EVALUATION OF LIPID PEROXIDATION PRODUCT, NITRITE AND ANTIOXIDANT LEVELS IN NEWLY DIAGNOSED AND TWO MONTHS FOLLOW-UP PATIENTS WITH PULMONARY TUBERCULOSIS

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Abstract. This case-control study followed by a longitudinal cohort study was undertaken to evaluate the level of lipid peroxidation product malondialdehyde (MDA) and nitrite as an indirect measurement of nitric oxide vis-à-vis the levels of antioxidants vitamin C and vitamin E in pulmonary tuberculosis. Fifty-six sputum smear-positive cases of pulmonary tuberculosis based on Ziehl-Neelsen (ZN) staining and 50 healthy controls without any systemic disease were included in this study. Thirty-five cases were longitudinally followed up with standard antituberculosis chemotherapy (ATT) for two months. Serum levels of malondiadehyde (MDA), nitrite, and plasma levels of vitamins C and E were measured. The mean serum MDA level was significantly higher (8.1 ± 1.61 nmoles/ml) in PTB patients before commencement of ATT as compared to healthy controls (3.45 ± 1.7 nmoles/ml) (p=0.0001) and decreased significantly after 2 months of ATT (3.84 ± 1.28 nmoles/ml) (p=0.0001). The mean serum nitrite level (47.19 ± 18.44 μmol/l) was significantly elevated before ATT compared to healthy controls (32.89 ±11.94 μmoles/l) and decreased significantly after 2 months of ATT (27.71 ± 11.97 μmoles/l) (p=0.0001). The mean plasma levels of vitamins C (0.88 ± 0.33 mg/dl) and E (0.79 ± 0.24 mg/dl) in PTB patients before commencement of ATT were lower than healthy controls (1.42 ± 0.38 mg/dl) and (1.35 ± 0.35 mg/dl), respectively (p=0.001). There was a significant increase in vitamin C levels after 2 months of ATT (1.19 ± 0.40 mg/dl) compared to before ATT (0.83 ± 0.31 mg/dl) (p=0.0001), but no significant change in mean plasma vitamin E level before and after 2 months on ATT was found. Elevated malondialdehyde and nitrite levels with concomitant depressed vitamin C and E levels are indicative of lipid peroxidation and oxidative stress. The decrease in levels of malondialdehyde and nitrite with subsequent increase in vitamin C levels after two months of follow-up indicate a good response to treatment with standard ATT. Hence, the extent of oxidative stress in PTB can be evaluated by analyzing lipid peroxidation product, antioxidant and nitric oxide levels.

INTRODUCTION

Nearly one third of the global population (2 billion people) is infected with *Mycobacterium tuberculosis* (WHO, 2003) and an estimated 8.5 million people develop active TB disease each year resulting in approximately 2 million deaths. Mycobacteria are intracellular pathogens which grow and replicate in host macrophages. After phagocytosis survival of mycobacteria depend on their ability to avoid destruction by macrophages. The main candidates for the direct killing of *M. tuberculosis* are granulolysin produced by T-cells and ni-

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tric oxide and superoxide radicals produced by activated macrophages (Stenger and Modlin, 1999; Chan *et al*, 2001).

Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) are produced to fight foreign organisms especially through membrane bound NADPH oxidase as a consequences of phagocyte respiratory burst (Babior *et al*, 1973). The generation of lipid peroxides indicates the extent of lipid peroxidation in general and serves as marker of cellular damage due to free radicals. The most reactive and harmful ROS is the hydroxyl radical ($OH^{\bullet}$) which can be formed from hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$) alternatively, the reaction of superoxide with nitric oxide (NO) produces peroxynitrite ($OONO^-$), which decomposes to form NO_2 and $OH^{\bullet}$. NO plays an important role in host defense and homeostasis. The biological outcome of the NO mediated effects is complex and depends on the internal and external environment of the target and generation sites of the cells as well as the concentration of nitric oxide radical ($NO^{\bullet}$) generated.

Oxidative stress results when reactive oxygen species are not adequately removed and can lead to peroxidation of membrane lipids, depletion of nicotinamide nucleotides, rise in intracellular Ca^{2+} ions, cytoskeleton disruption and DNA damage (Halliwell and Aruoma, 1991). These ROS and RNI induce lipid peroxidation (LP), a chain process which affects unsaturated fatty acids mainly localized in cell membranes leading to generation of malondialdehyde (Wallis and Ellner, 1994). Lipid peroxidation products (LPPs) diffuse from the site of inflammation enters in circulation and can be measured in the blood.

Vitamin C is the most important extracellular antioxidant and has a crucial role in protection against lipid peroxidation. It scavenges $O_2^{\bullet -}$, H_2O_2 and thiol radicals and is a potent quencher of singlet oxygen (Vijayamalini and Manoharan, 2004). Vitamin E is a lipid soluble antioxidant that can convert $O_2^{\bullet -}$, $OH^{\bullet}$, and lipid peroxyl radicals to less reactive forms. Vitamins E, C and β-carotene protect cells against ROS produced during normal metabolism and after an oxidative insult. Antioxidant defense systems work cooperatively to improve the oxidative stress caused by enhanced free radical production. Any change in one of these systems may break this equilibrium and cause cellular damages. Vitamin C spares reduced glutathione (GSH) and together with vitamin E prevents the oxidation of GSH.

MATERIALS AND METHODS

Study population

The study population comprised of 56 patients with sputum smear-positive by Ziehl-Neelsen (ZN) staining for acid-fast bacilli newly diagnosed pulmonary tuberculosis patients attending the medicine out-patient department of B. P. Koirala Institutes of Health Sciences (BPKIHS), Dharan, Nepal, 50 healthy controls without any systemic diseases visiting from the same endemic area, and 35 follow-up patients on standard chemotherapy with isoniazid (INH 5 $mgkg^{-1}day^{-1}$), pyrazinamide (PZA, 25 $mgkg^{1}day^{-1}$), rifampicin (RFP, 10 mg $kg^{-1}day^{-1}$) and ethambutal (ETH, 15 mg $kg^{-1}day^{-1}$) daily for two months. Informed consent was obtained from the participants of this study and was conducted as per the ethical guidelines set by the Institute Research and Ethical Board of BPKIHS, Dharan, Nepal.

Blood collection

Fasting blood samples (6 ml) were collected from antecubital venipuncture. Four milliliters of blood were kept in a glass tube with anticoagulant and 2 ml in a plain glass tube without anticoagulant. Serum and plasma were separated by centrifugation (Remi Research centrifuge model R-23) at 2,500 rpm for 15 minutes at room temperature, collected in clean, dry sterile vials and stored at -20ºC.

Serum MDA estimation

Estimation of MDA in the serum was done by the method of Yagi (1987). The color produced by the reaction of thiobarbituric acid with MDA was measured spectrophotometrically (Shimadzu UV-1201 Spectrophotometer) at 533 nm. The results were expressed as nmoles/ml.

Serum nitric oxide determination

Serum nitric oxide was measured in terms of its product nitrite by the method of Griess (Green *et al,* 1982). This method is based on a two-step process. The first step is the conversion of nitrate to nitrite using cadmium metal granules and the second is the addition of sulphanilamide and N (-naphthyl) ethylenediamine (Griess reagent). This converts nitrite into a deep purple azo compound, which was measured spectrophotometrically at 540 nm. Nitric oxide products were expressed as μmoles/l.

Determination of plasma ascorbic acid

Determination of ascorbic acid was done in plasma by the method described by Sullivan and Clarke (1955) which depends on the reduction of ferric ion to ferrous ion by ascorbic acid as red-orange,α,α′-dipyridal complex. In the presence of orthophosphoric acid at a pH of 1-2 other reducing or interfering materials, *eg* reduction, glucosone, reductic acid, α-tocopherol, glutathione, cysteine, acetol, methyl glyxol, or creatinine, are inhibited. With this simple method it is possible to run a large number of samples in less than 2 hours, and with proper equipment it is possible to measure as small as 0.1 μg of ascorbic acid in as little as 0.01 ml of sample size.

Plasma vitamin E estimation

Vitamin E was estimated in plasma by the method of Bieri *et al* (1964), which is based on the reduction of ferric to ferrous ions by tocopherol, which then forms a red colored complex with 2,2′-dipyridyl that is read at 520 nm. The level of vitamin E was expressed as mg/dl.

Other estimations

Hemoglobin was estimated by the method of Drabkin and Austin (1932). The hemoglobin was oxidized to methemoglobin with alkaline cyanide reagent to produce a brown colored compound.

Total protein was estimated by the method of Reinhold (1953). This method is based on the reaction of Biuret reagent with peptide bonds of proteins. The violet color produced was measured at 540 nm.

Serum albumin was estimated by the method of Doumas *et al* (1971) in which BCG binds to albumin and gives a yellowish green colored compound which is measured colorimetrically at 630 nm.

Data analysis

The data were analysed by SPSS version 10. The results were expressed as mean ± SD. Statistical comparisons were carried out using the Student's *t*-test for unpaired data. The Student's paired *t*-test was used to compare data in pre-treatment and post-treatment patients. The null hypothesis was rejected for $p<0.05$.

RESULTS

Table 1 shows the basic anthropometric measurements of healthy individuals and the patients with pulmonary tuberculosis. The mean age (±SD) (40.11 ± 15.33 yrs), height (158.43 ± 7.16 cms), weight (45.61 ± 8.06 kg) and BMI (18.22 ± 2.93 kg/m^2) of the pulmonary tuberculosis patients were compared to the controls mean age (36.22 ± 13.30 yrs), height (160.48 ± 7.86 cms), weight (57.92 ± 6.72 kg) and BMI (22.52 ± 3.83 kg/m^2). There were highly significant differences in weight and BMI between these two groups ($p<0.001$), however, age and height were not significantly different between the two groups. The control group (n=50) consisted of 27 males and 23 females and the pulmonary tuberculosis cases (n=56) consisted of 34 males and 22

females.

Table 2 shows the levels of serum MDA, nitrite and plasma vitamins C and E in the control subjects and pulmonary tuberculosis patients. The extent of lipid peroxidation as evidenced by serum MDA was significantly higher in the pulmonary tuberculosis patients ($p<0.0001$) than in the control subjects. The serum nitrite levels were significantly higher in the pulmonary tuberculosis group ($p<0.001$) than the control group. The plasma levels of vitamin C and E, as indicators of antioxidants, were significantly lower in the pulmonary tuberculosis patients ($p<0.0001$).

Table 3 shows the levels of serum MDA, nitrite, plasma vitamins C and E in the control subjects, and in the pre- and post-treatment pulmonary tuberculosis patients. The extent of lipid peroxidation, as evidenced by the serum MDA level was significantly lower in the post-treatment pulmonary tuberculosis patients ($p<0.0001$) than the pre-treatment pulmonary tuberculosis patients. The level of serum nitrite was found to be significantly lower in the post-treatment pulmonary tuber-

Table 1
Basic anthropometric measurements of healthy individuals and patients with pulmonary tuberculosis (values are mean ±SD).

Variables	Controls (n=50)	Cases (n=56)
Age (yrs)	36.22 ± 13.3	40.11 ± 15.33
Sex		
Male	27 (54%)	34 (61%)
Female	23 (46%)	22 (39%)
Height (cms)	160.48 ± 7.86	158.43 ± 7.16
Weight (kg)	57.92 ± 6.72	45.61 ± 8.06[a]
BMI (kg/m^2)	22.52 ± 3.83	18.22 ± 2.93[a]

[a] $p<0.0001$

Table 2
Levels of serum MDA, serum nitrite and plasma vitamins C and E in healthy individuals and patients with pulmonary tuberculosis (values are mean ± SD).

Parameters	Controls (n=50)	Cases (n=56)
Serum MDA (nmoles/ml)	3.45 ± 1.70	8.10 ± 1.61[a]
Serum nitrite (μmoles/l)	32.89 ± 11.94	47.19 ± 18.44[b]
Plasma vitamin C (mg/dl)	1.42 ± 0.38	0.88 ± 0.33[a]
Plasma vitamin E (mg/dl)	1.35 ± 0.35	0.79 ± 0.24[a]

[a]$p<0.0001$, [b]$p<0.001$

Table 3
Levels of serum MDA, serum nitrite and plasma vitamins C and E in healthy individuals, pre-treatment and post-treatment pulmonary tuberculosis patients at follow-up after standard chemotherapy for two months (values are expressed as mean ± SD).

Parameters	Controls (n=50)	Pre-treatment (n=35)	Post-treatment (n=35)
Serum MDA (nmoles/ml)	3.45 ± 1.7	8.37 ± 1.55	3.84 ± 1.28 [a,c; NS,d]
Serum nitrite (μmoles/l)	32.89 ± 11.94	49.58 ± 19.80	27.71 ± 11.97 [a,c; NS,d]
Plasma vitamin C (mg/dl)	1.42 ± 0.38	0.83 ± 0.31	1.19 ± 0.40 [a,c; b,d]
Plasma vitamin E (mg/dl)	1.35 ± 0.35	0.85 ± 0.33	0.94 ± 0.32 [NS,c; a,d]

[a]$p<0.0001$, [b]$p<0.01$, NS-non significant
[c]Comparison between pre-treatment and post-treatment
[d]Comparison between control and post-treatment

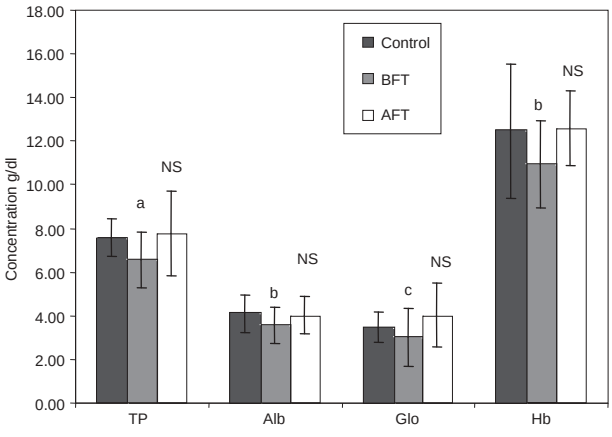

Fig 1–Levels of total protein (TP), albumin(Alb), globulin (Glo) and hemoglobin (Hb) in healthy individuals compared with pre-treatment and post-treatment pulmonary tuberculosis patients after two months of standard chemotherapy respectively (significance levels: a=p<0.001, b=p<0.005, c=p<0.05 and NS=non significant, p>0.05).

culosis group (p<0.0001) than the pre-treatment pulmonary tuberculosis group. The level of plasma vitamin C was significantly lower in the pre-treatment pulmonary tuberculosis patients (p<0.0001) than the post-treatment pulmonary tuberculosis patients. No significant difference in plasma vitamin E levels were found between the pre-treatment and post-treatment groups. A significantly lower plasma vitamin C level was found in the post-treatment pulmonary tuberculosis group (p<0.01) than the control group. A significantly lower plasma vitamin E level was found in the post-treatment pulmonary tuberculosis group than the control group (p<0.0001) without any significant differences in the serum MDA and nitrite levels.

Fig 1 shows the higher levels of total protein, albumin, globulin and hemoglobin in the controls, as compared to pre-treatment and post-treatment pulmonary tuberculosis patients. Levels of total protein, albumin, globulin and hemoglobin were found to be lower in the pre-treatment group, which gradually increased after 2 months of standard antitubercular chemotherapy.

DISCUSSION

Pulmonary tuberculosis, a disease associated with a wide range of respiratory symptoms, is socially and economically very cumbersome to control. Although tuberculosis morbidity and mortality has decreased to low levels in developed countries, it still remains one of the most common causes of morbidity and mortality in developing countries.

Mycobacteria are intracellular pathogens which grow and replicate in host macrophages. After phagocytosis survival of mycobacteria depends on their ability to avoid destruction by macrophages. Phagocytes (neutrophils, macrophages and monocytes) undergo respiratory burst after contact with microorganisms. These cells possess the capacity to generate huge amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are essential for the destruction of ingested microorganisms and also contribute to inflammatory injury to host tissue. Inflammation-related oxidative stress has been implicated in the pathogenesis of lung fibrosis and dysfunction in patients with pulmonary tuberculosis (Kwiatkowska *et al,* 1999).

There was a significantly low BMI in pulmonary tuberculosis patients than in the controls, which is consistent with the significantly lower weights of the patients groups. There was a higher level of MDA in the pulmonary tuberculosis patients than the controls, which decreased significantly after two months of treatment with standard chemotherapy. This result suggests that after two months of treatment there is a significant reduction in ROS generation and the extent of lipid peroxidation is diminished by chemotherapeutic destruction of mycobacteria. Reddy *et al* (2004) reported a significantly lower MDA concentra-

tion and higher antioxidant levels in patients with clinical improvement after chemotherapy. Similarly, Jack *et al* (1994) reported that several circulating markers of free radical activity were increased in pulmonary tuberculosis patients and in some of them it remain elevated even after completion of chemotherapy, which may contribute to the development of lung functional abnormalities.

The immune activation and enhancement of oxidative stress may be accountable for the interaction and destruction of ingested microorganisms contributing to inflammatory injury of the host tissue. MacMicking *et al* (1997) have recently demonstrated that among mice with the genetic inability to produce inducible nitric oxide synthase (i-NOS) replication of *Mycobacteria tuberculosis* is faster than in wild type animals. Serum levels of nitrite (NO_2^-) and nitrate (NO_3^-) are used to estimate the level of $NO^\bullet$ formation, since $NO^\bullet$ is highly unstable and has a very short half-life. Chronic inflammation can lead to the production of $NO^\bullet$, which in turn has the potential to mediate DNA damage directly or indirectly through the generation of more persistent RNS (Li and Hotchkiss, 1995). Schon *et al* (2004) reported the presence of i-NOS and nitrotryramine (Ntyr) reactive macrophages in granulomas, including Langerhans giant cells, indicating high out-put $NO^\bullet$ production at the primary site of disease in tuberculosis. The present study showed an initial higher level of nitric oxide, which may be accounted for killing of *M. tuberculosis* by mononuclear phagocytes. Lower $NO^\bullet$ levels in post-treatment may be due to a reduction in microbial load.

The levels of vitamins C and E were significantly reduced in patients compared to healthy controls. There was a significant increase in vitamin C levels in patients after two months of standard chemotherapy. Ascorbate is the first antioxidant to be depleted upon exposure to both environmental and inflammatory oxidants suggesting that it is the ultimate antioxidant either by directly scavenging these oxidants or trapping their intermediates. However, there was no significant increase found in vitamin E levels post-treatment at two months. Moreover, there were still significantly lower levels of vitamin C and vitamin E in the post-treatment group than the healthy controls. This may imply that supplementation with vitamins C and E may be beneficial in PTB patients for fast recovery of disease but further interventional trials are required to test this theory (Reddy *et al*, 2004). Some studies have reported that antituberculosis drugs induce formation of reactive oxygen species. Thus, patients with poor antioxidant mechanisms are at a greater risk for drug toxicity (Walubo *et al*, 1994; Chowdhary *et al,* 2001). Others studies have shown that antimicrobial chemotherapy supplemented with antioxidants improves cure rates, reduces the period of treatment, and shortens the period for cavity closure in patients with tuberculosis (Safarian *et al*, 1990). Vitamin E scavenges lipid peroxy free radicals and interrupts the chain reaction of lipid peroxidation, becoming oxidized itself in the process. Ascorbic acid present in aqueous compartments (*eg* cytosol, plasma and other body fluids) functions as a water soluble chain-breaking antioxidant, converts the tocopheroxyl radical back to active tocopherol, thereby replenishing the antioxidant activity of vitamin E (Winkler *et al*, 1994). It has been demonstrated that vitamin E acts as mobilizable antioxidant, being released from tissues stores and diverted to the lungs of PTB patients during oxidative stress radical mediated pulmonary fibrosis (Chow *et al*, 1993). The lipid peroxides formed at the primary site may be transferred through the circulation to other organs and tissues and provoke damage by propagating lipid peroxidation (Gutteridge, 1995). Therefore, high MDA concentrations and low levels of non-enzymatic antioxidant vitamins C and E may indicate depletion of

antioxidants due to excessive lipid peroxidation by ROS in PTB patients.

Some biochemical parameters were measured in PTB patients before and after two months of chemotherapy. There were significantly lower levels of total protein, albumin, globulin and hemoglobin in PTB patients than in controls. The combination of undernourishment with decreased supplementation of antioxidants which enhances ROS generation leading to increased utilization of these compounds represents a pathogenic loop that may result in markedly enhanced oxidative stress during pulmonary tuberculosis infection (Madebo *et al, 2*003). There were significantly higher levels of total protein, globulin and hemoglobin in PTB patients post-treatment than pre-treatment. This shows that parameters eventually increased as there was clinical improvement in patients after two months of chemotherapy. The higher lipid peroxide level had a toxic effect causing an elevated ROS produced by immune system during respiratory burst. There may have been synchronized release of ROS during hemoglobin degradation causing lower hemoglobin levels in the pre-treatment PTB patients. The increase in globulin level post-treatment may be accounted for by an antibody response boosted by the breakdown products of dead mycobacteria without necessarily playing a role in protection.

There was slight negative correlation between MDA and vitamins C and E levels observed in present study, which may have been due to the involvement of other enzymatic and non-enzymatic antioxidants besides vitamins C and E. The antioxidative micronutrients vitamins C and E have been reported in several studies to regulate the production and/or reactivity of phagocyte-derived free radicals (Anderson, 1991). A reduced level of serum vitamin C has been reported in patients with pulmonary tuberculosis (Awotedu *et al*, 1984).

The elevated levels of cytokines and enhanced free radical production, although designed to combat the invader, has the potential to damage host tissue (Kuo *et al*, 1996). However, the host tissue damage is limited by concurrent enhancement of antioxidant defense in the host. The present study shows that poor antioxidant defense may cause oxidative stress and host tissue damage, which is consistent with a number of other studies (Grimble, 1994; Jack *et al*, 1994; Plit *et al*, 1998; Evans and Halliwell, 2004).

The present study showed enhanced oxidative stress in patients diagnosed with pulmonary tuberculosis. Lower antioxidant levels of vitamins C and E and higher lipid peroxidation product MDA with activated immune response as indicated by nitrite levels show marked oxidative stress in patients compared to control subjects. With two months of intensive anti-tuberculosis treatment, there was lower levels of lipid peroxidation and nitrite and increased levels of antioxidants. Though a significant increase in vitamin E was not seen, this should be studied in future prospective with interventional trials.

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