Erythrocyte functional status and lipid profile of coal mine workers of West Bengal, India

Prasad SK.[1], Bhattacharjee A.[1], Bandyopadhyay P.[2], Pal S.[3], Syamal AK.[4], Mukherjee S.[1*]

[1] Department of Physiology, Serampore College, West Bengal, India
[2] Department of Physical Education, Kalyani University, Kalyani, Nadia, India
[3] Indian Institute of Chemical Biology, Kolkata, India
[4] Department of Physiology, Hooghly Mohsin College, Hooghly, India

ABSTRACT

Purpose: Despite people suffering from several forms of ill health, constant exposure to toxic wastes and chronic diseases as a result of mining, there is a tragic gap in the availability of 'scientific' studies and data on the health hazards of mining in India. This study was proposed to understand better the relationship between occupational exposure to coal, blood lipid profiles and red blood cell (RBC) functional status of coal workers.

Materials and methods: Blood samples were obtained from coal miners (n=32) of an underground mine in West Bengal. Blood lipid profiles and RBC functional status were determined. Students' *t*-test and Pearson correlation analyses were completed to analyze the data.

Results: Compared to the control subjects, significantly higher levels of cholesterol ($p<0.01$), triglycerides ($p<0.01$), LDL ($p<0.001$), and VLDL ($p<0.001$) were observed in coal miners. HDL, Hb, Na^+-K^+ATPase and SOD activity were significantly ($p<0.001$) lower in coal miners, whereas MDA levels ($p<0.001$) and osmotic fragility in coal miners were increased significantly ($p<0.01$).

Conclusions: Our study indicates that elevated MDA and antioxidant insufficiency caused disruption in the structural integrity of erythrocyte, which may be a pathophysiological mechanism in the progression of disease in coal miners. Also, cardiovascular disease risk factors were more prevalent in the coal miners.

Keywords: Coal miners, blood lipid profiles, erythrocyte osmotic fragility, oxidative stress, antioxidant insufficiency

* Corresponding author:
Dr Sandip Mukherjee
Department of Physiology, Serampore College
9 William Carey Road, Serampore, Hooghly – 712201
West Bengal, India
e-mail: sm_kdc@yahoo.co.in

INTRODUCTION

Mining is an ancient occupation, long recognized as being arduous and liable to injury and disease [1]. The lifecycle of mining consists of exploration, mine development, mine operation, decommissioning and land rehabilitation. Mining is a multi-disciplinary industry, drawing on several professions and trades. To ensure precision in clinical and epidemiological work, it is important to enquire about the details of tasks, as the term 'miner' is relatively non-specific.

An area of major concern in considering increased coal production and utilization is the health and safety of those who mine coal or subsequently process coal. Greatly increased production of coal in the United States under either the National Energy Plan or business as usual will expose larger numbers of workers to the health and safety hazards of coal mining. Such hazards in the past have been serious resulting in many mine-related accidental deaths and disabling injuries. Disability and death from chronic lung disease among coal miners have also been excessive [2].

Underground coal miners experience increased mortality from both occupationally induced lung diseases and accidental deaths. In addition, there is increasing concern in regard to a possible increased risk of death from stomach cancer among underground coal miners. Coal workers' pneumoconiosis and associated chronic bronchitis, emphysema, and airways obstruction, affect over a third of our currently employed underground coal miners. Surface miners have been found to have substantially less respiratory disease than underground coal miners. Two newly emphasized coal mining technologies, the use of diesel powered mining equipment and the use of long wall mining techniques, may be associated with potentially serious health effects. Coal-fired power plants are also associated with exposure to noise and respiratory disease risk from exposure to coal dust, SO_2, NO_2, and asbestos [2].

Coal mine dust is a heterogeneous and complex mixture of more than 50 organic and inorganic compounds and their oxides. Minerals contained in coal dust include silicates, oxides, carbonates, sulfites, and sulfates. A number of particles are respirable, and therefore increase the risks of disease in coal miners [3]. The diseases among the coal miners do not only comprise pulmonary disorders, such as pneumoconiosis, progressive fibrosis, chronic bronchitis, and

accelerated loss of pulmonary function [4] but also cardiovascular disease (CVD) and cytotoxicity.

Coal dust has been reported to produce reactive oxygen species (ROS), a critical factor for cytotoxicity [5]. Basically, two mechanisms by which coal dust exposure causes formation of ROS in vivo have been proposed: 1) the formation of ROS by intrinsic properties of the particles, i.e. non-cellular mechanisms, and 2) the excessive formation of ROS by the oxidative burst of macrophages and neutrophils activated during particle phagocytosis and persistent inflammation [6].

Oxidative stress is defined as a condition marked by an imbalance between free radicals and antioxidants on the cellular or individual level. Oxidative damage is one of the results of this imbalance, comprising oxidative modification of cellular macromolecules [7]. It has long been recognized that ROS are harmful for cells, because they injure lipids, thiol proteins or nucleic acids, which leads to structural and functional impairments. Intrinsic properties of particles and iron content of coal dust and the oxidative burst of macrophages and neutrophils activated during phagocytosis and persistent inflammation may be the plausible mechanisms by which coal dust exposure causes formation of ROS [8, 9].

Atherosclerosis is the underlying cause of heart attack, stroke and peripheral vascular disease. It is a major cause of morbidity and mortality worldwide. In addition, it has been demonstrated that an increased intracellular generation of ROS plays an important role in chronic inflammatory responses to atherosclerosis [10,11].

A study of the current health and safety record in coal mines and projections based upon this experience to estimate what might happen if increased coal production is not accomplished by adequate measures to protect workers are of concern. It is clear that strong preventive measures are necessary to reduce health and safety risks of miners, particularly as our reliance upon coal as an energy source increases in future years.

MATERIALS AND METHODS

Selection of subjects
Thirty two healthy miners from an underground mine of West Bengal, India were selected following a random sampling technique stratified based on the age. The subjects having a minimum work experience of five years were accustomed to work in heat. They had no report of

medical history as confirmed by the respective health centers of the coal mines. The miners selected were engaged in three different mining works, namely, drilling, shoveling, and carrying. The choice of miners from these three categories of work was based on the fact that these works demand a significant working time at a stretch in the allocated working areas; secondly, these activities can be quantified in terms of work output and finally these activities were supposed to be physically demanding. They had a mean age of 44.98 years (SD: 3.77). Thirty two age matched control subjects (mean age 44.80 years, SD: 3.50) were recruited from the same coal mines who were mostly office workers and did not have any exposure to underground mine environment. Before the study, interactions were carried out with the miners where they were explained about the objective of the study and the extent of their involvement. The selected miners agreed to render them voluntarily in accordance with the design of the experiment. This study was approved by the Human Ethics Committee of Serampore College. Written informed consent was obtained from each participant of this study.

Measurement of different physiological variables

Height (cm), weight (kg), resting heart rate, systolic and diastolic blood pressure were measured using standard procedure. Body Surface Area (B.S.A.) and Body Mass Index (B.M.I.) were calculated.

Sample collection and processing

Blood samples were taken from 32 coal miners working in the coal-mining industry and 32 healthy subjects. All of them were non smokers. Five ml of fasting venous blood sample (fasting time was > 12 hours) were taken from each donor before going to work. All samples were taken by venapuncture with anticoagulant (EDTA) evacuated tubes. Plasma was separated for measurement of lipid profile parameters. Then, plasma and rest of the samples were transferred to the laboratory within 4 hours using the ice bucket. In laboratory, centrifugation was performed at 4°C (10 min, 3000 rpm) and measurements were performed in erythrocyte and erythrocyte membrane.

Preparation of erythrocyte membrane

The blood was centrifuged at 2,000 g for 15 minutes to separate plasma. The layer of white blood cells above the packed red blood cells was removed, and discarded. The red blood cells were washed two times with Tris buffered saline (TBS), i.e., 20 mM Tris-HCl, pH 7.5 containing 145 mM NaCl by centrifuging at 2,000 g for 15 minutes. The washed packed red blood cells were lysed by diluting 10 fold in 10 mM Tris-HCl buffer. The lysed cells were kept

on ice for 15 minutes, followed by centrifugation at 12,000 g for 20 minutes. The supernatant was discarded, and the pellet was washed with lysis-buffer until the membrane became white.

Na^+- K^+ ATPase assay

ATPase activity was measures in a final volume of 1 ml as follows: Membranes (400ug) were preincubated for 10 min. at 37°C in a mixture containing 92 mmoL –HCl (pH=7.4), 100 mmol NaCl, 20 mmol KCl, 5 mmol, $MgSO_4.H_2O$ and 1 mmol EDTA. Assays were performed with or without 1 mmol Ouabain, a specific inhibitor of Na^+-K^+-ATPase. After incubation with 4 mmol ATP (Vanadate free, Sigma) at 37°C for 10 min. The reaction was stopped by adding of ice-cold trichloroacetic acid to a final concentration of 5%. After centrifugation at 4°C, 5500g for 10 min, the amount of inorganic phosphate in the supernatant was determined. Na^+- K^+ ATPase activity was calculated as the difference between inorganic phosphate released during the 10-minute incubation with and without ouabain. The phosphorus content in the supernatant was measured using Fiske and Subbarow [12].

MDA determination

In the sample of erythrocyte membrane, malondialdehyde (MDA) level was determined using the method of Draper and Hadley [13] based on the reaction of MDA with thiobarbituric acid (TBA) at 95°C. In the TBA test reaction, MDA and TBA react to form a pink pigment with an absorption maximum at 532 nm. The reaction was performed at pH 2-3 at 95°C for 15 min. The sample was mixed with 2.5 volumes of 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with 0.67% TBA in a boiling water-bath for 15 min. After cooling, the absorbance was read at 532 nm. Results were expressed as micromole per gram hemoglobin.

SOD activity determination

Total (Cu-Zn and Mn) SOD activity was determined according to the method of Sun et al. [14] and a slightly modified method by Durak et al. [15] and is based on the inhibition of NBT reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the lysate after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was also expressed as Units per gram hemoglobin.

Protein determination

The protein content of erythrocyte membrane was determined by the method of Lowry et al. [16].

Hemoglobin (Hb) measurement

Hemoglobin concentrations in blood as well as in erythrocytes were determined with the hemoglobin cyanide procedure [17]. In this method, cyanide and ferricyanide in an alkaline medium convert hemoglobin to the highly colored cyanme-themoglobin derivative measured spectrophotometrically at 540 nm. In brief, 0.02ml of whole blood or erythrocyte suspension and 5.0 ml of Drabkin solution were taken in a test tube, mixed well and allowed to stand for at least 15 minutes at room temperature (18-26°C). Then absorbance was read spectrophotometrically at 540 nm.

Erythrocyte osmotic fragility

Erythrocyte osmotic fragility was determined according to the method described by Oyewale (1992) [18]. Briefly, 0.02 ml of blood was added to tubes containing increasing concentration of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4 (0, 0.2, 0.3, 0.5, 0.7, 0.8 and 0.9%). The tubes were gently mixed and incubated at room temperature (29°C) for 30 minutes. The content in each tube was then centrifuged at 1500 rev/min for 10 minutes and supernatant decanted. Optical density of the supernatant was determined spectrophotometrically at 540 nm. Haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0% NaCl) as 100%.

Estimation of lipid profile

Total cholesterol, triglyceride, HDL, LDL and VLDL were measured using biochemical kits obtained from Accurex Private Limited, Mumbai, India.

Statistics

The results were analyzed for means and standard deviations with Statsdirect 2.7.2., a statistical package for Windows. The significance of difference in the mean between groups was calculated using two tail Student's *t*-test for unpaired samples. The Pearson correlation analyses were performed to test the type and significance of relations among selected parameters. A p value < 0.05 was considered statistically significant.

RESULTS

Different physical and physiological parameters of 32 control subjects and 32 coal miners were presented in Table 1. Both weight and height were higher but statistically insignificant in coal miners compared to control subjects. Similarly, body

surface area (BSA) and body mass index (BMI) were also found to be higher but statistically insignificant. Statistically significant higher resting heart rate (p<0.01), systolic blood pressure (p<0.05) and diastolic blood pressure (p<0.01) were observed among the coal miners compared to their control counterpart.

Erythrocyte membrane fluidity depends on Na^+-K^+ ATPase activity, which is reduced in hypertensive patients. In our study, the activities of Na^+-K^+ ATPase in the erythrocyte membrane was decreased significantly (p < 0.001) by 37.02% in the coal mine workers as compared to the control group (Figure 1). SOD activities, an anti-oxidative enzyme, was also found to be significantly (p < 0.001) lower in coal miners by 41.88 % as compared to control subjects (Figure 1).
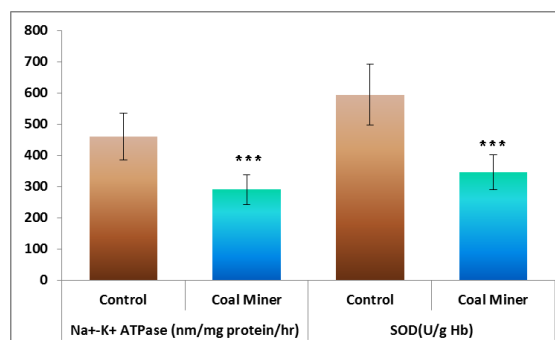

Figure 1. Erythrocyte membrane Na^+ K^+ ATPase (nm/mg protein/hr) and erythrocyte SOD activity (U/g Hb) of control and coal mine workers. Significance level based on Student's *'t'* test. ***p<0.001

The Figure 2 indicates MDA levels, which is an end product of fatty acid oxidation, and is often used as an indicator of lipid peroxidation, was significantly (p < 0.001) increased (39.41%) in the coal mine workers as compared to the control group.
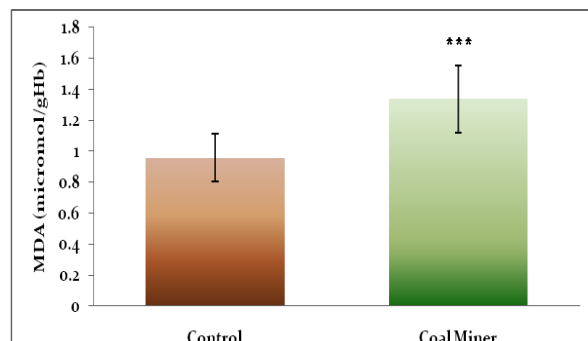

Figure 2. Malondialdehyde level (micromole/g Hb) of controls and coal mine workers. Significance level based on Student's *'t'* test. *** p < 0.001.

Hemoglobin level of the control group and coalmine workers was presented in Table 1. The hemoglobin

level in the control group was significantly (p< 0.001) higher (12.29 %) than the coal mine workers.

Table 1. Physical, physiological variables and haemoglobin level of controls and coal mine workers.

Parameters	Control (*n*=32)		coalminer (*n*=32)		Significance level
	Mean	SD	Mean	SD	P value
AGE (yrs)	44.12	3.33	44.24	3.67	p>0.05
WEIGHT (Kg)	63.26	10.38	68.88	12.07	p>0.05
HEIGHT (m)	1.60	0.071	1.63	0.072	p>0.05
Heart rate (beats/min)	72.59	7.56	80.24	8.24	p<0.001
SBP(mm Hg)	122.94	12.09	132.06	10.08	p<0.01
DBP (mm Hg)	75.76	7.26	83.71	7.53	p<0.001
BMI (Kg/m^2)	24.69	2.93	24.96	3.05	p>0.05
BSA (m^2)	1.68	0.20	1.70	0.162	p>0.05
Haemoglobin level (g/dL)	14.89	0.98	13.26	0.87	p<0.001

* Significance level based on Student's *t*-test

The Figure 3 represents the results of osmotic fragility measurement for erythrocyte collected from control group and coalmine workers, where the percentage of hemolyzed cells has been plotted as a function of the percentage concentration of NaCl. It is clear from the Figure 4 that there was a significant increase (p <0.01) in the haemolysis of RBCs of coal mine workers as compared with the control group.
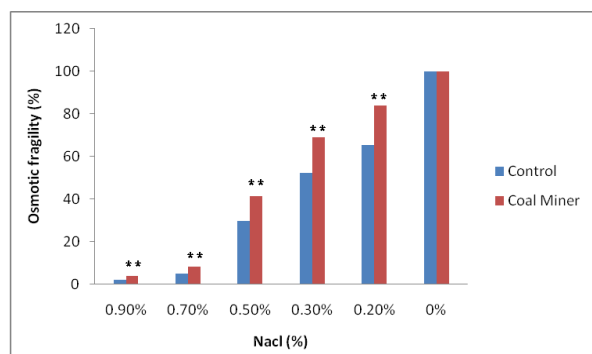

Figure 3. Osmotic fragility of control and coal mine workers. Significance level based on Student's '*t*' test. ** p < 0.01.

The Figure 4 shows lipid profile of control group and coalmine workers. The level of total cholesterol and triglycerides were found to be significantly (p< 0.01) higher (14.23 % and 19.76% respectively) in coal mine workers. Similarly, LDL and VLDL were also significantly (p<0.001 and p<0.001, respectively) higher (27.87% and 26.13%, respectively) in coal mine workers compared to control subjects.

Coal mine workers showed 14.69% lower value as compared to the control group which was statistically significant (p<0.001). The relation between the tested parameters of lipid profile with that of erythrocyte Na$^+$ - K$^+$ ATPase activity, MDA level and SOD activity of both control subjects and coal mine workers is shown in Table 2. Total cholesterol and triglyceride showed significant negative correlation with erythrocyte Na$^+$ - K$^+$ ATPase activity (r= - 0.52 and r= -0.51, respectively) and SOD activity (r=- 0.59 and r= -0.54, respectively) and insignificant but positive correlation with MDA level (r= 0.40 and r= 0.41, respectively).

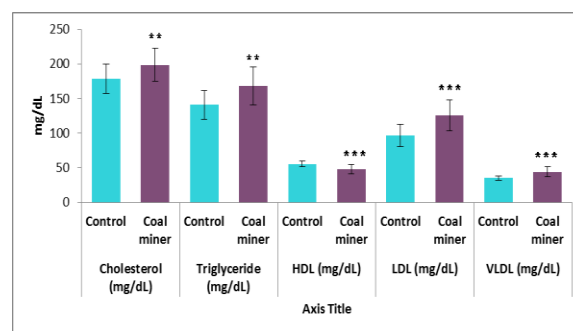**Figure 4.** Lipid profile of control and coal mine workers. Significance level based on Student's '*t*' test. ** p < 0.01, *** p < 0.001

LDL and VLDL had strong positive relation with MDA levels in coal mine workers (LDL: r=0.68; VLDL: r=0.64). In coal mine workers, LDL had significant negative relation with erythrocyte Na$^+$ - K$^+$ ATPase activity (r= -0.50) and insignificant negative relation with SOD activity (r= -0.33) whereas VLDL had insignificant negative relation with erythrocyte Na$^+$ - K$^+$ ATPase activity (r= -0.33) and significant negative relation with SOD activity (r= -0.58).

Table 2. Pearson correlation matrix in between lipid profile parameters with Na$^+$-K$^+$ ATPase and Oxidative stress parameters of coal mine workers.

Parameters	Worker (*n*=32)					
	Na$^+$-K$^+$ ATPase		MDA		SOD	
	r value	P value	r value	p value	r value	p value
Cholesterol	-0.52	0.0159	0.40	0.0543	-0.59	0.0071
Triglyceride	-0.51	0.0199	0.41	0.0509	-0.54	0.0134
HDL	0.41	0.0531	-0.52	0.0178	0.40	0.0566
LDL	-0.50	0.0209	0.68	0.0016	-0.33	0.0955
VLDL	-0.33	0.0955	0.64	0.0032	-0.58	0.0079

HDL had insignificant but direct relation with erythrocyte Na$^+$ - K$^+$ ATPase activity (r= 0.41) and SOD activity (r= 0.40). Whereas HDL had a insignificant inverse (negative) relation with MDA level (r= -0.26) in control subjects and significant positive relation with MDA level (r= -0.52) in coal mine workers.

DISCUSSION

The prevalence of occupational health hazards and mortality has been reported to be unusually high among people of India. In different occupations, several hazardous substances contaminate the work environment. In different industries and mines the nature of the small inhalable particles are different and causing different types of occupational diseases.

In this study it is evident that coalminers and control group of subjects had no significant association in terms of weight, height, BSA and BMI. So, in this study both groups of subjects were standardized in terms of weight, height, BSA and BMI. Further on analyzing the physical characteristics of the subjects, it was observed that the mean BMI value of both control and coal miners were within the normal range of BMI (Normal BMI: 18-25 kg/m^2), as per classification. In addition, heart rate and systolic and diastolic blood pressure were significantly higher in coal miners compared to control (Table 1).

Red blood cells (RBC) precipitate intraplaque hemorrhage, which leads to plaque instability. Large extra cellular cholesterol crystals in unstable atherosclerotic plaques come from the membrane cholesterol of senescent RBC after intraplaque hemorrhage [19]. Circulating RBC are able to dampen vessel oxidative damage by means of their antioxidant machinery (glutathione), a physiological role closely related to their ability to uptake, carry and release nitric oxide (NO), thus enhancing local vasodilatation and prevention of ischemia [20]. However, when local inflammatory and oxidative stimulus is strong enough to surpass their capability, RBC behave as "oxidative bullets", extending the oxidative damage by ONOO- transport [21].

The present study demonstrates that erythrocyte MDA levels are increased in a group of coal miners when compared to control group. Erythrocytes are more vulnerable to lipid peroxidation due to constant exposure to high oxygen tension and richness in polyunsaturated fatty acid [22]. The observed increase in lipid peroxidation in the present study might be due to increased oxidative stress caused by the coal dust induced generation of free radicals and increased formation of lipid hydroperoxides or due to other products, as reported by others [8]. Peroxidation of membrane lipids not only alters the lipid milieu and structural as well as functional integrity of cell membranes, but also affects the activity of various membrane-bound enzymes, including Na$^+$-K$^+$ ATPase [23]. Since the Na$^+$-K$^+$ ATPase is an essential enzyme of the plasma membrane of animal cells, it has been suggested to represent an important target of ROS induced membrane damage. ROS are known as to inhibit Na$^+$-K$^+$ ATPase activity. Decrease in erythrocyte Na$^+$-K$^+$ ATPase activity (Fig. 1) associated with increased lipid peroxidation (Fig. 2) in our study, which disturbs the phospholipids moiety that is essential for the functioning of the enzyme, could be related to an impairment in the optimal interaction of Na$^+$-K$^+$ ATPase with membrane phospholipids, considering that its activity is modulated by the microenvironment given by the physicochemical properties of the membranes into which it is inserted. The alteration of phospholipid composition of the membrane due to coal dust might be an important factor in the decrease of enzyme activity.

In this study, we observed a significant reduction in erythrocyte SOD activities in coal miners (Fig. 1). Reduction in SOD activity as observed by us may be due to an increased endogenous production of ROS as evidenced by increased MDA. This decrease in antioxidant enzyme may be related to the consumption of activated enzymes against oxidative

stress. This inhibition is probably achieved through the process of lipid peroxidation, which disturbs the phospholipid moiety that is essential for the functioning of Na^+-K^+ ATPase. Depletion of antioxidants in coal workers may also be a contributing factor to the decrease in Na^+- K^+ ATPase activity. Also, decreased SOD activity in coal miner might be a marker of diminished antioxidant defence system which was caused by dust.

Moreover, alterations of the antioxidant status and increased lipoperoxidation have been also proposed as a cause of Na^+-K^+ ATPase reduction in erythrocyte membranes [24]. Results of our study can suggest but cannot prove that an initial damage in cell membranes can lead to future complications, like erythrocyte Na^+-K^+ ATPase activity reduction and hypertension development. Furthermore, it cannot determine by itself whether the diminution of the Na^+ - K^+ ATPase activity found in this study is cause or consequence of the hypertension.

By-products of lipid peroxidation have been shown to cause profound alterations in structural organizations and functions of the cell membranes, including decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes and loss of essential fatty acids. Erythrocytes and erythrocyte membrane are more vulnerable to lipid peroxidation due to constant exposure to high oxygen tension and richness in polyunsaturated fatty acid. The osmotic fragility used as an indication of oxidative hemolysis measures the sensitivity of the erythrocytes to changes in osmotic pressure and has been used to measure the integrity [25] and metabolism [26] of the erythrocytes. In this study RBC osmotic fragility was found to be higher in coal mine workers as compared to control subjects (Fig. 3). Anaemia may result from peroxidation of red cell membrane. At the same time, anaemia renders the red cells more accessible to oxidative injury, which increases more the degree of anaemia and a vicious circle is created. The results of present study showed a significant decrease of haemoglobin concentration in coal mine workers as compared to the control. Therefore, in hyperlipidemia, lipid peroxidation and free radicals promote oxidation of haemoglobin and reduce its concentration. The decreases in haemoglobin concentration in the hyperlipidemic coal miners reflect the presence of anemia. Hyperlipidemia, especially in the form of increased plasma cholesterol and LDL, is a major determinant of CHD risk in the developed countries [27] and constitutes one of the major components of the metabolic syndrome [28]. However, on inspection, our data showed that coal miners had increased tendency toward higher blood total cholesterol, TG, LDL and VLDL; and low levels of HDL (Fig. 4)

compared with the other groups. The validity of these observations will need to be tested in enlarged cross-sectional study. Lipid peroxidation due to ROS is thought to be involved in a number of pathological processes. Oxidative modification of LDL is the best-substantiated example of in vivo lipid peroxidation. Oxidized LDL is either formed in the circulation and diffuses through the vascular endothelium or secreted as an oxidative metabolites from macrophages. Oxidized LDL in turn stimulates the formation of foam cells which are typically seen in the development of atherosclerosis. Since the membranes of living cells mainly consist of different types of polyunsaturated fatty acids, it is the different cellular membranes that are damaged by lipid peroxidation. Lipid peroxidation is free radical chain reaction, by which the esterified polyunsaturated fatty acids are oxidatively degraded into several products.

The present study, demonstrated that the elevated MDA and insufficiency of antioxidant potential in plasma and erythrocytes cause a decrease in erythrocyte Na^+-K^+ ATPase enzyme activity in coal miners as compared to normal subjects, which might be responsible for the alteration in lipid profiles of coal mine workers.

CONCLUSION

Our study indicates that elevated MDA and antioxidant insufficiency caused disruption in structural integrity of erythrocyte, which may be a pathophysiological mechanism in progression of disease in coal miners.

Conflicts of interest
The authors declare that they have no conflicts of interest.

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