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ORIGINAL ARTICLE

Antioxidant Capacity and Lipid Peroxidation Product in Pulmonary Tuberculosis

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Abstract: *Background:* Severe oxidative stress has been reported in pulmonary tuberculosis (TB) patients because of increased production of reactive oxygen species (ROS) secondary to phagocyte respiratory burst, malnutrition and poor immunity but there is limited knowledge available for antioxidant status and its relation to lipid peroxidation in tuberculosis patients, particularly in developing countries. The purpose of this study was to determine the Trolox Equivalent Antioxidant Capacity (TEAC), Superoxide Dismutase (SOD) activity and Malondialdihyde (MDA), a marker of lipid peroxidation in TB patients. *Methods:* The study samples comprised of healthy volunteers (n=38) as control group and TB patients (n=85) undergoing anti-tuberculosis therapy (ATT). Trolox equivalent antioxidant capacity (TEAC) was determined by ABTS 2, 2□-azinobis-(3-ethylbenzeno-thiazoline-6-sulphonic diammonium salt), SOD activity by Potassium Metabisulphite-NADH-Nitroblue Tetrazolium (PMS-NADH-NBT) assay and MDA by Thiobarbituric Acid (TBA) method. *Results:* TEAC values (mmol/l) were decreased significantly in TB patients (0.84 ± 0.10 mmol/l) in comparison with control group; 1.49 ± 0.03, P<0.05. Percent inhibition of SOD in TB patients was 11.77 ± 1.98 which was found to be markedly lower than 23.89 ± 0.92 in controls. The levels of lipid peroxidation products; MDA was significantly high in TB patients (4.04±0.25 μmol/l) then control group; 2.03±0.15 μmol/l, P<0.05. *Conclusion:* The study showed that in TB patients, total antioxidant capacity in terms of TEAC value and SOD in terms of percent inhibition was low while lipid peroxidation products were significantly high. Adequate nutritional supplementation may prove beneficial, representing an efficient approach to fast recovery.

Keywords: Total antioxidant capacity, pulmonary tuberculosis, Oxidative Stress, Malondialdehyde.

Introduction

Pulmonary tuberculosis, an infectious disease caused by Mycobacterium tuberculosis, is one of the world's leading causes of death [1]. The pathogenesis of TB is multifactorial and includes the effects of oxidative stress [2-4] Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) are induced by mycobacteria through the activation of phagocytes [5-7] by respiratory burst mechanism [8] which is crucial to host defense but may promote tissue injury, inflammation [4,9] and may further contribute to immunosuppression [10-12]. Pulmonary fibrosis and dysfunction in TB are thought to be a consequence of chronic inflammatory events involving pro-inflammatory cytokines, activated macrophages and ROS that stimulate fibroblast proliferation and mononuclear cell DNA damage [9, 13-14].

In developing countries, infection and malnutrition form a vicious cycle, [15-16] hence TB is often said to be the disease of the malnourished. Lipid peroxidation, a general mechanism of tissue damage by free radicals, is known to be responsible for cell damage and may induce many pathological events [17]. The process of lipid peroxidation is one of oxidative conversion of polyunsaturated fatty acids to lipid peroxidation products of which MDA is the most widely studied, biologically relevant, free radical reaction. MDA itself, owing to its high cytotoxicity and inhibitory action on protective enzymes, is suggested to act as a tumor promoter and a co-carcinogenic agent. MDA is a well-characterized mutagen that reacts with deoxyguanosine to form a major endogenous adduct found in the DNA of human liver [18]. TB patients have been reported to have decreased concentrations of antioxidants, enhanced generation of ROS [3] and increased levels of lipid peroxidation product as a consequence of impaired activity of scavenging enzymes [19]**.** Superoxide, a free radical is produced during cellular metabolic reactions. SOD, an antioxidant enzyme, changes superoxide anion into hydrogen peroxide and oxygen. Superoxide dismutase is expressed at high levels in mammalian lungs compared with other tissues [20]**.**

Patients and Methods

This study was conducted as per good clinical practice (GCP) guidelines (declaration of Helsinki). A cross-sectional analytical study design was employed for this study. Patient samples were collected from Gulab Devi Chest Hospital, Ferozepur Road Lahore, Pakistan where formal approval from Ethics Committee of the Hospital and informed consent was also taken from all the study subjects. Study samples comprised of normal healthy volunteers as control(n=38) and pulmonary tuberculosis patients; TB patients (n=85) undergoing ATT. Eighty five patients with age ranging from 15 to 55 years (mean age 36.22 ± 15.06 years) were enrolled if they had proven pulmonary tuberculosis based on at least two positive Ziehl–Neelsen sputum specimens, cultures positive for Mycobacterium tuberculosis, clinical and radiographic abnormalities consistent with pulmonary TB and being treated by ATT. Patients having any of the following conditions were excluded: active smoker, cardio vascular disease, diabetes, asthma, any kind of malignancy, concurrent use of vitamin supplements especially containing vitamin E and selenium, illicit drug addiction (e.g. hashish, heroin), alcohol drinking, and signs of adverse effects of ATT during the treatment. Blood samples were collected and isolated plasma was stored at -80 ^{o}C till analysis. Study parameters were analyzed at department of Biochemistry, Central Park Medical College Lahore in collaboration with department of Chemistry, Government College University Lahore.

Determination of Trolox equivalent antioxidant capacity of Plasma by ABTS method: TEAC value was determined by ABTS 2, 2□-azinobis-(3-ethylbenzeno-thiazoline-6-sulphonic diammonium salt) assay protocol as developed by Re *et al* [21]. ABTS was dissolved in water to concentration of 7 mM. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM freshly prepared potassium per-sulfate.

The mixture was allowed to stand in the dark at room temperature for 14 hours before use. For the study of antioxidant activity of plasma, the ABTS stock solution was diluted with PBS buffer (7.4 pH) to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 ^{o}C. The reduction in absorbance of a 2.99 ml of ABTS radical cation solution ($A_{734\ nm}$ = 0.700 ±0.02) after adding 10 µl of plasma sample at 30 ^{o}C was noted using UV-1700 PharmaSpect UV-Visible Spectrophotometer (Shimadzu, Japan) exactly after one minute following initial mixing. Subsequently, readings were noted after every minute for a total of 8 minutes. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants and Trolox for the standard reference data. TEAC was calculated by defining the concentration of Trolox in mmol/l having the equivalent antioxidant capacity to a 1.0 mmol/l sample of the plasma under investigation.

Plasma Superoxide Dismutase (SOD) activity: SOD activity was determined in plasma by PMS-NADH-NBT assay as described by Liu *et al* [22] with minor modifications. Superoxide radicals were generated in 3.0 ml of Tris-HCL buffer (16 mM, pH 8.0), which contained 78 µM *β*-nicotinamide adenine dinucleotide (reduced form, NADH), 50 µM nitroblue tetrazolium (NBT), 10 µM phenazine methosulfate (PMS), and 50 µL of plasma sample. The color reaction of superoxide radicals and NBT was detected at 560 nm using UV-1700 PharmaSpect UV-Visible spectrophotometer (Shimadzu, Japan). SOD activity was reported as % inhibition.

Plasma MDA level: Plasma MDA level was determined by the method of Satoh [23]**.** To 0.5 ml volume of plasma, 0.5 ml of 35% Trichloroacetic acid was added. After vortex, 0.5 ml of Tris–HCl buffer (50 mM; pH 7.4) was mixed, followed by incubation at room temperature for 10 min. To this, 1 ml of 0.75% TBA in 2 M Na_2SO_4 was added and then the mixture was heated at 100 °C for 45 min. After cooling, 1 ml of 70% TCA was added, vortexed and centrifuged at 950 g for 10 min. The absorbance of the supernatant was measured at 532 nm. Total TBA-reactive materials were expressed as MDA, using a molar absorptivity for MDA of 1.56×105 cm^{-1} M^{-1}. The results were expressed as µmol/l. The absorption of the colored product was determined by using UV-1700 PharmaSpect UV-Visible spectrophotometer (Shimadzu, Japan).

Statistical analysis: Microsoft EXCEL 2007 for windows was used for all statistical analysis. Differences between TB Patients and control group were evaluated by using t-test. p-value less than 0.05 was considered to be statistically significant.

Results

Age range of control group was 25-55 years with a mean of 34.20 years. Age range of TB group was 19-57 years with a mean of 36.22 years. There was no significant age difference between the two groups.

TEAC values (mean, 0.84 ± 0.10 mmol/l) in TB patients were found to be significantly lower (P<0.05) than the control group (1.49 ± 0.03 mmol/l) as shown in table 1. A comparison of TEAC values of TB patients and control group is shown in fig.1.

Table-1: TAC values, % inhibition of SOD and MDA levels in study group			
Parameter	**Control Group n=38 Mean±SD**	**TB Patients n=85 Mean±SD**	**P-Value***
TEAC value(mmol/l)	1.49±0.03	0.84±0.10	<0.05
% inhibition of SOD	23.89±0.92	11.77±1.98	<0.05
MDA (µmol/l)	2.03±0.15	4.04±0.25	<0.05
* p-value <0.05 is considered to be statistically different.			

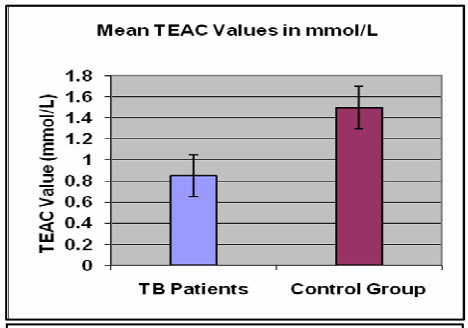

Fig-1: TEAC value in mmol/l of TB patients and control group

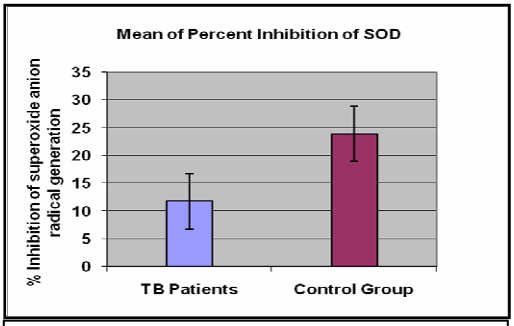

Fig-2: Percentage inhibition of superoxide anion radical generation activity of 10 µl plasma sample of TB patients and control group by PMS-NADH-NBT assay

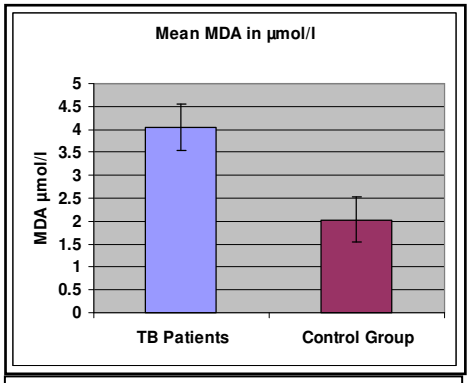

Fig-3: Plasma MDA levels in µmol/l in TB patients and control group by TBA method.

Percent inhibition of SOD in TB patients was 11.77 ± 1.98 which was found to be markedly lower than 23.89 ± 0.92 in control group as shown in table 1. A comparison of the percent inhibition (% inhibition) of plasma samples of TB Patients and control group is shown in fig.2.

Plasma MDA level in TB patients was 4.04 ± 0.25 (µmol/l), that is significantly higher (P<0.05) than 2.03 ± 0.15 (µmol/l) in control group as shown in table 1. Comparison of Plasma MDA level of TB Patients and control group is shown in fig.3.

Discussion

The intensity of oxidative stress is measured not only by the production of free radicals but also by antioxidant enzymes. Antioxidant enzymes help in decreasing the oxidative stress by inactivating the ROS. The results of present study indicate that TB patients are under oxidative stress demonstrated by decreased levels of total

antioxidant capacity in terms of trolox equivalent antioxidant capacity and decreased superoxide dismutase activity in terms of % inhibition. Our results are in accordance with various other studies [24-25] in which different methodologies were used to assess the total antioxidant capacity. Reduced total antioxidant capacity has been documented in all these studies in patients with pulmonary tuberculosis. Very recently, a study has also reported the same results of reduced total antioxidant capacity and has extended the study by finding out the correlation of antioxidant and oxidants in pulmonary tuberculosis patients [26]. Total antioxidant status in pleural fluid in pulmonary tuberculosis patients has also been elaborated by comparing untreated and patients under treatment. The antioxidant levels increased significantly in under treatment cases in contrast with untreated cases [17] however the results were not compared with the control group. Our results of reduced antioxidant capacity in patients undergoing ATT are based on comparison with the control group. Moreover, our study has the advantage of plasma sampling in contrast to the invasive pleural aspiration employed in the above mentioned study. Reduced concentrations of vitamin A and of the anti-oxidant vitamins C and E were previously reported in patients with tuberculosis [27-29]. We extended these findings by showing a significant reduction in SOD activity which is an important enzymatic anti-oxidant. Similar kind of results have also been reported [17] where SOD levels were significantly lower in all TB patients in comparison to control group and then increased gradually with clinical improvement following ATT. As our study is limited because we only studied patients undergoing ATT at a given point of time and follow up was not conducted. We document significantly high levels of plasma MDA in patients with pulmonary tuberculosis as compared to the control group, which is in consensus with earlier studies [26]. Increased MDA levels have also been detected in pleural fluid of TB patients [17]. In this study we report the same with non invasive technique.

Our results indicate increased oxidative stress in plasma of tuberculosis patients undergoing ATT as compared to normal healthy controls. Oxidative stress and resultant lipid peroxidation may lead to increased levels of ROS, which may be responsible for pulmonary damage and lung fibrosis during tuberculosis. Furthermore, tuberculosis and oxidative stress compliment each other in development of debilitating complications resulting from this precarious disease. Nutritional supplementation may signify effective approach for limiting the oxidative damage and ultimately helping in swift recuperation. Clinical trials need to be instituted to further investigate the therapeutic benefit of the exogenously administered antioxidants like vitamin C, tocopherols etc.

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