OXIDATIVE STRESS AND ANTIOXIDANTS IN TUBERCULAR MENINGITIS

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ABSTRACT

Oxidative stress is implicated in the etiopathogenesis of a variety of human diseases. Therefore, in the present study, erythrocyte lipid peroxidation, percentage hemolysis, antioxidant enzymes viz., glutathione reductase, glutathione peroxidase, superoxide dismutase and plasma antioxidants viz., ceruloplasmin, vitamins A, E and C have been determined in 19 patients with tubercular meningitis (TBM) and 50 normals. Six patients who were treated with antibiotics were considered for the follow up. The statistical analysis was carried out by Mann Whitney U test and Wilcoxon rank sum test. Lipid peroxidation ($P < 0.02$), percentage hemolysis ($P < 0.001$) and plasma ceruloplasmin ($P < 0.0001$) of TBM patients were significantly higher, whereas erythrocyte glutathione reductase ($P < 0.05$) and plasma antioxidant vitamins A, E and C ($P < 0.01$, $P < 0.05$ respectively) were significantly lower than those of the controls. In the follow up patients the glutathione reductase and catalase levels were significantly high ($P < 0.05$) compared to their pre-treated condition. Vitamin C and E levels have attained normal range. This study indicated that the blood antioxidant status of TBM patients which was low compared to controls improved after treatment, suggesting the role of free radicals in TBM.

KEY WORDS

Antioxidant enzymes, antioxidant vitamins, lipid peroxidation, tubercular meningitis.

INTRODUCTION

Tubercular meningitis (TBM) is one of the most common type of neurological disorders found in India and other socioeconomically deprived countries. Mycobacterium tuberculosis infection leads to the inflammation of the duramater resulting in TBM. The bacteria infecting mammals are dealt by polymorphonuclear leukocytes which produce bacteriocidal agents like superoxide and other

oxidants like hydroperoxy radicals and hydrogen peroxide (H_2O_2) after engulfment of bacteria (1). Formation of superoxide ions by respiratory burst has been observed not only in macrophages and neutrophils but also in the microglial cells of the brain. During infections of the central nervous system (CNS), microglia are known to be stimulated and migrate to area of injury where they phagocytize debris (2).

Under pathological conditions much larger amounts of oxygen free radicals are formed than normal and these can overwhelm the defences of the cell and lead to lipid peroxidation and even death of the cell (3,4). To mitigate the harmful effects of free radicals, all aerobic cells are endowed with extensive antioxidant defence

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mechanisms which include vitamin A, vitamin E, vitamin C, glutathione (GSH), and enzymes viz., glutathione reductase (GR, E.C.1.6.4.2), glutathione peroxidase (GP, E.C.1.11.1.9), superoxide dismutase (SOD, E.C.1.15.1.1) and catalase (CT, E.C.1.11.1.6) (5).

Accumulating evidence implies that reactive oxygen species produced by polymorphonuclear leukocytes and possibly other sources may contribute to the complex pathogenesis of the diseases associated with inflammation (6). Since very few reports are available to indicate the changes of antioxidant status in meningitis, the present work aims at studying antioxidant parameters in blood of TBM patients, then note variation in their levels before and after therapy.

MATERIALS AND METHODS

Nineteen patients with TBM were studied. The results were compared with those of 50 age and sex matched controls. Lumbar puncture was performed and CSF analysis was done to confirm meningitis. The diagnostic criteria of TBM are as follows: Clinical symptoms and signs such as fever, persistent headache, neck stiffness, vomiting, focal deficit, mental changes and confusion. Supporting evidence obtained by examination of CSF included the presence of ≥ 20 cells/ μ l, predominantly lymphocytes, protein concentration > 1 mg/ml, glucose less than 60% of corresponding blood glucose. In those few patients who presented with focal signs on neurologic examinations, CT scan was obtained prior to lumbar puncture. All the procedures followed were in accord with the ethical standards on human experimentation. All the patients considered for the study were prescribed treatment with a combination of isonix (300 mg/day) and rifampicin (450 mg/day) for a period of one year and pyrazinamide (1500 mg/day) for a period of two months. Posttreatment studies were performed in six of the above patients after fifteen days therapy. Random blood samples were collected in EDTA bottles from normal subjects and TBM patients. Blood was centrifuged at 3000g for 10 minutes. Plasma was separated, buffy coat was carefully removed and separated erythrocytes washed thrice with 0.01M saline phosphate buffer pH 7.4 (containing 0.15M NaCl), then diluted 1:2 with the same buffer. The haemoglobin content of the erythrocytes was determined by

cyanmethemoglobin method. Erythrocyte enzymes were estimated in appropriately diluted hemolysates prepared by addition of distilled water. Erythrocyte GR and GP activity was determined by recording the decrease in absorbance due to depletion of NADPH for a period of 5 minutes at 340nm (7,8). SOD was determined according to the method of Beauchamp and Fridovich (9) based on inhibition of nitroblue tetrazolium reduction. CT activity in the hemolysate was determined by the method of Brannan *et al.* (10). The assay is based on the disappearance of H_2O_2 in the presence of enzyme source at 26°C. The lipid peroxidation and oxidative hemolysis of RBC were determined by incubating RBC suspension in saline phosphate buffer containing 0.44M H_2O_2 at 0 hour and 2 hours. Aliquots were withdrawn from the above mixture at 0 hour and at 2 hours for studying both these parameters (11,12). Lipid peroxidation in RBC was determined by estimating malondialdehyde (MDA) produced using thiobarbituric acid (11).

Plasma ceruloplasmin (E.C.1.16.3.1) was determined by its p-phenylene diamine oxidase activity (13). Plasma alpha tocopherol was measured by Emmerie Engel reaction given by Bieri *et al.* (14). Vitamin A was determined by reading the extinction at 327nm before and after exposure to UV light (15). Plasma vitamin C was determined chemically using dinitrophenyl hydrazine as a colour compound (16).

Data was analysed statistically by Mann Whitney U test and Wilcoxon rank sum test. The differences of $P < 0.05$ were considered significant.

RESULTS

Erythrocyte lipid peroxidation in TBM patients before and after treatment was significantly high after 2 hours of incubation of RBC with H_2O_2 compared to controls. The oxidative hemolysis values in both pretreated and posttreated TBM patients were significantly increased over control subjects (Table 1, Table 2).

GR activity decreased significantly in TBM patients compared to normals. The enzyme activity increased significantly in posttreated patients when compared to corresponding pretreated subjects (Table 3, Table 4). A comparison of erythrocyte

GP, SOD and CT levels in TBM patients to that of control showed no significant change. However, CT activity was significantly high in posttreated patients compared to their pretreated condition (Table 3, Table 4).

Plasma ceruloplasmin was significantly high both in pretreated and posttreated patients compared to controls (Table 3, Table 4). The mean vitamin A, E and C levels were significantly low compared to controls. Though vitamin E and C levels in the follow up cases have increased to attain normal range, vitamin A tended to decrease further and has attained a significantly low value compared to normals (Table 3, Table 4).

DISCUSSION

As a defence against invading microorganisms in normal phagocytosis, cytotoxic oxygen species are produced to destroy them (17,18). Erythrocytes might act as "sinks" for H_2O_2 and superoxide ions produced. Rosly *et al* (19) have reported an increase in serum lipid peroxidation products in patients with meningococcal meningitis which correlated with the severity of the disease. Increased lipid peroxidation in blood was observed in pneumococcal infection. (20) The results of present study are also in agreement with these findings, where both lipid peroxidation and percentage hemolysis in TBM patients increased significantly compared to normal. Lipid peroxidation may be a major factor that causes alterations of biophysical properties of membrane thereby leading to hemolysis.

At physiological rates of H_2O_2 generation the GP/GSH/GR system is more important in catabolising H_2O_2 in RBC and leukocytes (21). This system is very important in the protection of phagocytic leukocytes against their own products. In the present study, erythrocyte GR activity in TBM is significantly low compared to controls. Decreased GR activity may be a predominant cause for GSH depletion within the RBC leading to serious consequences like increased lipid peroxidation and hemolysis. A similar observation was made by earlier workers also. Decrease in GR activity and a rise in GP activity was reported in thrombocytes of patients with meningococcal meningitis (22). Prasnikova *et al* (23) demonstrated

an imbalance in redox system of GSH in RBC during the disease period in meningococcal infections and meningitis. However, in this study other antioxidant enzymes viz., GP, SOD and CT remained unchanged. In the present study, the plasma ceruloplasmin level of TBM patients showed a pronounced increase compared to controls. This observation is similar to those made by Immanuel *et al* (24) who reported high serum ceruloplasmin levels in patients with pulmonary tuberculosis, abdominal tuberculosis and TBM. The ferroxidase action of ceruloplasmin allows it to inhibit iron dependent lipid peroxidation. Ceruloplasmin also binds copper ions and can inhibit copper stimulated hydroxy radical formation and lipid peroxidation (25).

Regarding vitamins, the 2 groups under this study belong to a homogenous population with no difference in their cultural or socio-economic status. Hence, nutritional differences between the 2 groups are minimal. Observations in the present study indicate a significant decrease in plasma antioxidant vitamins A, E and C in TBM patients compared to controls. One of the earlier reports indicate a low vitamin E level in serum of patients with recurrent infections (26). A significant decrease in CSF vitamin C has been reported in cases of purulent meningitis in children (27). H_2O_2 in combination with ascorbate (28) or certain metal ions serve as microbicidal agents in phagocytic leukocytes apart from superoxide. Van den Bergh *et al* (29) have reported synergistic role of vitamin E and C in protection against peroxidation in human erythrocyte membrane. Vitamin C can restore the antioxidant properties of oxidized vitamin E by recycling vitamin E radical (30). In the present study decrease in plasma vitamin C may be due to increased utilization for bacteriocidal activity, which in turn may have lead to decreased vitamin E regeneration.

Six TBM patients who received anti-tubercular drugs as treatment were considered for followup studies at the end of fifteen days. The RBC GR activity in the post-treated TBM patients rose significantly compared to their pretreatment condition. The enzyme activity reached normal range. This indicates that the RBC is regaining its antioxidant capacity to overcome oxidative stress. A significantly increased CT activity in post treated condition may suggest an increased H_2O_2

production. A possible cause for increased H_2O_2 production may be the action of rifamycin, which gets oxidized in presence of transition metal ions to give superoxide ions(1). Dismutation of superoxide ions lead to production of H_2O_2 . Biological importance of H_2O_2 arises from its ability to diffuse through hydrophobic membranes with relative ease (1). Protective action of CT has been illustrated by Pfister *et al* (31) in an experimental rat model of pneumococcal meningitis. Though plasma ceruloplasmin activity has gradually decreased in post treated TBM patients in the present study, it still remains significantly high compared to that of normals. This finding agrees with one of the earlier reports on tuberculosis patients, where the ceruloplasmin level which was high at the time of admission decreased from 78% to 50% at the end of treatment (24).

Free radical reactions have been suggested to be involved in the toxic effects produced by several antibiotics (1). As the blood antioxidants have come back to normal, increased *in vitro* lipid

peroxidation of RBC and oxidative hemolysis found in post treated TBM patients may be attributed to the side effects of antibiotics. Many antibiotics have a propensity to oxidize and produce reactive intermediates capable of damaging the molecular structure of cells.

Plasma vitamin C and E in the post treated TBM patients have increased compared to their pretreated condition. Frei *et al* (32) reported that pathologically relevant lipid hydroperoxide formation consequent to acute or chronic leukocyte activation can be prevented by ascorbate supplementation. Both vitamin E and C have attained normal range in post treated TBM patients.

The results of the above study indicate that a disturbed prooxidant antioxidant balance in TBM patients tend to return to normal after treatment. Furthermore, almost similar changes were observed in our earlier study in cases of epilepsy, a neurological disorder (33).

Table 1
ERYTHROCYTE LIPID PEROXIDATION AND HEMOLYSIS LEVELS IN TBM
(Mean $\pm$ SEM)

	Normal (n=50)	TBM (n=19)
MDA, (n mol/ dl RBC)		
0 hour	1426 $\pm$ 86	1649 $\pm$ 131
2 hour	3159 $\pm$ 193	4479 $\pm$ 498 ^c
% Hemolysis		
0 hour	1.47 $\pm$ 0.19	3.46 $\pm$ 0.69 ^b
2 hour	8.18 $\pm$ 0.79	15.46 $\pm$ 2.33 ^a

n = Sample size

^aP < 0.001, ^bP < 0.01, ^cP < 0.02 significantly different from normal
(Mann-Whitney U test)

Table 2

FOLLOW UP STUDIES OF *IN VITRO* LIPID PEROXIDATION AND PERCENTAGE
HEMOLYSIS IN TBM (MEAN $\pm$ SEM)

		Before treatment (n=6)	After treatment (n=6)
MDA, (n mol/ dl RBC)	0 hour	1812 $\pm$ 390	1930 $\pm$ 266
	2 hour	4749 $\pm$ 689	4973 $\pm$ 799 ^b
% Hemolysis	0 hour	4.98 $\pm$ 2.26	4.97 $\pm$ 1.27 ^a
	2 hour	19.79 $\pm$ 4.70	18.80 $\pm$ 6.30

n = Sample size

^aP < 0.02, ^bP < 0.05 significantly different from normal

(Mann-Whitney U test and Wilcoxon rank sum test)

TABLE 3

COMPARISON OF ANTIOXIDANTS IN TBM PATIENTS AND CONTROLS

(Mean $\pm$ SEM)

	Normal (n=50)	TBM (n=19)
Erythrocyte GR (μ mol NADPHoxidised/min/g Hb)	1.08 $\pm$ 0.07	0.74 $\pm$ 0.13 ^c
Erythrocyte GP (μ mol NADPHoxidised/min/g Hb)	7.94 $\pm$ 0.47	8.47 $\pm$ 0.99
Erythrocyte SOD (U/g Hb)	5235 $\pm$ 239	4548 $\pm$ 342
Erythrocyte CT (U/g Hb)	148550 $\pm$ 16686	129585 $\pm$ 18714
Plasma ceruloplasmin (mg/dl)	17.47 $\pm$ 0.86	32.30 $\pm$ 2.43 ^a
Plasma vitamin A (μ g/dl)	70.14 $\pm$ 6.3	34.86 $\pm$ 6.40 ^b
Plasma vitamin E (mg/L)	10.56 $\pm$ 0.69	6.84 $\pm$ 0.59 ^b
Plasma vitamin C (μ mol/L)	28.02 $\pm$ 2.12	19.23 $\pm$ 2.30 ^c

n = Sample size

^aP < 0.001, ^bP < 0.01, ^cP < 0.05 significantly different from normal

(Mann-Whitney U test)

Table 4

COMPARISON OF ANTIOXIDANTS IN TBM PATIENTS BEFORE AND AFTER TREATMENT

(Mean $\pm$ SEM)

	Before treatment (n=6)	After treatment (n=6)
Erythrocyte GR (μ mol NADPHoxidised/min/g Hb)	0.67 $\pm$ 0.15	0.92 $\pm$ 0.17*
Erythrocyte GP (μ mol NADPHoxidised/min/g Hb)	11.67 $\pm$ 2.54	11.54 $\pm$ 1.06
Erythrocyte SOD (U/g Hb)	4723 $\pm$ 586	4248 $\pm$ 525
Erythrocyte CT (U/g Hb)	95716 $\pm$ 14709	154738 $\pm$ 12619*
Plasma ceruloplasmin (mg/dl)	39.90 $\pm$ 5.60	29.67 $\pm$ 3.97 ^a
Plasma vitamin A (μ g/dl)	26.50 $\pm$ 10.94	31.33 $\pm$ 5.48 ^b
Plasma vitamin E (mg/L)	7.89 $\pm$ 1.04	9.93 $\pm$ 1.47
Plasma vitamin C (μ mol/L)	17.35 $\pm$ 2.48	31.52 $\pm$ 8.11

n = Sample size

^aP < 0.01, ^bP < 0.02 significantly different from normal

(Mann-Whitney U test)

*P < 0.05, significantly different from pre-treatment values

(Wilcoxon rank sum test).

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