OXIDATIVE STRESS AND ANTIOXIDANT STATUS IN BRONCHOALVEOLAR LAVAGE FLUID, PLASMA AND ERYTHROCYTE OF CRITICALLY MIXED ILL WITH RESPIRATORY FAILURE

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Aim: Increased oxidative stress is a significant part of pathogenesis of various lung diseases. In this trial, it is aimed to determine the role of oxidative stress in patients receiving mechanical ventilation for respiratory failure (RF).

Methods: The oxidative stress was evaluated by determining plasma, bronchial fluid and erythrocyte levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), ascorbic acid, alpha-tocopherol, uric acid (UA) and nitric oxide (NO) in 25 critically ill patients with RF. Twenty five patients without RF was evaluated as control group.

Results: Oxidative stress (MDA, NO) levels of plasma, erythrocyte and BAL fluid in mixed critically ill with respiratory failure were higher than control group. Antioxidant levels of plasma, erythrocyte and BAL fluid in mixed critically ill with respiratory failure were lower than control group. In all parameters, there was no statistically difference as exitus and survivors in critically mixed patients.

Conclusion: Oxidative stress was higher in respiratory failure than control group.

Key words: Respiratory failure, oxidative stress, antioxidant status

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INTRODUCTION

Lung represents a unique tissue for oxidant stress among most organs because it is directly exposed to higher oxygen tensions (1). In the resting state, the balance between antioxidants and oxidants is sufficient to prevent the disruption of normal physiologic functions; however, either increases in oxidants or decreases in antioxidants can disrupt this balance. The state of imbalance is collectively referred to as oxidative stress and is associated with diverse airway pathologies (2). The major oxidants in airways are reactive oxygen and reactive nitrogen species (ROS/RNS). ROS include superoxide, hydrogen peroxide, and hydroxyl radical. RNS include nitric oxide (NO) (3). Antioxidants are the primary defense against ROS/RNS. The antioxidant effect can be either enzymatic (superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)) or nonenzymatic (vit C, vit E and uric acid) (4).

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Oxidative stress during critical illness may be related to activation of phagocytes, production of NO, and release of iron and copper ions and metalloproteins. Critical illnesses, such as sepsis or acute lung injury/ARDS, are characterized by a severe production of ROS and other radical species with consequent oxidative stress (5,6).

The aim of the present study was to investigate the status of oxidative stress and antioxidant in mixed critically ill patients with respiratory failure as assessed by the malondialdehyde (MDA), NO, SOD, GSH-Px, vit C, vit E and uric acid measurements in bronchoalveolar lavage (BAL), erythrocyte and plasma.

MATERIALS AND METHODS

A total 50 subjects were examined, 25 with respiratory failure (average 65.6±12.7 years, 17 males and 8 females) and 25 patients without respiratory failure (average 55.4±9.36 years, 19 males and 6 females). The patients

Table 1. Characteristics of patients and control group

	Mixed critically ill patients	Control group
n	25	25
Sex (M/F)	17/8	19/6
Age, years	65.6±12.7	55.4±9.36
Length of ICU stay, day	13.35±8.4	-
APACHE II	23.42±5.6	4.7±1.2
PCO_2, mmHg	67.41±24.6	43.2±4.1
PO_2, mmHg	53.7±12.8	81.5±8.4
HCO_3, mmol/L	33.25±15.4	22.1±2.1
pH	7.32±0.15	7.42±0.1
WBC, K/uL	14678.5±7361.8	9237.4±2531.2

with respiratory failure including chronic obstructive pulmonary disease (COPD) (n: 15), pneumonia (n: 7) and congestive heart failure (n:3) were divided into two groups as exitus (n:15) and survivor (n: 10) patients. Control group was including patients who performed diagnostic bronchoscopy (lung cancer (n: 18), COPD (n: 7)). Bronchoscopy was performed as described previously (7). Control group is heavy smoker.

BAL; BAL was obtained via endotracheal tube. BAL was performed by Combicath© (Plastimed Division, Prodimed, Saint-Leu-La-Foret Cedex, France). About 15 ml of fluid recovered from each subject were centrifuged for 10 min at 300 x g to separate the BAL cells from the acellular BAL fluid.

MDA; Malondialdehyde levels were estimated by the double-heating method of Wasowitz (8). MDA, an endproduct of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a coloured complex. The principle of the method is the spectrophotometric measurement of the colour generated by the reaction of TBA with MDA. The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex (absorbance coefficient e = 1.56 × 10^5 L/mol per cm) and is expressed as µmol / L for plasma and BAL, and nmol/gr Hb for erytrocyte.

Total SOD; Total SOD activity (Cu/Zn and Mn) was determined according to the method of Sun et al (9). Briefly, the principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/XO system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1.0 mL ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged at 4000 g for 30 min at 4 ^{0}C. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the rate of NBT reduction. Activity was expressed as U/ml for plasma and BAL, and U/mg Hb for erytrocyte in 560 nm wavelent.

Erythrocyte GSH-Px; Erytrocyte Glutathione peroxidase activity was measured according to the method of Paglia and Valentine (10). The enzymatic reaction in the tube that contained reduced nicotinamide adenine dinucleotide phosphate, reduced glutathione, sodium azide and glutathione reductase was initiated by the addition of hydrogen peroxide (H_2O_2) and the change in absorbance at 340 nm was monitored with a spectrophotometer. Hb determination was done by spectrophotometric cyanmethemoglobin method (Drapkin's solution) and results were given gr/dl . Erytrocyte GSH-Px activity is given in U/gr Hb. All samples were assayed in duplicate.

Plazma E vitamin; The estimated procedure of plasma alpha tocopherol is based upon the original Emmerie-Engel tocopherol assay (11). 0.5 ml of plasma, 0.5 ml of absolue ethanol and 1 ml of n-heptan were pipetted into a glass-stoppered 15-ml centrifuge tube. The contents of tube were shaken vigorously by hand for 2 min and centrifuged to completely separate the phases. 0.5 ml of the n-heptan phase (upper layer) was pipetted into another centrifuge tube, and then 0.3 ml α- αý-dipyridyl and 0.1 ml $FeCl_3$ were pipetted tube. Produced red

Table 2. Characteristics of Exitus and survivor patients

	Exitus	Survivor
n	15	10
Age, years	66.6±13.0	62.4±8.09
Length of ICU stay, day	10.0±3.8	21.7±11.5
Mortality rate, %	53.69±17.2	33.9±19.5
APACHE II	25±5.2	19.5±5.5
PCO_2, mmHg	64.24±21.56	75.35±33.5
PO_2, mmHg	52.09±14.6	57.7±6.8
HCO_3, mmol/L	33.25±15.4	32.1±12.1
pH	7.30±0.15	7.37±0.13
WBC, K/uL	14678.5±7361.8	14567±5531.2

color was measured at 510 nm as %T. The results were given in µg/dl.

Plazma C vitamin; Plasma ascorbate levels were determined photometrically with 2,4-dinitrophenylhydrazine to form the red bis-hydrazone. Ascorbic acid in plasma is oxidized by Cu^{+2} to form dehydroascorbic acid, which reacts with acidic 2,4-dinitrophenylhydrazine to form a red bis-hydrazone, which is measured at 520 nm (12).

Uric acide; Uric acid in the plasma was measured using enzymatic spectrophotometric kit method (GD086500, Globe Diagnostics, Milan, Italy). Uric acid is transformed by uricase into allantoin and hydrogen peroxide which, under the catalytic influence of peroxidase, oxidizes the chromogen (4 aminopherazone/ESPT) to form a purple quinoneimine whose intensity of colour is proportional to the concentration of uric acid in the sample. The results were given in mg/dl.

Plazma NO; The levels of plasma NO were measured using colorimetric kit method (Cat. No. CM780001, Cayman Chemical Company, USA). The best index of total NO production is the sum of both $NO2^-$ and $NO3^-$. The Cayman Chemical Nitrate/Nitrite Assay Kit provides an accurate and convenient method for measurement of total nitrate/nitrite concentration in a simple two-step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of the Griess Reagents which convert nitrite into a deep purple azo compound. 3 Photometric measurement of the absorbance due to this azo chromophore accurately determines $NO2^-$ concentration. The levels of plasma NO were given µM/ L.

RESULTS

Table 1 shows characteristics of patients and control group. Table 2 shows characteristics of exitus and survivor patients. The mean MDA levels of plasma, erythrocyte and BAL fluid in mixed critically ill with respiratory failure were 1.61±0.41 µmol/L, 25.6±8.02 µmol/L and 1.29±0.29 µmol/L, respectively. The mean MDA levels of plasma, erythrocyte and BAL fluid in mixed critically ill with respiratory failure were higher than control group (1.39±0.31 µmol/L, 11.8±3.09 µmol/L and 0.94±0.32 µmol/L, respectively) ($p<0.05$, $p<0.0001$, $p<0.01$). The mean NO levels of plasma and BAL fluid in mixed critically ill with respiratory failure were 55.6±11.7 µM/L and 44.5±9.4 µM/L, respectively. The mean NO levels of plasma and BAL fluid in mixed critically ill with respiratory failure were higher than control group (33.95±8.4 µM/L and 27.16±6.8 µM/L, respectively) ($p<0.0001$ and $p<0.0001$).

Mean SOD levels of plasma, erythrocyte and BAL fluid in mixed critically ill with respiratory failure were 6.99±1.27 U/ml, 3.13±1.33 U/mg Hgb and 6.9±2.29 U/ml,

Table 3. The mean levels of oxidants and antioxidants parameters in exitus and survivor patients.

	Exitus	Survivor
MDAp, µmol/L	1.62±0.37	1.59±0.39
NOp, µM/L	53.7±10.9	58.5±9.9
SODp, U/ml	7.46±0.73	6.28±1.38
C vitp, mg/dl	0.39±0.16	0.42±0.2
Evitp, µg/dl	0.58±0.17	0.65±0.19
UAp, mg/dl	4.6±1.5	5.8±1.1
MDAe, nmol/gr Hgb	24.2±6.5	27.7±9.0
SODe, U/mg Hgb	3.39±1.3	2.7±0.4
GSH-Pxe, U/gr Hgb	88.5±24.7	85.2±9.4
MDAlv, µmol/L	1.29±0.3	1.29±0.2
NOlv, µM/L	44.5±8.0	44.2±9.7
SODlv, U/ml	6.8±1.7	7.1±2.4

p; plasma, e; erythrocyte, lv; lavage

respectively. The mean SOD levels of plasma, erythrocyte and BAL fluid in mixed critically ill with respiratory failure were lower than control group (7.93±1.0 U/ml, 4.02±1.25 U/mg Hgb and 7.7±1.48 U/ml, respectively) ($p<0.05$, $p<0.05$, $p<0.05$). The mean of uric acid, vitamin C, vitamin E levels of plasma in mixed critically ill with respiratory failure were 4.94±1.52 mg/dl, 0.40±0.19 mg/dl and 0.61±0.18 µg/dl, respectively. The mean of uric acid, vitamin C, vitamin E levels of plasma in mixed critically ill with respiratory failure were lower than control group (7.35±2.03 mg/dl, 0.66±0.27 mg/dl and 1.09±0.31 µg/dl, respectively) ($p<0.0001$, $p<0.01$, $p<0.0001$).

Mean GSH-Px level of erythrocyte in mixed critically ill with respiratory failure was 87.2±22.5 U/gr Hgb. The mean of GSH-Px level of erythrocyte in mixed critically ill with respiratory failure was lower than control group (111.16±32.7 U/gr Hgb) ($p<0.05$). In all parameters, there was no statistically difference as exitus and survivors in critically mixed patients (Table 3).

According to all data, oxidative stress was higher in mixed critically ill with respiratory failure than control group. Antioxidant status was lower in mixed critically ill with respiratory failure than control group.

DISCUSSION

Oxygen-derive free radicals play an important role in the development of disease in critically ill patients. Oxidative stress during critical illness may be related to activation of phagocytes (neutrophils, monocytes, macrophages, eosinophils), production of NO, and release of iron and copper ions and metalloproteins. Critical illnesses, such as sepsis or acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) are characterized by a severe production of ROS and other radical species with consequent oxidative stress (13).

The most frequently used method to assess lipid peroxidation is the measurement of thiobarbituric acid-reactive substances (TBARSs) since MDA and other aldehydes react with thiobarbituric acid to give a pink fluorescent colour, which can then be measured. Several studies have confirmed the presence of increased TBARSs in patients with systemic inflammatory response syndrome and multiple organ failure (14,15). Bela et al. (16) reported that critically ill patients irrespective of the disease process indicated significantly very high serum levels of MDA at the time of admission. In an other study, the severity of illness by APACHE III was proportionally related to the degree of oxidative stress (17). In our study, the mean MDA and NO levels as evidences of oxidative stress were higher in patients with respiratory failure (RF) than control

group. APACHE II score and mortality rate were 23.4±5.7 and 48.0±19.5% in patients with RF, respectively. There is substantial experimental evidence that reactive oxygen species (ROS) and reactive nitrogen species (RNS) may be involved in pulmonary epithelial injury in a variety of pathologic situations. The induction of immune complex alveolitis in rat lungs results in increased alveolar epithelial permeability, which is associated with the presence of elevated concentrations of NO decomposition products in BAL fluid (18). Sittipunt et al (19) found that NO concentrations were significantly higher than normal in the BAL fluid from patients who were at risk for developing ARDS, as well as in the BAL fluid of those with ARDS. In patients who were at risk for ARDS, the NO concentration in BAL fluid was significantly higher than in healthy subjects (19). Sepsis results in a large increase in the production of nitric oxide and superoxide anions within body (20). Strand et al. (21) found a mean level of 144±39μmol/L in septic patients as compared to 20±3 μmol/L in control subjects. In an other study authors found similar findings (22). In our study, there were a patient with sepsis and two patients with ARDS. NO levels of serum and bronchial lavage fluid were higher in mixed critically ill patients than control group.

ROS are balanced by the activities of enzymes and other molecules called antioxidants, which delay or inhibit oxidation of a substrate. Endogenous antioxidant defences are both non-enzymatic (e.g. uric acid, glutathione, bilirubin, thiols and albumin) and enzymatic (e.g. superoxide dismutase, catalase and the glutathione peroxidase). In the normal subject the endogenous antioxidant defences balance ROS production (23,24).

There is a complex endogenous defense system designed to protect tissues from ROS/RNOS induced cell injury. Special enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, glutathione, and vitamins form a network of functionally overlapping defense mechanisms. In critically ill patients there are reduced stores of antioxidant, reduced plasma or intracellular concentrations of free electron scavengers or cofactors, and decreased activities of enzymatic systems involved in the detoxifications of ROS in critically ill patients (25). The circulating antioxidant levels decrease rapidly after insult, trauma, or surgery and remain below normal levels for several days or even weeks (26).

In Bela's study, serum level of superoxide dismutase was lower in critically ill patients (16). Flaring et al. (27) found that plasma glutathione remains depleted in whole blood in ICU. Selected antioxidants were measured including plasma ascorbate, a major plasma antioxidant, and were significantly decreased in patients with ongoing ARDS when compared to healthy control subjects. Interestingly, the levels of α-tocopherol, an additional plasma antioxidant, were unchanged when the two groups were compared (28). Schorah et al (29). reported that lower levels of ascorbic acid in patients of critical care were associated with severity of the illness and were not prevented by parenteral nutrition with ascorbic acid. In our study, the levels of ascorbate and α-tocopherol in plasma were lower in mixed critically ill than control group. Another important antioxidant In our study, the levels of antioxidants (SOD, GSH-Px, Vit C, Vit E, and Uric acid) in plasma, erythrocyte and bronchial lavage were lower in mixed critically ill than control patients. In a study, samples of BAL fluid and epithelial lining fluid from patients with ARDS were analyzed for the presence of GSH, and levels of GSH were found to be decreased when compared to samples from healthy control subjects (30). In another study, levels of catalase were found to increase in patients with sepsis (31). In our study, levels of SOD in BAL fluid were decreased in mixed critically ill with respiratory failure when compared to those of control patients.

In conclusion, oxidative stress is a feature of most respiratory diseases, particularly when inflammation is prominent. Both an increase in ROS/RNS and depletion antioxidants are thought to contribute the pathogenesis of oxidative stress; however it is still unclear which species are the most active in respiratory failure. Decreasing of antioxidant levels (SOD, GSH-Px, Uric acid, Vit C and Vit E) in plasma, erythrocyte and BAL fluid was seen in mixed critically ill with respiratory failure when compared to control group. In

parallel higher concentration of plasma, erythrocyte and BAL fluid oxidants (MDA and NO) was seen in mixed critically ill with respiratory failure when compared to control group. The imbalance of antioxidant and oxidants was related with high APACHE scores. Several antioxidants, alone or in combination, have been tested in different small randomized, double blind, placebo-controlled trials. However, antioxidant therapy may be useful in decreasing the mortality of respiratory failure. Clearly, additional studies in this area of research are needed.

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