Comparative Study of Impact of Smoking in Healthy and Pulmonary Tuberculosis patients measured by Indices of Oxidative Stress

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Abstract

Pulmonary tuberculosis (PTB) infection possesses the capacity to generate toxic free radicals for the destruction of microorganisms and inflammatory injury. This study was undertaken to see severe impact produced due to oxidative stress in PTB patients as a consequence of smoking habit. This prospective study included confirmed cases of Smoker PTB (n=25) and Non smoker PTB (n=31) from the Tuberculosis Centre of B.P. Koirala Institute of Health Science, Dharan, Nepal. Age and sex matched Healthy Smoker (n=25) and Healthy Non-smoker Control (n=31) from the same endemic area were also enrolled. Serum Malondialdehyde (MDA), serum Nitrite, Plasma Vitamin C & Vitamin E levels were assayed from study groups and are used as indices of oxidative stress. The one way analysis of variance (ANOVA) revealed the mean MDA and Nitrite levels were higher among smoker PTB in comparison to Healthy Smokers and Non Smokers groups ($p<0.0001$). Moreover, Vitamin C & E were significantly higher in Non smokers compared to Healthy Smokers and Smoker PTB ($P<0.0001$). Among Smokers, the MDA and Nitrite levels were higher with concomitant lower level of Vitamin C & E in PTB patients compared to without PTB. These observations reflect increased oxidative stress which interplays between smoking and PTB infection.

KEYWORDS: Pulmonary tuberculosis, Smoker, Non smoker, oxidative stress, antioxidants

Introduction:

Tuberculosis is a bacterial disease caused by tubercle bacilli which includes mostly *Mycobacterium tuberculosis.* Globally, approximately 16 million people are suffering from active tuberculosis (TB) disease, with estimated 8.5 million persons developing active TB disease each year resulting in approximately 2 million deaths.[1] Estimated cases of TB in Nepal are 47,315 and estimated new smear positive TB being 21,245.[2]

Today, about one in three adults, or 1.2 billion people, smoke and of these, about 82 percent lives in low-and middle-income countries. Overall 29 percent of the world adults smoke. Gender wise more men (47%) smoke than their female counterparts (12%). In contrast fewer men (39%) but about one in five women (22%) smoke in the high-income countries. In low/middle income

countries nearly half (49%) adult men smoke and about 9 per cent women do so.[3] Active smoking is significantly associated with TB infection and disease while second hand smoking is associated with tuberculosis infections in children and younger people.[4, 5] Up to one in five deaths from TB could be avoided if patients were not smokers.[6] Smoking attributable annual deaths for Nepal is estimated at nearly 14,000 (9,000 male deaths and 5,000 female deaths) for population aged 35 and over.[3]

Free radicals in smoke are present both in the gas phase and in the particulate phase (tar). The inhaled gas components contain approximately 10^{15} free radicals per puff.[7] Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are oxidants which are highly toxic to all types of biological molecules including DNA, lipids, proteins and carbohydrates. The end product of the lipid peroxidation process is malondialdehyde (MDA) as well as the antigenic challenge producing nitric oxide measured in terms of nitrite whose significant presence in serum indicates the tissue damage. Antioxidants are compounds that are involved in effective scavenging of free radicals and in suppressing the actions of ROS and RNS. Vitamin E and C are most effective natural free radical scavengers available from dietary sources. The present study was undertaken to study severe impact produced due to oxidative stress in pulmonary tuberculosis patients as a result of smoking habits. The serum MDA, nitrite and Vitamins E & C levels were used as indices of oxidative stress. It was therefore hypothesized that in pulmonary tuberculosis there is altered oxidant and antioxidant status in association with smoking habit.

Materials and Methods:

The study population comprised of:

1. Thirty one (31) newly diagnosed Non smokers PTB patients with sputum smear-positive on Ziehl Neelsen (ZN) staining, attending medicine out-patient department of B. P. Koirala institutes of health sciences (BPKIHS), Dharan, Nepal.
2. Twenty five (25) newly diagnosed Smoker PTB patients with sputum smear-positive on Ziehl Neelsen (ZN) staining, attending medicine out-patient department of B. P. Koirala institutes of health sciences, Dharan, Nepal.
3. Thirty two (32) Healthy Non smokers (Control) without any systemic diseases visiting from same endemic area.
4. Eighteen (18) Healthy Smokers without any systemic diseases visiting from same endemic area.

Non-smokers did not have exposure to active and passive smoking. The smoking history was evaluated by the unit a “pack-year” which was defined as smoking of a pack of 20 cigarettes or 80 beedies per day for one year.[8] The number of pack-years was calculated as:

$$\frac{\text{The number of cigarettes smoked per day}}{20} \times \text{the number of years smoked}$$

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.

Sample collection and processing: Blood sample (6ml) was collected through antecubital venipuncture. 4ml of blood was kept in a glass tube with anticoagulant and 2ml in a plain glass tube without anticoagulant. Serum and plasma were separated by centrifuging (Remi Research centrifuge model R-23) at 2500 rpm for 15 minutes at room

temperature. Plasma and serum were separated into clean dry and sterile vial and stored at -20°C.

Estimation of Malondialdehyde: MDA in serum was estimated by employing the method of Yagi.[9] The color produced by the reaction of thiobarbituric acid with MDA was measured at 533 nm using Shimadzu, UV-1201 Spectrophotometer. The results were expressed as nmoles/ml serum.

Estimation of nitrite: Serum nitric oxide (NO) was measured in terms of its products nitrite (NO_2) by the method of Griess.[10] 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/dl serum.

Estimation of ascorbic acid: Ascorbic acid in plasma was estimated by method described by Sullivan[11] which depends on the reduction of ferric ion to ferrous ion by ascorbic acid as red-orange,α,α'-dipyridal complex. In presence of orthophosphoric acid at pH 1-2, other reducing or interfering material, e.g., reduction, glucosone, reductic acid, α-tocopherol, glutathione, cysteine, acetol, methyl glyxol, or creatinine, are inhibited. The level of vitamin C was expressed as mg/dl serum.

Estimation of Vitamin E: Vitamin E in plasma was estimated by the method of Bieri[12] 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 serum.

Estimation of Total Protein, Albumin & Globulin: Total protein is estimated by the Biuret method[13] based on Copper binding to peptide bonds in alkaline condition measured in 540 nm. Similarly, Albumin is measured by Bromo Cresol Green (BCG) dye binding method[14] in acidic condition measured in 630 nm. Globulin is calculated indirectly by deducting albumin from total protein value. The unit of expression for these variables is g/dl.

Estimation of Hemoglobin: Hemoglobin is estimated by Drabkin's method[15] based on the formation of methaemoglobin with alkaline cyanide to produce brown color compound reagent which is measured at 540 nm.

Statistical analysis of data: The data were analyzed by using SPSS version 17. The results were expressed as mean ± SD. Statistical comparisons were carried out using Student's *t*-test, one way analysis of variance (ANOVA) and Pearson's correlation. The null hypothesis was rejected for $p<0.05$.

Results:

Table 1 shows basic anthropometric measurement of study population. The mean age (±SD) (40.11 ± 15.33 yrs), height (1.58 ± 0.07 m), weight (45.61 ± 8.60 Kg) and BMI (18.22 ± 2.93 Kg/m^2) of Non smoker PTB patients (n=32, M:F=18:14) was compared to that of Healthy Non smoker Control (n=31, M:F=16:15) age (35.59 ± 3.59 yrs), height (1.60 ± 0.08 m), weight (57.92 ± 2.32 Kg) and BMI (22.52 ± 3.83 Kg/m^2) respectively. There were

significant differences in weight and BMI between these two groups ($p< 0.001$). Similarly, The mean age (±SD) (40.80 ± 14.29 yrs), height (1.56 ± 0.06 m), weight (42.11 ± 9.27 Kg) and BMI (17.30 ± 2.32 Kg/m^2) of smoker PTB patients (n=25, M:F=18:7) was compared to that of Healthy Smoker (n=18,M:F=13:5) age (37.33 ± 12.17 yrs), height (1.61 ± 0.06 m), weight (52.67 ± 4.23 Kg) and BMI (20.31 ± 3.18 Kg/m^2) respectively. There were significant differences in weight and BMI between these two groups ($p<0.001$). PTB smokers had a higher smoking habit (28.00 ± 6.00 pack-years) than healthy smokers without PTB (8.00 ± 2.00 pack-years) ($p<0.0001$).

Table 2 shows the level serum MDA, nitrite, plasma vitamins C & E in study population as indices of oxidative stress. The mean serum MDA (±SD) (8.15 ± 1.82 nmol/ml), Nitrite (31.22 ± 10.64 µmol/dl), plasma Vitamin C (0.93 ± 0.34 mg/dl) and Plasma Vitamin E (0.81 ± 0.23 mg/dl) of Non smoker PTB patients (n=31, M:F=18:14) was compared to that of Healthy Non smoker Control (n=32, M:F=16:15) serum MDA (3.30 ± 1.63 nmol/ml), Nitrite (47.58 ± 18.67 µmol/dl), plasma Vitamin C (1.42 ± 0.37 mg/dl) and Vitamin E (1.39 ± 0.29 mg/dl) respectively. There were significant differences in MDA, Nitrite, Vitamin C and Vitamin E between these two groups ($p<0.001$). Similarly, The mean serum MDA (±SD) (8.13 ± 1.37 nmol/l), Nitrite (47.58 ± 18.67 µmol/dl), Vitamin C (0.93 ± 0.34 mg/dl) and Vitamin E (0.81 ± 0.23 mg/dl) of Smoker PTB patients (n=25, M:F=18:7) was compared to that of Healthy Smoker (n=18,M:F=13:5) MDA (3.71 ± 1.83 µmol/dl), Nitrite (49.58 ± 19.80 µmol/dl), Vitamin C (1.41 ± 0.39 mg/dl) and Vitamin E (1.27 ± 0.34 mg/dl) respectively. There were significant differences in MDA, Nitrite, Vitamin C and Vitamin E between these two groups ($p<0.001$). The one way analysis of variance (ANOVA) revealed the mean MDA and Nitrite levels were higher among Smoker PTB in comparison to Healthy Smoker and Non-smoker groups ($p<0.0001$). Moreover, Vitamin C & E were significantly higher in Non-smoker compared to Smoker PTB and Non smoker PTB ($P<0.0001$). The nitrite was found to be still significant between Healthy non smoker and healthy smoker ($p<0.001$) and also in between Non smoker PTB and Smoker PTB ($p<0.001$).

Table 3 shows the nutritional status of study population. The mean serum Total protein (±SD) (6.70± 1.33 g/dl), serum Albumin (3.50 ± 0.93 g/dl), serum Globulin (3.21 ± 1.53 g/dl) and Hemoglobin (10.71 ± 1.81 g/dl) of Non smoker PTB patients (n=31, M:F=18:14) was compared to that of Healthy Non smoker Control (n=32, M:F=16:15) serum Total protein (7.40 ± 0.91 g/dl), Albumin (4.12 ± 0.93 g/dl), Globulin (3.56 ± 0.67 g/dl) and Hemoglobin (12.15 ± 3.07 g/dl) respectively. There were significant differences in Total protein, albumin and hemoglobin between these two groups ($p<0.01$). The mean serum Total protein (±SD) (6.32 ± 1.26 g/dl), Albumin (3.60 ± 0.77 g/dl), Globulin (2.72 ± 1.03 g/dl) and Hemoglobin (10.91 ± 2.24 g/dl) of Smoker PTB patients (n=25, M:F=18:7) was compared to that of Healthy Smoker (n=18,M:F=13:5) Total protein (7.83 ± 0.72), Albumin (3.98 ± 0.73 g/dl), Globulin (3.28 ± 0.77 g/dl) and Hemoglobin (12.96 ± 3.10 g/dl) respectively. There were significant differences in Total protein, Albumin

and Hemoglobin between these two groups ($p<0.01$). Total protein, Albumin and Hemoglobin level decreases in Smoker and Non smoker PTB patients compared to Healthy Non smoker Control ($p<0.05$).

Table 4 shows the Pearson's correlation among indices of oxidative stress. The significant negative correlation was observed between MDA and Vitamin C, MDA and Vitamin E, MDA and Albumin, Nitrite and Vitamin C, Nitrite and Vitamin E. Moreover, the significant positive correlation was observed between Vitamin C and Vitamin E, Nitrite and MDA, Vitamin C and hemoglobin, Vitamin C and albumin and Vitamin E and albumin.

Discussion:

Tobacco smoking is the solid etiological factor for the accelerated decline in the lung function.[16] Pathophysiology of tobacco smoking associated TB may be related to several possible mechanisms, such as impairment in the immune response, CD4 lymphopenia[17], hormonal imbalances, disruption of cilia function[18], morphological and functional changes in alveolar macrophages[19],so it may be a causal factor for an individual to be easily infected with mycobacterium tuberculosis leading to pulmonary tuberculosis. However, the exact pathophysiology of smoking associated tuberculosis is still not well understood. The immune activation and enhancement of oxidative stress may be accountable for the interaction and destruction of ingested microorganisms contributing to inflammatory injury to the host tissue. Jack et al reported that several circulating markers of free radicals activity were increased in pulmonary tuberculosis patients and in some of them it remain elevated even after completion of chemotherapy that might contribute to the development of lung functional abnormalities.[20] Lung is a vulnerable organ to oxidant damage because of its location, anatomy and function.[21] Lungs epithelium is constantly exposed to oxidants generated internally as a part of normal metabolism as well as to oxidants in the ambient air, microbes, cigarette smoke etc.

In our earlier study, the serum MDA and nitrite were significantly increased and vitamin C & E were significantly reduced in patients with PTB.[22] In the present study, Healthy Smoker and Smoker PTB had higher MDA & Nitrite levels compared to Healthy Non-smoker Control and Non smoker PTB cases. Among smokers, the MDA and Nitrite levels were higher in PTB patients compared to without PTB. These observations reflect enhanced lipid peroxidation because of oxidative stress due to smoking. The significant negative Pearson's correlation was observed between oxidants and antioxidants.

The present study showing initial increased level of nitric oxide may be accounted for killing *M. tuberculosis* by mononuclear phagocytes. Nitric oxide (NO), a free radical physiologically produced by the organism following antigenic stimulation, is also present in cigarette smoke. This compound reacts quickly with the superoxide anion ($O_2^{-\cdot}$) to form peroxynitrite and with peroxyl radicals to form alkyl peroxynitrites.[7] The significant difference in nitrite level in present study in the healthy non smoker and healthy smoker ($p<0.001$) and in between PTB Non-smoker and PTB smoker ($p<0.001$) could be due direct presence of nitric oxide related

compounds in the smoke besides PTB infection.

Uzun et al have shown diagnostic role of MDA by measuring its level in the serum of patients with lung diseases with various etiologies also found significantly higher MDA level than control.[23] ROS have also been suggested to play a role in smoking induced diseases such as COPD[24, 25] and in addition to that human lung fibroblast recruit in respond to smoke extract, which may suggest that ROS have a role in other cigarette smoke associated fibrotic lung disease.

Similarly in the present study the mean level of Vitamin C & E were significantly lower in smokers compared with non smokers. Moreover, among smokers the mean Vitamin C & E were decreased in PTB patients as compared to without PTB. Antioxidants are involved in effective scavenging of free radicals and in suppressing the action of ROS.[26] Ascorbic acid is the first antioxidant to be depleted upon exposure to both environmental and inflammatory oxidants suggesting that it is the ultimate antioxidants either by directly scavenging these oxidants or trapping their intermediates. It may, therefore, imply that supplementation of vitamin C and E could be beneficial for PTB patients for fast recovery of disease but further interventional trials are required to approve this fact.[27] Further research is required to see the effect smoking in smoker and non-smoker PTB patients with antioxidants supplementation to know the association between smoking habit and PTB.

There were significantly lower levels of total protein, albumin and hemoglobin in smokers PTB patients than in control. The combination of undernourishment with decreased supplementation of an antioxidants which enhances ROS generation leading to increased utilization of these compounds represent a pathogenic loop that may result in markedly enhanced oxidative stress during pulmonary tuberculosis infection.[28] Therefore high MDA concentrations and low level of non-enzymatic antioxidant vitamin C and E respectively may indicate depletion of antioxidants due to excessive lipid peroxidation by ROS and RNS in PTB patients due to oxidative stress produced by mycobacterium tuberculosis nevertheless, smoking as well**.**

Conclusion:

Smoking of cigarette is one of the important factors for oxidative stress which interplay between pulmonary tuberculosis and smoking habits.

Conflict of Interest:

The authors have declared that there is no conflict among all authors and no compelling interest exist.

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Table 1: Basic anthropometric measurement and smoking status of study population (Values are Mean ± SD)

Variables	Healthy Non smoker (Control) (n=32)	Healthy smoker (n=18)	Non smoker PTB (n=31)	Smoker PTB (n=25)	p-value one way ANOVA
Age (years)	35.59 ± 3.59	37.33 ± 12.17	40.09 ± 16.09	40.80 ± 14.29	0.485
Sex (M)	18	13	16	18	0.084
(F)	14	5	15	7	
Height (m)	1.60 ± 0.08	1.61 ± 0.06	1.58 ± 0.07	1.56 ± 0.06	0.073
Weight (kg)	57.92 ± 6.72	52.67 ± 4.23	45.61 ± 8.06	42.11 ± 9.27	0.0001
BMI (Kg/m^2)	22.52 ± 3.83	20.31 ± 3.18	18.22 ± 2.93	17.30 ± 2.32	0.0001
Smoking habit (pack-years)	--	8.00 ± 2.00	--	28.00 ± 6.00	0.0000

Table 2: Level of serum MDA, Nitrite and plasma Vitamins C & E, Indices of oxidative stress of study population (Values are expressed in Mean ± SD)

Variables	Healthy Non smoker (Control) (n=32)	Healthy smoker (n=18)	Non smoker PTB (n=31)	Smoker PTB (n=25)	p-value one way ANOVA
MDA (nmol/ml)	3.30 ± 1.63	3.71 ± 1.83	8.15 ± 1.82	8.13 ± 1.37	0.0001
Nitrite (µmol/dl)	32.89 ± 11.94	49.58 ± 19.80	31.22 ± 10.64	47.58 ± 18.67	0.001
Vitamin C (mg/dl)	1.42 ± 0.37	1.41 ± 0.39	0.86 ± 0.32	0.93 ± 0.34	0.0001
Vitamin E (mg/dl)	1.39 ± 0.29	1.27 ± 0.34	0.77 ± 0.23	0.81 ± 0.23	0.0001

Table 3: Level of serum Total protein, Albumin, Globulin and Hemoglobin, the nutritional status of study population (Values are expressed in Mean ± SD)

Variables	Healthy Non smoker (Control) (n=32)	Healthy smoker (n=18)	Non smoker PTB (n=31)	Smoker PTB (n=25)	p-value one way ANOVA
Total Protein (g/dl)	7.40 ± 0.91	7.83 ± 0.72	6.70± 1.33	6.32 ± 1.26	0.0001
Albumin (g/dl)	4.12 ± 0.93	3.98 ± 0.73	3.50 ± 0.93	3.60 ± 0.77	0.023
Globulin (g/dl)	3.56 ± 0.67	3.280 ± 0.77	3.21 ± 1.53	2.72 ± 1.03	0.043
Hemoglobin (g/dl)	12.15 ± 3.07	12.96 ± 3.10	10.71 ± 1.81	10.91 ± 2.24	0.01

Table 4: Pearson's Correlation among indices of oxidative stress and nutritional parameter

Parameters	MDA	Nitrite	Vitamin C	Vitamin E	Albumin	Hb
MDA	1	0.34*	-0.55**	-0.62**	-0.2*	-0.15
Nitrite	0.34*	1	-0.23*	-0.31*	-0.11	0.1
Vitamin C	-0.55**	-0.23*	1	0.41**	0.41*	0.25**
Vitamin E	-0.62**	-0.31*	0.41**	1	0.33**	0.13
Albumin	-0.2*	-0.11	0.41**	0.33**	1	0.04
Hb	-0.15	-0.15	0.25**	0.13	0.04	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).