), a selective inhibitor of *M. tuberculosis* GS.[4] Again, there had been sodium cyanide resistant highly elevated serum SOD activity in TB (both PTB and EPTB) patients. This NaCN resistant serum SOD activity clearly indicates that they were of host origin.[5] A recent publication had elaborated marked inhibition of serum cholinesterase (ChE) activity in both PTB and EPTB patients.[6] So, demonstration of increased serum concentration of *M. tuberculosis* origin SOD and GS; and decreased serum concentration of host origin ChE might confer the specificity to diagnose TB at the earliest.[6]

The higher requirement of L-glutamine for *M. tuberculosis* is for the formation of a poly-L-glutamate/glutamine cell wall structure contributing 10% of cell wall mass.[7] The high GS level allows the enhanced growth of pathogenic mycobacteria under restrictive conditions *in vivo*.[3] For *M. tuberculosis*, iron is the obligate cofactor for at least 40 different enzymes including SOD as encoded in its genome.[8] Again, only pathogenic mycobacteria can survive and proliferate inside macrophages whereas *M. tuberculosis* survives nutrient starvation by using the β-oxidation pathway.[9]

The phagosome with the help of its membrane-bound NADPH oxidase system reduces O_2 to superoxide anion ($O_2^{\cdot}$) and oxidative burst is initiated.[10,11] The imbalance between oxygen-derived reactive oxygen species (ROS) and the antioxidant system to scavenge it, will develop oxidative stress.[12] Wild et al. had reported a significantly lower total antioxidant status (TAS) in TB patients.

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It had also been inferred in the same paper that the TAS had increased in the same individuals with A-TB drug treatment.[13] The extracellular release of SOD in the vicinity of the organism enables them to neutralize toxic superoxide before they might reach the outer wall of the mycobacteria.[14] Nevertheless, *M. tuberculosis* stations itself within mature macrophages which are deficient in myeloperoxidase (MPO) activity unlike the phagocytes; and thus prevents the formation of highly toxic ROS, hypochlorous acid (HOCl) within mature macrophages, and thus host system is unable to kill pathogenic intracellular *M. tuberculosis* within macrophages.[15]

Being a catalase-positive and obligatory aerobe organism, *M. tuberculosis* uses up catalase to fend off harmful H_2O_2 by breaking into water and oxygen and thus enabling mycobacteria to survive within the host tissue.[16] By this reaction, *M. tuberculosis* also receives oxygen for its survival. However, studies with virulent *M. tuberculosis* suggest that nitric oxide (NO) is more important than ROS in the killing of mycobacteria.[17] $O_2^{\cdot}$ might react with NO to form peroxynitrite anion (ONOO–) which on pronated forms hydroxyl radical and nitrogen dioxide and ultimately nitrate.[18] Thus, it is possible that SOD by acting as O_2^{-} scavenger might inhibit the production of RNI rendering a basal level of NO secreted by non-activated D9 macrophages and the mycobacteria might save themselves from the onslaught.[17]

Mycobacterium tuberculosis excretes out only one catalase (*kat*G), which is heat-labile, H_2O_2-inducible, hyperoxidase I (HPI) type catalase-peroxidase.[19] Also, as reported there was a diminished level of survival of *kat*G deletion mutants of *M. tuberculosis* in the spleen of mice and guinea pigs pointing to *kat*G catalase-peroxidase of *M. tuberculosis* responsible for the persistence in infected tissues.[20]

Glutathione peroxidase (GPx), the generic name of the peroxidase family enzymes, is to protect the organism from oxidative damage by reducing the peroxides generated by ROS at the expense of glutathione.[21] Glutathione peroxidase catalyzes the oxidation of reduced glutathione (GSH) by H_2O_2 to an oxidized form of glutathione (GSSG). Superoxide dismutase, GPx, and catalase are three major antioxidant enzymes in humans and their mode of action is well correlated to one another.[22] It had been reported that the activities of blood glutathione, GPx, and glutathione reductase were significantly decreased in PTB and EPTB subjects.[23] Keeping into view of all those facts this author had undertaken the research work to interpret the ratio of two parameters (SOD and GPx) to get an idea to use that as a possible diagnostic marker for TB.

Materials and Methods

The cohort study was conducted on about 190 participants (aged 8–62 years). The study protocol to collect blood samples from human subjects was approved in writing by the Institutional Ethics Committee. The purpose of the study was explained to all participants (subjects) and before the collection of blood informed verbal consent was obtained from each of the subjects. The subjects were divided into three groups (Gr):

Group I

Normal control subjects-The healthy relatives of the TB patients having no clinical signs, symptoms, or X-ray findings suggestive of TB or any sort of diseases were considered for this group. They were sputum negative for acid-fast bacilli.

Group II

Lung disease control subjects—It included the patients who had attended the Out Patient Department (OPD) of B S Medical College And Hospital, Bankura-722 102, West Bengal, India and had been suffering from respiratory tract infection (RTI) or bronchial asthma or chronic obstructive pulmonary disease (COPD) or bronchiectasis or bronchial carcinoma. None of them had any clinical signs or symptoms of TB. They were under individual treatment procedure (not under the anti-TB drug regime).

Group III

Tuberculosis patients attending the OPD of the said Institute and also being admitted in the Isolation Ward of the same hospital were taken into account. Based on prior diagnosis, the tubercular subjects were categorized as Sub-group A: Pulmonary and Sub-group B: Extrapulmonary. For all these TB subjects, the anti-TB (A-TB) drug therapy was started between 0 and 15 days. Multi-drug-resistant TB (MDR-TB) cases were not included in the Result section but for them, serial measurement of the parameters was continued even after 1 month. All TB subjects were kept under the Directly Observed Treatment (DOT) program under the Revised National Tuberculosis Control Program (RNTCP).

Nota Bene

All the subjects under study (groups I–IIIA and IIIB) had normal serum glucose levels and also normal liver and kidney function tests. This inclusion criterion was a must for this study as a lower GPx level was reported in type 2 diabetes patients with micro-albuminuria; which did correlate to the stage of diabetic nephropathy.[24]

Collection of Blood

Morning blood samples were collected from the subjects by venipuncture. Heparinized blood (containing 200 µg of heparin per mL of blood) was used for assaying GPx in whole blood. For estimating serum SOD, the blood sample without any anticoagulant was kept in a plain vacutainer. The samples were allowed to clot for about two hours and the serums were obtained by centrifugation at 2,200 rpm for 15 minutes. After centrifugation, the serum was transferred to the respective clean and sterile Eppendorf tube. All the samples were stored at 2–4°C until further analysis.

For SOD

Serum SOD was measured by using a reagent kit of Randox Labs Ltd, USA. The measurement process employed xanthine and xanthine oxidase to generate superoxide radicals which on reaction with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) had formed a red formazan dye. The absorbance was measured at 505 nm by a spectrophotometer. Superoxide dismutase was measured from the degree of inhibition of this reaction and expressed as units/mL (U/mL). One unit of SOD is equal to the amount of SOD required to cause 50% inhibition of reduction rate that is the change of absorbance per minute.[25]

For GPx

The concentration in whole blood was measured by using a reagent kit of Randox Labs Ltd., USA. In this assay procedure, GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH(H^+) oxidized glutathione is immediately converted to its reduced form with concomitant oxidation of NADPH(H^+) to $NADP^+$. The decrease in absorbance at 340 nm was measured spectrophotometrically. The concentration of the enzyme in the whole blood was expressed as units/liter (U/L).[26]

Statistical analysis

The results were analyzed using statistical software for Statistical Package for Social Sciences (SPSS, version 21.0). The level of significance was calculated using Student's *t*-test. $p < 0.05$ was considered significant statistically.

Results

It is observed that there had been a significant increase in serum SOD activity in both pulmonary and extrapulmonary tubercular subjects ($p < 0.05$). After an additional treatment with A-TB drugs for 1 month, there was a significant decrease in serum SOD activity ($p < 0.05$) in tubercular subjects (Table 1). It is also observed that whole blood GPx level in pulmonary as well as extrapulmonary tubercular subjects at the beginning (A-TB drugs started between 0 and 15 days), had shown significantly decreased value ($p = 0.01$) when compared with that of in normal control and lung disease control subjects (Table 2). With an additional 1 month's A-TB drug therapy, there was a significant recovery in whole blood GPx activity ($p = 0.01$) in tubercular subjects.

Discussion

Pathogenic mycobacteria exports extracellularly major amounts of iron-cofactored SOD (encoded by sodA).[27] This multimeric ubiquitous enzyme along with the other multimeric enzyme GS, both having no leader peptides; are secreted extracellularly in abundance by pathogenic mycobacteria by bacterial leakage or autolysis and they are highly expressed extracellularly and have higher extracellular stability.[2,27] *Mycobacterium tuberculosis* also excretes out mycobactin and carboxymycobactin forms of siderophore (lower molecular weight ferric iron specific chelating agent) having a high affinity for iron and thus scavenging iron ions from host extracellular transferrin, lactoferrin, and intracellular iron pools.[28,29]

The siderophore ligand has a strong affinity for Fe^{+3} iron whereas the weak complexing with Fe^{+2} facilitates release by reduction inside the cell.[30] It is an interesting observation that in the absence of siderophores *M. tuberculosis* cannot use iron salts.[31] *Mycobacterium tuberculosis* had been shown to have high resistance to killing by H_2O_2 as mediated by the mycobacterial catalase-peroxidase protein (KatG) and the alkyl hydroperoxide reductase protein (AhpC) which are encoded by the genes *katG* and *ahpC*, respectively.[32] The mechanism of the reaction of GPx, a selenoenzyme involves first the oxidation of selenol of selenocysteine residue by H_2O_2 to the selenenic acid group. The selenocysteine residue forms a "catalytic triad" with tryptophan and glutamine at the active site, thus activating the selenium moiety for efficient reduction of harmful hydroperoxides with thiol cofactors.[33]

Now, glancing at the experimental outcome as depicted in the Result section, it is noticed that at the beginning endogenous serum SOD activity in TB subjects (both pulmonary and extrapulmonary) was more than eight times as that of in normal control and lung disease control subjects. With the introduction of A-TB drug therapy for 1 month in tubercular subjects, serum SOD level had fallen by an average of 31% in PTB subjects and 29% in EPTB subjects. In a previous study, it had revealed that there was a very little increase in intracellular SOD (SOD in RBC) in TB subjects, not significant at all ($p = 0.02$) when compared with that of in normal control as well as lung disease control subjects.[14] This intracellular increase of SOD is of host origin and Cu–Zn cofactored, which does contain leader peptides.[34] On the other hand, the whole blood GPx level in tubercular subjects (both PTB and EPTB) was significantly lower than that of normal control and lung disease control subjects ($p = 0.01$). This can be explained by the fact that in tubercular subjects there was already a deficit of reducing substances like NADPH(H^+) and reduced glutathione (GSH) because of oxidative burst as a result of infection. So, there is a jolt in the conversion of oxidized glutathione (GSSG) to its reduced form (GSH) by glutathione reductase for want of reducing substance NADPH(H^+). To detoxify the high concentration of H_2O_2 generated by dismutation reaction in TB subjects, catalase comes into action as it has a higher K_m value.[14] It is well established *in vitro* study that the virulent *M. tuberculosis*

Table 1: Serum superoxide dismutase (SOD) activity before and after

		Before treatment		*After one month's usual treatment*		
Group	*Subjects*	*n*	*Serum SOD (U/mL)*	*n*	*Serum SOD (U/mL)*	*P value*
I	Normal control	30	126 ± 37	–	–	
II	Lung disease control	27	142 ± 31	27	131 ± 23	0.1
III	Tubercular					
IIIA	Pulmonary	42	1413 ± 103*	42	978 ± 72*	0.03*
IIIB	Extra pulmonary	34	1226 ± 76*	34	869 ± 57*	0.04*

The table as depicted has been taken from previous published paper of this author.[5]
The serum SOD activities had been extrapolated from the published paper of index author.[5]

Table 2: Whole blood glutathione peroxidase (GPx) activity before and after

		Baseline value		*After one month's additional treatment*		
Group	*Subjects*	*n*	*Whole blood GPx (U/L)*	*n*	*Whole blood GPx (U/L)*	*p value*
I	Normal control	40	8632 ± 1806	–	–	
II	Lung disease control	40	4970 ± 750	40	7460 ± 625	0.1
III	Tubercular					
IIIA	Pulmonary (PTB)	40	982 ± 214*	40	2665 ± 367*	0.01*
IIIB	Extra pulmonary (EPTB)	40	918 ± 196*	40	2442 ± 325*	0.01*

Table depicting whole blood GPx activities had been assayed for this Paper.

shows sensitivity to GSH at physiological concentration.[35] It had also been reported that the growth of *M. tuberculosis* was reduced significantly by the treatment with *N*-acetyl cysteine (NAC) or with rGSH formulated in liposomes and that had been achieved by enhancing the level of GSH in human macrophages.[36,37] It is also to mention that the categorical report of Venketaraman et al. had stated that GSH level was significantly reduced in mononuclear cells and red blood cells in TB patients.[37] Also, the siderophore iron in TB subjects might bind with the thiol group at selenium rather than sulfur hampering the formation of catalytically active selenol of GPx. Again, superoxide anion (produced in excess in TB subjects) might dampen GPx activity by its reaction with selenium at the active site of the enzyme. The oxidative stress and antioxidant defense mechanism have key roles during TB infection and treatment.[38] With A-TB drug therapy in TB subjects, there was a decrease in the mycobacterial load resulting in a decrease in dismutation reaction along with recovery of reducing substances like NADPH(H^+). Due to less amount of H_2O_2 so produced there was a recovery in the activity of GPx as it has a lower K_m value and also there is a decrease in respiratory burst increasing the level of reducing substances like NADPH(H^+). The recovery of whole blood GPx level in TB subjects with treatment might also be due to a decrease in siderophore iron load resulting in decreased binding of iron with thiols at selenium — the active site of the enzyme GPx; and thus finally the enhanced activity of whole blood GPx. *Mycobacterium tuberculosis* is relatively resistant to killing by H_2O_2 and other organic peroxides. As reported by Manca et al., the extent of peroxidase activity correlated significantly with a resistance of mycobacterial strains to H_2O_2 mediated killing.[39] In fact, whole blood GPx activity correlates positively with total antioxidant capacity in the body but correlates negatively with SOD activity.[40] So, the magnitude of the ratio of serum SOD activity and whole blood GPx activity in TB subjects might be a measure to be used as a diagnostic for TB.

With the result as depicted in the Tables, the statistical computation was looked up to engross the lowest cutoff value for serum SOD and the highest cutoff value for whole blood GPx in TB patients and also to calculate the ratio of these two parameters to diagnose TB. Statistically, the area under the normal curve between $(\bar{X} - 3SD)$ and $(\bar{X} + 3SD)$ is 99.74% of the total area under the curve [$\bar{X}$ is mean and SD is standard deviation]. As serum SOD in TB patients is markedly elevated, the value as depicted in the left half of the normal curve has been considered for statistical computation, and for that purpose, three times of SD value has been subtracted from the mean value. On the other hand, the whole blood GPx activity in TB patients had shown marked inhibition and for statistical computation for calculating the GPx activity, the right half of the normal curve has been considered and three times of SD value has been added to the mean value. Similarly, for statistical computation in normal control and lung disease control subjects, the right half of the normal curve has been considered for serum SOD and the left half of the said curve for whole blood GPx. Now, if from the mean value of serum SOD level of TB subjects (A-TB drugs started between 0 and 15 days) is deducted three times of standard deviation (SD) value, serum SOD level in PTB subjects becomes 1,104 U/mL and in EPTB subjects 998 U/mL. In normal control and lung disease control subjects, if three times of SD is added to the mean SOD level, it becomes 237 U/mL in normal control and 235 U/mL in lung disease control subjects. Also, if three times of SD value is added to the mean value of whole blood GPx level, it becomes 1,624 U/L in PTB subjects and 1,506 U/L in EPTB patients. With the deduction of three times of SD value from the mean value in normal control subjects; whole blood GPx is 3,224 U/L and in lung disease control subjects is 2,720 U/L.

The magnitude (ignoring the unit whatsoever) of this ratio as calculated from the data is as follows:-

Normal control—0.07; Lung disease control—0.08; PTB subjects—0.68; and EPTB subjects—0.66. So, the ratio in both PTB and EPTB subjects is more than nine times and more than eight times, respectively, than those of normal control and lung disease control subjects. So, the higher magnitude of this ratio might be a faithful diagnostic aid for TB, both pulmonary and extrapulmonary especially before the institution of any treatment. With additional treatment for 1 month with A-TB drugs in TB subjects, there was a significant decrease in the said ratio. So, by serial measurement of this ratio during the course of A-TB drug therapy, the effectiveness of treatment may be well monitored. The TB patients later diagnosed as MDR-TB were omitted from the field of study, but for them, serial measurement of serum SOD and whole blood GPx and calculation of the ratio of these two were continued even after 30 days. Some of those patients had shown no decrease in the ratio from the very beginning. These subjects might be diagnosed as primary drug-resistant TB patients. On the other hand, the rest of the subjects with A-TB drugs had a noteworthy decrease initially of the said ratio but later on showed a rebound increase of the said ratio. Those patients might be diagnosed as secondary drug-resistant TB subjects.

Conclusion

The measurement of the ratio of serum SOD to whole blood GPx might help in the early diagnosis of TB. Also by serial measurement of the said ratio, it is useful to monitor the sensitiveness of *M. tuberculosis* toward drug therapy and diagnose drug-resistant cases.

Clinical Significance

Superoxide dismutase is ubiquitous in aerobes, but *M. tuberculosis* is unique in exporting an extracellularly great quantity of iron-cofactored SOD which is NaCN resistant while in other bacterial species and non-pathogenic mycobacteria, SOD is essentially intracellular, not iron-cofactored and non-resistant to NaCN. Gr-II subjects under lung disease control subjects which also included COPD patients had recorded no significant increase of serum SOD. The receiver-operating characteristic (ROC) analysis is a useful tool for evaluating the performance of diagnostic tests; the corresponding empirical ROC curve was drawn by a non-parametric method using SPSS software. This curve and the corresponding area under the curve (AUC) established that the concerned ratio as a biomarker had predictive ability to differentiate TB patients from normal and other lung disease control subjects. For measurement of serum SOD and whole blood GPx, kits are available; also preparation of working reagents from ingredients might be made and assay procedure is not so complicated.

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