Antioxidants and Tumor Necrosis Factor-α in Patients with Pulmonary Tuberculosis

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ABSTRACT

Background: Tuberculosis (TB), is one of the major common air born infectious bacterial diseases which remains a major worldwide health problem with global mortality. **Objective:** To evaluate the efficiency of serum samples compared to sputum for the early diagnosis of TB, and to evaluate the levels of superoxide dismutase (SOD), catalase (CAT), total antioxidant status (TAS) and tumor necrosis factor-α (TNF-α) in patients with pulmonary tuberculosis (PTB). **Methods:** One hundred patients with clinically suspected PTB and 25 healthy individuals were enrolled in the study. According to the bacteriological results, 78 patients were diagnosed as having PTB infection. These cases were categorized into 69 culture positive cases [sputum and serum PCR positive patients (n=42), sputum PCR positive and serum PCR negative patients (n=16) and sputum and serum PCR negative patients (n=11)] and 9 culture negative and sputum PCR positive cases with radiological lung abnormalities suggestive for PTB. For these 78 cases, erythrocyte SOD, CAT, serum TAS and TNF-α were determined. Twenty two patients were culture negative and negative for both sputum and serum PCR. They had no PTB and were not involved in biochemical studies. **Results:** In all 78 PTB patients, erythrocyte SOD, CAT and serum TAS levels were statistically lower than controls ($p<0.05$), while TNF-α was highly significantly increased (p=0.001). There was a significant direct linear correlation between SOD and CAT and TAS ($p<0.0001$, r= 0.78; $p<0.0001$, r= 0.88; $p<0.0001$, r=0.80 respectively) and a significant reverse linear correlation between TNF-α and SOD, CAT and TAS level (p<0.0001, r=-0.55; p<0.0001, r=-0.51; p<0.0001, r=-0.65 respectively). **Conclusion:** Although the sputum culture is still the gold standard for the diagnosis of patients with PTB, sputum PCR is an efficient method that could be used as an alternative to the culture for the rapid identification of PTB cases. The lower levels of SOD, CAT and TAS may be improved by the antioxidant therapy which may help in better prognosis. Anti TNF-α therapy may help in decreasing the elevated level of TNF-α shown in all PTB patients.

Key words: Catalase, pulmonary tuberculosis, superoxide dismutase, total antioxidant status and tumor necrosis factor-α.

INTRODUCTION

Tuberculosis (TB) is still a serious health problem of global importance caused by infection with *Mycobacterium tuberculosis* (MTB). An estimated one third of the world's population is recently infected; there are 2-3 million deaths from tuberculosis

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annually.[1] MTB is an intracellular pathogen which after inhalation is internalized mainly by alveolar macrophages, phagocytes, then undergoes respiratory burst resulting in the production of capacious amount of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) which are not only essential for the destruction of ingested microorganisms, but also contribute to inflammatory injury of the host tissue.[2] Thereby, excess amounts of ROS and RNI can cause protein oxidation, lipid peroxidation and DNA damage.[3]

Pulmonary fibrosis and dysfunction in TB are thought to be a consequence of chronic inflammatory events involving pro-inflammatory cytokines, activated macrophages and toxic oxygen radicals that stimulate fibroblast proliferation and mononuclear cell DNA damage. In TB, oxidative stress is a result of tissue inflammation, poor dietary intake of micronutrients due to illness, ROS and anti-tuberculosis drugs. These free radicals may in turn contribute towards pulmonary inflammation if not neutralized by antioxidants.[4]

The total antioxidant status (TAS) of TB infected patients would be the net result of the protective effect of antioxidants such as antioxidant enzymes, small molecule antioxidants (glutathione) and dietary antioxidant micronutrients (vitamin C and vitamin E) on one hand,[5] and free radicals generated from TB infection on the other hand. The TAS of these patients would be a better indicator of the net free radical burden which, if indicated, could be manipulated by dietary supplementation.[6]

Tumor necrosis factor-α (TNF-α) is a multipotent cytokine produced predominantly by monocytes / macrophages, but it can also be produced by many other cells, including mast cells, endothelial cells, neuronal tissue and lymphoid cells such as T and B lymphocytes, and natural killer (NK) cells. TNF-α is an essential component of the innate defense mechanism of the host against

pathogenic challenge. Unfortunately, it can also play a major role in the pathology of certain diseases such as tuberculosis. This disease is a striking example of the role of TNF-α as a 'double-edged sword', because apart from its role in controlling the MTB infection, it can also cause severe tissue damage.[7]

Laboratory diagnosis of TB involves several bacteriologic, immunologic or molecular approaches.[8] However, conventional microbiology methods still constitute the principal tool for the diagnosis of TB in developing countries. Direct testing has low sensitivity and culture requires long culturing time.[9] Nucleic acid amplification-based diagnostic approaches may provide very sensitive, specific, and rapid detection of MTB.[10]

For diagnosis of TB by PCR, specimens from the site of infection are required. In certain cases it is difficult to get the specimens from the site of infection and in such situations, some researchers have tried to detect the DNA of MTB complex from the blood of these patients.[11]

So, the aim of this study was to evaluate the efficiency of serum samples compared to sputum for the early diagnosis of TB. In addition to evaluate the levels of superoxide dismutase (SOD), catalase (CAT), TAS and TNF-α in patients with PTB.

SUBJECTS AND METHODS

Patients

One hundred clinically suspected patients for PTB, regardless of age, sex or occupation, and before starting any anti-tuberculosis therapy, were enrolled as a case group. The control group consisted of 25 healthy individuals. Patients were admitted to EL-Mamoura Hospital, Alexandria.

According to the bacteriological results, 78 patients were diagnosed as having PTB infection and categorized into 69 culture positive cases [sputum and serum PCR positive patients (n=42), sputum PCR positive and serum PCR negative patients (n=16) and

sputum and serum PCR negative patients (n=11)] and 9 culture negative and sputum PCR positive cases with radiological lung abnormalities suggestive for PTB.

Twenty two patients were culture negative and negative for both sputum and serum PCR. They had no PTB and were not involved in biochemical studies.

Patients were excluded if they met any of the following criteria: pregnancy, lactation, use of supplements containing vitamin E and selenium, alcohol drinking, other respiratory disorders, HIV positive, diabetes mellitus, coronary artery disease or rheumatoid arthritis. An informed consent was taken from patients prior to participation. The study was approved by the Ethical Committee of Medical Research Institute.

Collection of samples:

Early morning sputum samples were collected from each patient in a sterile, disposable plastic container and immediately transported to the laboratory.

Also, 5 ml of blood were collected from all patients and controls. Three ml of blood were processed and the serum was separated in a sterile eppendorf and stored immediately at -20 ºC. Two ml were kept in EDTA vial for immediate separation of erythrocytes.

Sputum sample processing:

1. **Microscopical examination and culture**: Immediately after collection, sputum samples were treated with N-acetyl-L-cysteine-NaOH (NALC-NaOH). From the pellet, part was subjected to direct microscopy examination by Ziehl-Neelsen stain (ZN), another part was inoculated on to Lowenstein-Jensen (LJ) and incubated at 37ºC for 10 weeks. The remaining part of the sputum pellet was further processed for DNA extraction.

2. **PCR**:

A- **DNA extraction**: DNA was extracted using the QIAamp viral DNA mini kits (Qiagen, GmbH, Germany) according to the manufacturer's instructions. Briefly, 200 µl

serum or the sputum pellet were mixed with 180 µl of lysozyme (20 mg/ml lysozyme) 20mM Tris-HCl pH 8.0; 2mM EDTA; 1.2% Triton and incubated at 37°C for 24 hrs then 20 µl proteinase K and 200 µl AL buffer were added and incubated for 30 minutes at 56°C then boiled for 10 minutes, then 200 µl ethanol were added and the samples were applied to the QIAamp spin column in a 2 mL collection tube.

B- Amplification of MTB DNA: A 123 bp DNA fragment was amplified using IS6110 specific primers P1 5′CCT GCG AGC GTA GGC GTC GG 3′ and P2 5′CTC GTC CAG CGC CGC TTC GG 3′. Ten µl of eluted DNA were used. PCR was performed in a total volume of 25 µl using 2x PCR master mix (Qiagen) containing 0.05 units/ml of Taq DNA polymerase, PCR buffer, 2 mM $MgCl_2$, 0.2 mM of dNTPs, with 50 picomole of each primer. Amplification was performed in a Perkin-Elmer 9600 thermo cycler with an initial denaturation for 10 minutes at 94°C, then 35 cycles of denaturation for 1 minute at 94°C, annealing

for 50 seconds at 67°C and extension for 50 seconds at 72°C and final extension for 10 minutes.[12]

PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gel stained with ethidium bromide. The sizes of the PCR products were estimated by comparison with 100-bp DNA size markers (Fermentes).

Preparation of haemolysate

Erythrocytes were separated from whole blood by centrifugation at 3000 rpm for 15 minutes at 4°C. Plasma and Buffy coat were discarded. Packed blood cells were washed 3 times with 0.9 g/L NaCl, and then lysed by adding cold deionized water. For SOD, haemoglobin was precipitated by the addition of chloroform: ethanol (1.5:1) then diluted with 500 ml of distilled water and centrifuged at 3000 rpm for 15 minutes at 4°C. The clear top layer was used for SOD determination. For CAT, lysed cells were frozen and thawed 3 times within 10 minutes followed by centrifugation at 3000

rpm for 15 minutes at 4°C. The supernatant was used for CAT assay.

Biochemical assays:

1- Erythrocyte SOD: The method depends on the spontaneous auto-oxidation of pyrogallol at alkaline pH, resulting in the production of O_2, which in turn enhances auto-oxidation of pyrogallol.

The assay mixture consisted of 1 ml of tris-HCL buffer (50 mM containing 1 mM diethylene - triamine penta acetic acid, pH=8.2), 10 µl of pyrogallol (20 mM) and 10 µl of diluted haemolysate (1:10). After mixing, the rate of increase in absorbance/min was measured at 420 nm. A blank was similarly treated but without adding haemolysate. The enzyme unit expressed was calculated as the amount of the enzyme required to inhibit 50% of auto-oxidation of pyrogallol.[13]

2- Erythrocyte CAT: On decomposition of H_2O_2 with catalase, the absorption decreases with time and from this decrease the enzyme activity can be calculated.

In the experimental cuvette, 1 ml H_2O_2-phosphate buffer (0.07 M, pH=7) was mixed with 0.01 ml of the sample. The time required for a decrease in the optical density from 0.450 to 0.400 nm was used for the calculation. One IU of catalase is the enzyme which decomposes 1 mM of H_2O_2/minute at 25°C.[14]

3- Serum TAS was determined by the method of Blios (1958),[15] using free radical, a,a-diphenyl-b-picrylhydrazyl (DPPH) (Sigma Aldrich, USA), at a concentration of 0.2 mM in methanol. One ml serum was deproteinated by the addition of 1 ml methanol, vortexed for 30 seconds then centrifuged at 3000 rpm for 30 minutes to separate the proteins. To the clear supernatant 1.5 ml of methanol and 0.5 ml DPPH solution were added, mixed thoroughly and the absorbance was read at 517 nm against blank, prepared in an identical way, but without the addition of serum. Ascorbic acid was used as a reference standard.[15]

4- Serum TNF-α cytokine was analyzed on the immulite 1000 analyzer by the solid-phase, chemiluminescent immunometric assay. A serum sample (haemolysis and lipemic samples were excluded) was introduced to each patient and control after introduction of the quality control with each run and calibration when indicated. Calibration range: up to 1.000 pg/ml and analytical sensitivity: 1.7 pg/ml.[16]

Statistical analysis

Statistical analysis was done using the Software Statistical Package for Social Science (SPSS) version 11.5. Results were presented as mean values with a standard error (mean ± SE). Statistical difference between groups and correlation were estimated by using Mann Whitney test and Spearman's correlation respectively. The results were considered statistically significant at $p \leq 0.05$.

RESULTS

Out of 100 clinically suspected TB patients, 63 were ZN positive and 69 were culture positive.

From Tables 1 and 2, out of 100 clinically suspected PTB patients, 63% were positive by both sputum PCR and culture, whereas 27% were negative by both methods compared to 42% who were positive by both serum PCR and culture and 27% were negative by both serum PCR and culture. However, 6% were culture positive but sputum PCR negative compared to 27% who were culture positive but serum PCR negative, and 4% were culture negative and both sputum PCR and serum PCR positive. Considering culture results as the gold standard, the sensitivity of sputum PCR was 91%, with 87 % specificity compared to 60% sensitivity and 87% specificity in case of serum PCR.

Table 1. The percentage of sputum culture and sputum PCR for all clinically suspected PTB patients (n=100)

Sputum PCR	TB culture positive		TB culture negative		Total	
	No.	(%)	No.	(%)	No.	(%)
Positive	63	91.3	4	12.9	67	67
Negative	6	8.7	27	87.1	33	33
Total	69	100	31	100	100	100

Table 2: The percentage of sputum culture and serum PCR for all clinically suspected PTB patients (n=100)

Serum PCR	TB culture positive		TB culture negative		Total	
	No.	(%)	No.	(%)	No.	(%)
Positive	42	60.9	4	12.9	46	46
Negative	27	39.1	27	87.1	54	54
Total	69	100	31	100	100	100

Out of 69 culture positive TB patients, 42 (61%) were positive for both sputum and serum PCR, 11 (16%) were negative by both sputum and serum PCR, 16 (23%) were sputum PCR positive but serum PCR negative, and none was sputum PCR negative and serum PCR positive (Table 3). Out of 31 culture negative cases, 3 (10%) were positive by both sputum and serum PCR, 22 (71%) were negative for both sputum PCR and serum PCR, 6 (19%) were sputum PCR positive and serum PCR negative, and none was sputum PCR negative and serum PCR positive (Table 4). PCR positive and culture negative results cannot be ignored as these patients (n=9) were clinically suspicious as having TB and thus were considered as TB infected patients and were further assessed by other biochemical tests, while PCR negative and culture negative cases (n=22) were not considered as having PTB and were excluded from other biochemical tests.

Table 3. Comparison between serum PCR and sputum PCR for all culture positive patients (n=69)

Serum PCR	Sputum PCR positive		Sputum PCR negative		Total	
	No.	%	No.	%	No.	%
Positive	42	72.4	0	0	42	60.9
Negative	16	27.6	11	100	27	39.1
Total	58	100	11	100	69	100

Table 4. Comparison between serum PCR and sputum PCR for all culture negative patients (n=31)

Serum PCR	Sputum PCR positive		Sputum PCR negative		Total	
	No.	%	No.	%	No.	%
Positive	3	33.3	0	0	3	9.7
Negative	6	66.7	22	100	28	90.3
Total	9	100	22	100	31	100

The data of Table 5 revealed that erythrocyte SOD, CAT and serum TAS levels were statistically low (p<0.05) in all TB patients in comparison to the control group.

A highly significant increase in serum TNF-α level had been observed in all TB patients when compared to that in the healthy subjects (p=0.001) (Table 5).

Table 5. Mean and standard deviation values of SOD, CAT, TAS and TNF-α levels in TB patients and control group.

Parameters	Control group (n=25)	TB patients (n=78) Culture +ve patients (n=69)			Culture -ve patients (sputum PCR +ve) (n=9)
		Sputum & serum PCR +ve (n=42)	Sputum PCR +ve & serum PCR –ve (n=16)	Sputum & serum PCR –ve (n=11)	
SOD (U/g Hb) (Mean ± SD)	88.72 ± 1.34	54.52 ± 1.96*	46.75 ± 3.05*	41.55 ± 0.37*	45.75 ± 1.28*
CAT (U/g Hb) (Mean ± SD)	14.02 ± 0.27	11.31 ± 0.55*	10.12 ± 0.94*	11.58 ± 0.48*	11.01 ± 0.33*
TAS (µM/ml) (Mean ± SD)	0.715 ± 0.01	0.435 ± 0.02*	0.402 ± 0.04*	0.389 ± 0.04*	0.338 ± 0.02*
TNF-α (pg/ml) (Mean ± SD)	9.33 ± 0.28	48.17 ± 1.48*	36.89 ± 1.00*	27.88 ± 0.59*	26.13 ± 0.99*

*Statistically significant at *P* ≤ 0.05 compared with control group using Mann Whitney test.

There was a significant direct linear correlation between SOD and CAT and TAS (p<0.0001, r= 0.78; p<0.0001, r= 0.88; p<0.0001, r=0.80 respectively) (Figure 1a, 1b and 2a respectively).

A significant reverse linear correlation between TNF-α and SOD, CAT and TAS level (p<0.0001, r=-0.55; p<0.0001, r=-0.51; p<0.0001, r=-0.65 respectively) (Figure 1c, 2b and 3 respectively) was observed.

(a)

r = 0.78
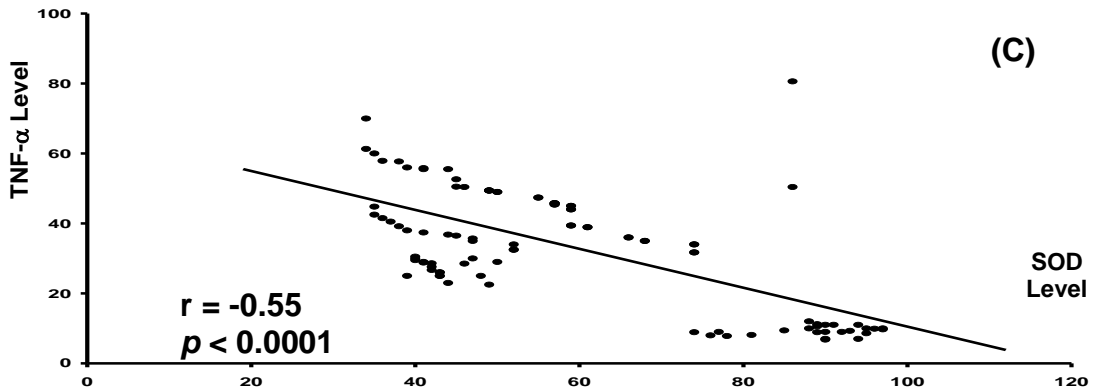*p* < 0.0001

(b)

r = 0.88
p < 0.0001
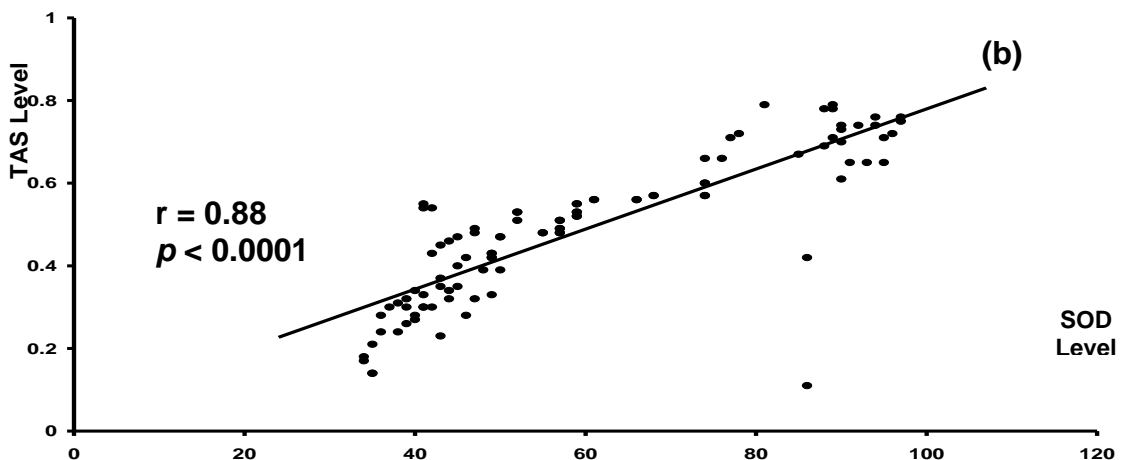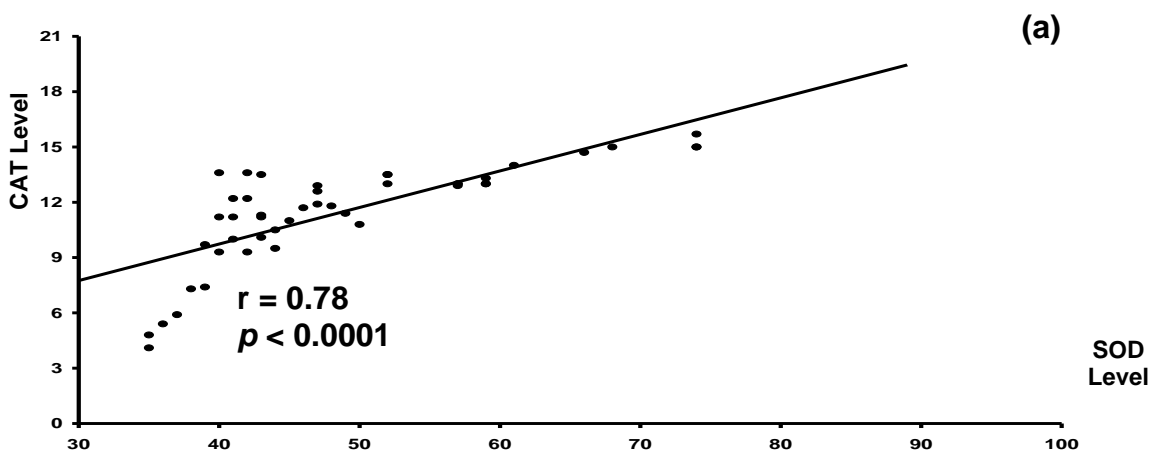
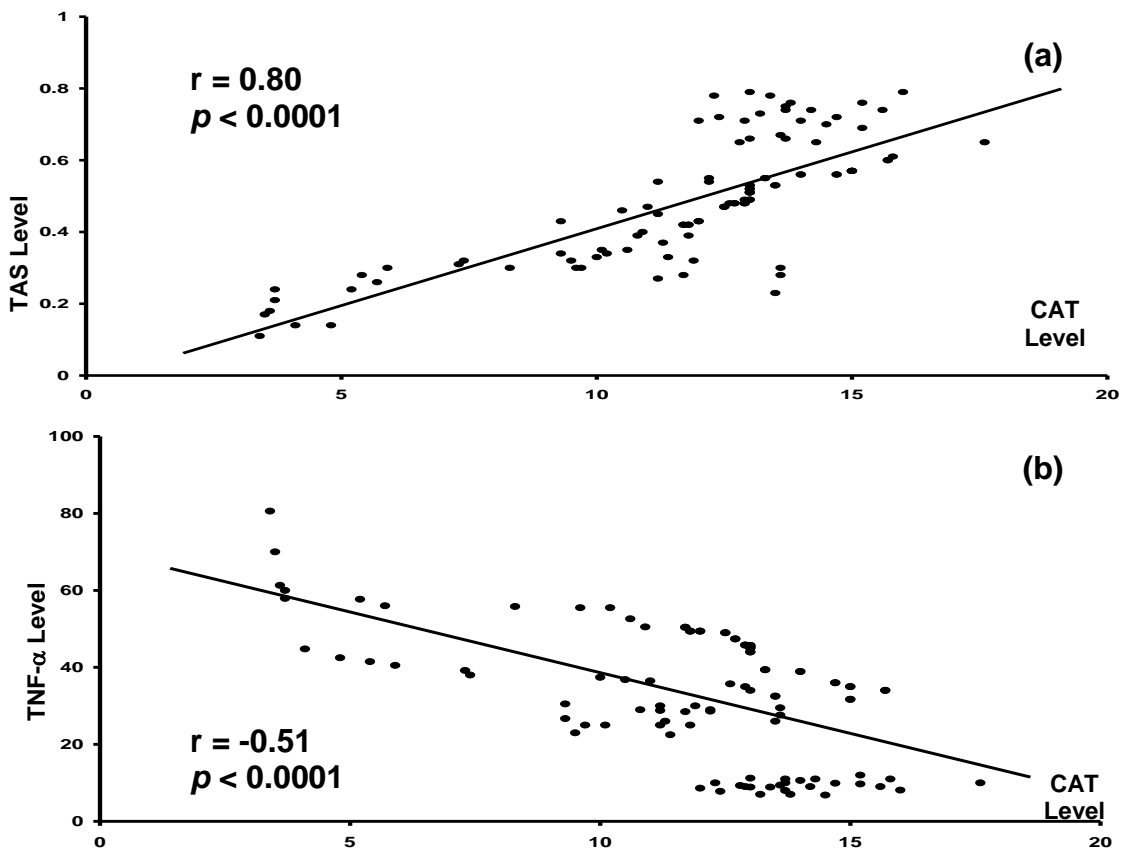**Figure 1. Spearman correlation between SOD and (a) CAT, (b) TAS and (c) TNF-α levels**

Figure 2. Spearman correlation between CAT and both of (a) TAS and (b) TNF-α levels
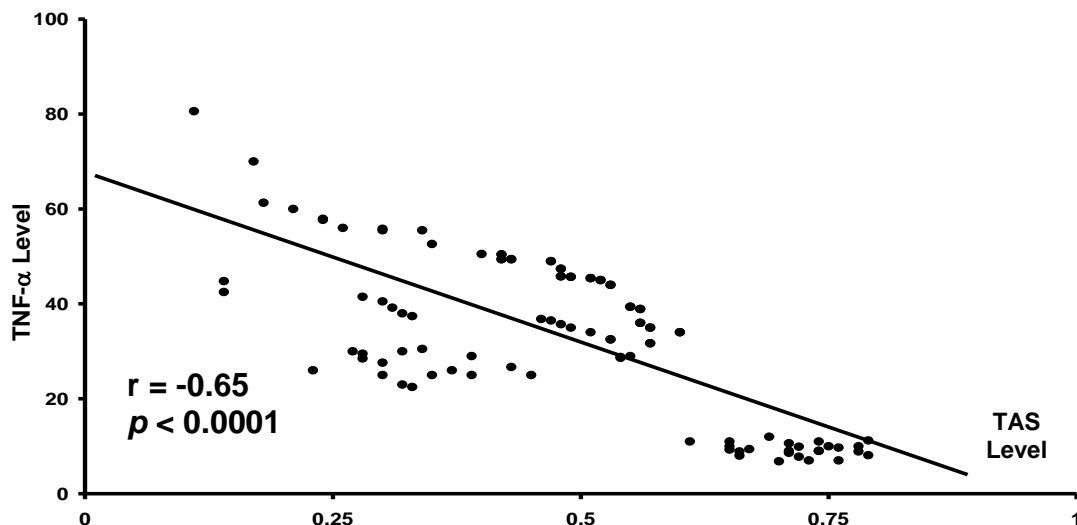
Figure 3. Spearman correlation between TAS and TNF-α levels

DISCUSSION

Tuberculosis has emerged as the greatest danger to people, threatening the health of millions. PTB is the most common form with insidious onset and illness remains unnoticed for sometime.[17] So our study, evaluated the sensitivity of PCR (sputum and serum) for diagnosis of PTB compared to culture which is still considered as the gold standard method for the diagnosis of tuberculosis worldwide.

The sensitivity of sputum PCR (91%) obtained was in agreement to that of culture,

as stated in most studies comparing these two methods,[18,19] where both culture and sputum PCR were superior to the direct testing method. This is possibly due to the greater sensitivity of culture and sputum PCR for bacillus detection in paucibacillary sputum samples. In this study, there were 4% of cases negative by culture but positive by sputum PCR and/or serum PCR yet they had radiological abnormalities and clinical symptoms suggestive for TB. This was reported by other investigators,[19,20] and might

have resulted from a failure of growth in culture due to insufficient or non cultivable bacteria in the samples. Furthermore, the extreme sensitivity of PCR was able to detect organisms that are present in low numbers. Also in this study, 6% of cases were culture positive but negative by sputum PCR compared to 27% serum PCR and this might be attributed to the presence of PCR inhibitors.[18,19,21] This observation was similar to that of Khan et al (2006).[21]

In the present work, serum PCR correctly identified 61% of culture positive cases and only 13% of culture negative cases (60% sensitivity, 87% specificity) which was similar to other workers.[11] This was lower than that for sputum PCR that identified 91% and 13% respectively (91% sensitivity, 87% specificity). It can be concluded that sputum PCR is preferred in diagnosis of PTB, however, serum PCR can be used in the diagnosis of extra PTB with reported sensitivity up to 70%,[22] or in cases when it is inconvenient to obtain sputum sample. Lastly, a positive predictive value of 94% and 91% for both sputum and serum PCR respectively makes this test very useful in strengthening the clinical suspicion in our high prevalence setting. The sensitivity, specificity and speed of molecular test in the diagnosis of tuberculosis should encourage the routine use of this test in clinical practices.

Lower erythrocyte SOD and CAT activities in all TB patients than in healthy persons were documented and are in agreement with previous studies.[4,23]

CAT is generally considered to be the dominant hydrogen peroxide-scavenging enzyme in the lung,[24] whereas the first enzymatic system decomposing superoxide radicals to H_2O_2 is the SODs. Several factors such as low food intake, nutrient malabsorption and inadequate nutrient release from the liver, acute-phase response and infections and inadequate availability of carrier molecules may influence circulating antioxidant concentrations.[25] Furthermore, MTB encodes a type of SOD which detoxifies exogenous ROS and contributes to the

survival of bacteria.[26] MTB self-produced SOD may further interfere with the production of the host's SOD by depleting the host's Zn resources.[23] Also, increased utilization of antioxidants by ROS works as an important contributing factor to lower antioxidants concentration in TB patients.[27]

The present study demonstrates a lower TAS as an indicator of an enhanced oxidative stress in PTB patients. The lower TAS in PTB could be partially due to depleted levels of non-enzymatic antioxidants such as Zn, vitamin E and C in these patients.[4,28] Vitamin E and C are the first antioxidants to be depleted upon exposure to both environmental and inflammatory oxidants.[28] Reduced serum levels of Zn in TB patients could be a result of redistribution of Zn outside plasma into other tissues, reduction of the hepatic production of the Zn-carrier protein (α2-macroglobulin), and/or rising of the metallothionein production. Poor nutritional intake is another important factor that

reduces the level of antioxidant micronutrients such as Zn, vitamin E and C in patients with PTB.[29] There are other factors that are associated with, or can have a similar presentation as poor nutritional intake, poor socio-economic status, anorexia, impaired absorption of nutrients, or increased liver metabolism.[30] Also, the disease contributes to the depleted TAS of the person probably through the inflammatory processes of the disease.[4]

Our observation of increased TNF-α level in all TB patients compared with healthy persons is complementary with previous findings.[7,31]

TNF-α is a potent activator of monocytes/macrophages, which acts in synergy with interferon- γ (IFN- γ) to induce antimicrobial activity via the induction of reactive oxygen and nitrogen intermediates (ROIs and RNIs). RNIs are believed to be particularly important for killing intracellular MTB.[32]

A major secretory component of actively replicating MTB, which is referred to as the 30 kDa antigen (Ag85B), has been revealed to enhance the production of TNF-α by monocytes through an interaction with fibronectin. Furthermore, lipoarabinomannan (LAM), a MTB glycolipid, has also been shown to potently stimulate the induction of TNF-α.[33]

Toll-like receptors (TLRs) have been strongly implicated in regulating immune response to mycobacterial infection.[34] In a report by Underhill et al (1999),[35] it was demonstrated that upon exposure to MTB, macrophages were stimulated to produce TNF-α in a TLR-dependent manner. Thus, TLR2 appeared to be the principal mediator of macrophage activation and TNF-α production in response to mycobacteria.

TNF-α itself can amplify its own production indirectly by enhancing MTB gene expression within newly infected monocytes leading to more secreted Ag85 and further production of TNF-α.[35] This creates a positive-feedback loop for the production of TNF-α during an MTB infection. Also, within MTB lesions characteristic of the disease, TNF-α production can be induced by IFN-γ, produced either in the autocrine fashion by the alveolar macrophages themselves or by the T lymphocytes.[36]

The present study demonstrates that PTB is associated with lower erythrocyte SOD and CAT activities which are positively correlated with TAS level. SOD is the primary enzyme involved in the enzymatic antioxidant defense system, since it reduce ROS to H_2O_2 which is further reduced by catalase to H_2O.[37]

The lung inflammation which occurs during TB infection is a continuous source of free radical generation. Increased ROS level leads to an increase in the consumption of antioxidants,[27] and can also lead to an increase in the gene transcription of an array of genes involved in oxidative stress and immunity, including TNF-α (negative correlation with antioxidants).[38]

From the present work we can conclude that: although the sputum culture is still the gold standard for the diagnosis of patients with PTB, sputum PCR is an efficient method that could be used as an alternative to the culture for the rapid identification of PTB cases. The lower levels of SOD, CAT and TAS may be improved by the antioxidant therapy which may help in better prognosis. Also, anti TNF-α therapy may help in decreasing the elevated level of TNF-α shown in all PTB patients.

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