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ORIGINAL ARTICLE

EVALUATION OF ERYTHROCYTE Na^+, K^+ -ATPASE AND SUPEROXIDE DISMUTASE ACTIVITIES AND MALONDIALDEHYDE LEVEL ALTERATION IN COAL MINERS

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The purpose of the present study was to determine the structural integrity of red blood cells in coal miners by assessing the concentration of malondialdehyde and the activities of superoxide dismutase and, Na^+, K^+ -ATPase in erythrocytes. Occupational exposure to coal mine dust can result in a wide range of lung diseases, which include pneumoconiosis, bronchitis, and emphysema. Pathophysiological mechanisms of the diseases are not fully understood; however, cytotoxicity is related to reactive oxygen substances which are produced by coal dust. The study population consisted of 40 coal workers previously known not to have any pulmonary disease and 34 healthy subjects who were randomly selected from the population register or recruited from the hospital staff. The activities of Na^+, K^+ -ATPase in the erythrocyte membrane was significantly decreased in the coal workers as compared to the control group. Serum potassium and iron concentrations were significantly higher whereas serum sodium was moderately decreased in coal workers as compared to control. MDA levels of all samples were significantly increased in the coal workers as compared to the control group. SOD activity in serum and erythrocyte was significantly lower in the coal miner group as compared to the control group. The present study demostrated that the elevated MDA and iron levels and insufficiency of antioxidant potential in serum and erythrocytes cause a decrease in erythrocyte Na^+, K^+ -ATPase enzyme activity in coal miners as compared to normal subjects.

Key words: Coal miners, eythrocyte, Na^+, K^+ -ATPase, MDA, SOD, iron, ROS, and coal dust

INTRODUCTION

Occupational exposure to coal mine dust can result in a wide range of lung diseases, which include pneumoconiosis, bronchitis, and emphysema (1). Pathophysiological mechanisms of the diseases are not fully understood. However, cytotoxicity is related to reactive oxygen substances (ROS) which are produced by coal dust (2). Basically, two mechanisms by which coal dust expose causes formation of ROS have been proposed, (i) the generation of ROS by intrinsic properties of particles and iron content of coal dust, non-cellular mechanism, and (ii) the excessive formation of ROS by the oxidative burst of macrophages and neutrophils activated during phagocytosis and persistent inflammation (3). In addition to these mechanisms, coal dust causes production of ROS via increaced metabolism of arachidonic acid in cell membranes (4). 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 (5,6). An extensive system of cytosolic and mitochondrial enzymatic and nonenzymatic antioxidants function to scavenge ROS in physiological processes and in pathological conditions (7).

The Na^+,K^+-ATPase is the intrinsic membrane protein responsible for pumping Na^+ and K^+ against their membrane gradient (8). In almost all animal cells, including human erythrocytes, Na^+, K^+ -ATPase is an essential protein transporter under normal physiological conditions. Na^+, K^+ -ATPase catalyzes the hydrolysis of ATP that is coupled to the active transport of Na^+/K^+ across the cell membrane (9). It mediates the transport of three Na^+ ions out of the cell and two K^+ ions into the cell per ATP molecule. The purpose of the present study was to determine the structural integrity of red blood cells in coal miners by assessing the concentration of malondialdehyde and the activities of superoxide dismutase and Na^+, K^+ -ATPase in erythrocytes.

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Table 1. Demographic characteristics of coal miners and control groups (mean±SD).

Groups	n	Age years	Exposure years	Smoker/ non-smoker	Smoking Pack-year
Coal miner	40	39.6±4.4	12.9±4.2	27/13	13.4±7.2
Control	34	40.8±5	-	21/11	14.4±8.1
p	ns	ns		n.s	n.s

MATERIAL AND METHODS

Subjects

The study population consisted of 40 coal workers previously known not to have any pulmonary disease and 34 healthy subjects who were randomly selected from the population register or recruited from the hospital staff. All cases were male and Caucasians. Healthy subjects were totally normal and had no pulmonary disease or complaints. The coal and coal mine characteristics Zonguldak Coal Basin has five areas for coal production. All coal workers were from the same area, namely Karadon. The workers had been working for at least 5 years (range 5-21 years). Mean coal chemical features of Karadon mines are as follows; 55% carbon, 26% volatile substances, 11% ash, 8% damp. All coal workers selected were from sections (coal face, mining, stope) that had coal dust exposure changing from low to high concentrations (0.5 to 12.3 mg/m^3) from time to time. All data were obtained from the Turkish Coal Company. Informed written consent was obtained from all subjects.

Sample collection and Processing

Blood samples were taken from 40 coal miners working in the Zonguldak coal mining industry and 34 healthy subjects. Ten ml of blood samples were taken from each donor after 8 hr work shift. All samples were taken by venipuncture with and without and anticuagulant (EDTA) evacuated tubes. Sample tubes were transferred to our laboratory within 2 hours for evaluation. Centrifugation was performed at 4 ^{0}C (10 min, 3000 rpm) and measurements were performed in serum, ertyhrocyte and erytrocyte 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 spinning 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 (It usually required 3-4 washes).

Na^+, K^+ -ATPase assay

The activity of Na^+, K^+ -ATPase in the erthrocyte membrane was measured by the method of Kitao-Hattori (10). The reaction mixture contained 200 µ ghost, and 800 µL medium fluid which included NaCl, KCl, EDTA, and MgCl. The reaction was started at 37 ^{o}C by the addition ATP. After 60 minutes, the reaction was stoppered by the addition of SDS. The samples were centrugated at 1,000 g for 15 minutes. The phosphrus content in the supernatant was measured using the inorganic phosphors estimation kit from Roche Diagnostica.

MDA determination

In the samples, which are serum, erythrocyte, and eyhrocyte membrane, malondialdehyde (MDA) levels were determined using the method of Draper and Hadley (11) based on the reaction of MDA with thiobarbituric acid (TBA) at 95^{o}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^{o}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 centrifigation and an aliquot of supernatant was reacted with 0.67% TBA in a boiling water-bath for 15 min. After cooling, the absorbance was read at 532 nm. Arbitrary values obtained were compared with a series of standard solutions (1,1,3,3 tetramethoxypropane). Results were expressed as nanomole per gram hemoglobin, mg protein, and mililiter.

Table 2. Serum sodium, potassium and iron levels in coal miners and controls. All measured values were given as mean ± SD

Groups	Na (mmol/L)	K (mmol/L)	Iron (µg/dl)
Coal miner	139±5.1	4.63±0.4	114±29
Control	142±5.2	4.38±0.5	88±20
p	≤0.05	≤0.05	≤0.001

Protein determination

The protein content of erythrocyte membrane was determined by the method of Lowry et al. (12).

SOD activity determination

Total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al (13) and a slightly modified method by Durak et al (14). The principle of the method is based on the inhibition of NBT reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assassed in the ethanol phase of the lyzate 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 or milliliter.

Hemoglobin (Hb) measurement

Hemoglobin concentration in erythrocytes was determined with the haemoglobin cyanide procedure (15).

Iron measurement

The serum concentrations of sodium, potassium and iron are measured with Roche diagnostica kit by using the same otoanalyzer (Roche Diagnostica).

Statistical Analysis

All values were expressed as means and standard deviations (SDs). SPSS for windows 11.0 was used for statistical analysis. Student's t-test was used to estimate the significance between parameters. The differences were considered to be significant when p was less than 0.05.

RESULTS

The Table 4 shows erythrocyte Na^+, K^+ -ATPase activity in the coal miner and control groups. The activities of Na^+, K^+ -ATPase in the erythrocyte membrane was significantly decreased in the coal workers as compared to the control group. Table 2 indicates the mean values and standard deviations (SDs) of the concentrations of serum sodium, potassium, and iron in the coal miner and control groups. Serum potassium and iron concentrations were significantly higher, whereas serum sodium was moderately decreased in coal workers as compared to controls.

Table 3 indicates lipid peroxidation in erythrocyte, erythrocyte membrane and serum of control and coal miner goups. MDA levels of all samples were significantly increased in the coal workers as compared to the control group. The table 4 shows SOD activities of serum and erythrocytes. SOD activity in serum and erythrocyte were significantly lower in coal miner group as compared to the control group.

Table 3. Erythrocyte, Erythrocyte membrane and serum MDA levels in coal miners and controls. All measured values were given as mean ± SD

Groups	Serum MDA (µmol/L)	Erythrocyte MDA (µmol/g Hb)	Erythrocyte membrane MDA (nmol/mg protein)
Coal miner	1.55±0.76	1.54±0.40	6.87±1.99
Control	1.14±0.51	1.26±0.36	5.54±1.45
p	≤0.01	≤0.01	≤0.01

Table 4. Serum SOD activity, SOD and Na $^{+}$, K^{+} -ATPase activities of erythrocyte in coal miners and controls. All measured values were given as mean ± SD

Groups	Serum-SOD (U/L)	Erythrocyte-SOD (U/g Hb)	Na $^{+}$, K^{+} -ATPase (µmolPi/mg prot/10 min)
Coal miner	4.03 ± 0.44	1134 ± 234	3.61 ± 1.01
Control	4.28 ± 0.38	1325 ± 308	4.69 ± 1.07
p	≤0.05	≤0.01	≤0.001

DISCUSSION

Lipid peroxidation is thought to be involved in a number of pathlogical processes. ROS have been implicated in the pathogenesis of various conditions including lung disease (16). The involvement of free radical induced lipid peroxidation and the role of antioxidants in several diseases are well established. MDA is an end product of fatty acid oxidation, and is often used as an indicator of lipid peroxidation.

The present study demonstrates that erythrocyte and erthrocyte membrane MDA levels are increased in a group of coal miners when comparated to a control group. Some animal studies have indicated that lipid peroxidation is enhanced in different tissues of rats exposed to coal dust and its derivates. For example, Srivastava et al showed that fly ash and fly ash residue increased the formation of conjugated dienes and the levels of oxidized glutathione (GSSG) and reduced the levels of reduced glutathione (GSH) in lung and liver, whereas fly ash extract administration had no effect on the formation of conjugated dienes and glutathione levels in lung and liver (17). Our findings are in agreement with the results obtained from some other studies (18, 19). 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 (3,20).

Erytrocytes and erytrocyte membrane are more vulnerable to lipid peroxidation due to constant exposure to high oxygen tension and richness in polyunsaturated fatty acid (21). By-products of lipid peroxidation have been shown to cause profound alterations in the structural organization and functions of the cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes and loss of essential fatty acids (22).

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 to inhibit Na^{+}, K^{+} -ATPase activity. Previous studies have shown that the activity of the enzyme is strongly and irreversibly reduced in the presence of ROS such as hydroxyl radicals, superoxide anion radical, or singlet oxygen (24). Jamme et al (25) reported that the inhibition of Na^{+}, K^{+} -ATPase activitiy by generated $^{\cdot}OH$ and peroxy radicals is also related to lipid peroxidation induced disruption of membrane integrity that results in conformational changes, leading to inactivation of membrane bound proteins. It has also been suggested that oxidant agent induced inactivation of Na^{+}, K^{+} -ATPase activitiy is due to lipid peroxidation-induced reduction in affinity for Na and K (26). The pump also appeared to be sensitive to changes in membrane phospholipids. Because phospholipid molecules, which are components of both types of Na^{+}, K^{+} -ATPase subunits, have a significant role in modulation of enzyme activity, changes in their composition are expected to alter Na^{+}, K^{+} -ATPase function (27).

This study demonstrated that Na^{+}, K^{+} -ATPase activity in erythrocyte membranes was lower in coal miners compared to a control group, whereas MDA level in erythrocyte membrane in coal workers group was was found to be higher than the control group. Decrease in erythrocyte Na^{+}, K^{+} -ATPase activity associated with increased lipid peroxidation, 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. Also activation of lipooxygenase by coal dusts, which results in enzyme inhibition, might have added to the decrease in Na^+, K^+ -ATPase activity (28).

It has been suggested that serum Na and K concentrations reflect inhibition of Na^+, K^+ -ATPase (29). The observed increase in plasma potassium and decline in sodium accompanied by lowered activity of erythrocyte membrane Na^+, K^+ -ATPase can disturb cellular ion homeostasis. ROS-induced oxidative damage to membrane Na^+, K^+ -ATPase and increase in potassium efflux from the cell have been assumed to be crucial for cell lysis. Hence, the disturbance in ionic homeostasis in the group of coal miners may result from elevated lipid peroxidation of erythrocytes and erythrocyte membrane and decreased Na^+, K^+ -ATPase activity, resulting in increased membrane permeability.

Increased serum iron concentrations reflected that coal miners are exposed to excessive iron, which is a transition metal ion, and appears to be an important mediator of oxidative damage in vivo/in vitro together with coal dust. High serum iron concentration shows that workers are exposed to high concentration of this metal, together with coal dust. Transition metal ions, especially iron, appear to be important mediators of oxidative damage in vivo. The iron present in medium may be involved in the formation of hydroxyl radicals via the Fenton-reaction. The iron content may also play an important role in the toxicity of coal dust (30). Fenton reaction type formation of hydroxy radicals was found to be positively correlated with the iron content of coal dust (31). The hydroxyl radical that may be produced by this reaction at the surface of the coal is a potent oxidant, which can initiate lipid peroxidation of cell membranes and oxidatively inactivate essential cell proteins (32).

The antioxidant defense system is known to inhibit lipid peroxidation in erythrocytes by destroying some ROS that have an important role in the initiation of lipid peroxidation processes. The antioxidant defense system operates through enzymatic and nonenzymatic components. The system is affected by coal dust and its derivates.

The studies show that reports about antioxidan parametres in coal miners are contradictory. While, coal dust and its derivates lead to decreased GSH levels and selenium concentration which is strongly an antioxidant mineral, and SOD activity (17,33,34). The other antioxidant enzymes are affected differently by coal dust. Both increase (35) and decrease (33) in activity has been reported. SOD activity was reported to be significantly reduced in coal miners with pneumoconiosis as compared to those without pneumoconiosis (34,35). Our results support these findings. We observed a significant reduction in erythrocyte SOD activities in coal miners. Reduction in SOD activity as observed by us may be due to an increased endogenous production of ROS as evidenced by increased MDA and products of ROS. 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.

Plasma MDA level has been used in many diseases as an indirect indicator of tissue lipid peroxidation and general oxidant stress. High serum plasma levels in our study suggest that coal dust, by exerting oxidant effect on tissues, especially the lungs, causes an increase in lipid peroxidation.

This agrees with the suggestion of Halliwell and Gutteridge (16) who proposed that lipid peroxidation in human diseases can be better explanained by the following reaction sequence: diseases or toxins induce cell damage or death; and lipid peroxidation then increases because disrupted tissues undergo peroxidation more quickly than healthy ones. Plasma concentration of antioxidants also has been used as a biomarker of oxidative stress in several diseases (36). A lower level of antioxidant as a consequence of a past situation of oxidative stress will correspond to a higher membrane lipid peroxide and MDA. Both are products of the lipid peroxidation process and presumably the result of ROS action (37). An increased serum MDA level in our group of coal miners may indicate a general oxidant effect of coal dust. Also, decreased SOD activity in coal miner might be a marker of diminished antioxidant defense system which was caused by dust.

Increased lipid peroxidation products in circulation, for instance MDA, may interact with erythrocyte membrane components such as lipid and protein, and demolish membrane bound enzyme activities and cellular functions.

The present study, thus, demostrated that the elevated MDA and iron levels and insufficiency of antioxidant potential in serum and erythrocytes cause a decrease in erythrocyte Na^+, K^+ -ATPase enzyme activity in coal miners as compared to normal subjects.

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