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Impact of Some Environmental Factors and Microbes Causing Respiratory Diseases on Antioxidant Levels in Small Ruminants

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Abstract: The present study was aimed to investigate some oxidative stress indices in the erythrocytes of two sheep groups (healthy and diseased) at North Western Coastal Zone of Egypt which may be affected with some environmental factors {levels of some antioxidant trace elements copper (Cu), iron (Fe), manganese (Mn), selenium (Se) and zinc (Zn) in water and ration } and microbes causing respiratory diseases (nasal swabs were taken from animals for bacterial identification) which the most dominant diseases in desert due to its sever climatic conditions. *Mycoplasma ovipneumoniae, Pasteurella multocida, Klebseilla pneumoniae, Staphylococcus aureus,* Shigella spp. and *E. coli* were isolated to define the role of bacteria in oxidative stress. *Klebseilla pneumoniae* was the dominant isolated bacteria. The same trace elements were estimated in the serum samples of the two groups. Oxidative stress in the infected sheep was evaluated by determining various serum biomarkers as the enzymatic activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) in erythrocyte haemolysate and catalase (CAT) in serum, the mean values of which were 225.67±15.34 U/L, 145.33±15.52 U/L and 713.50±34.92 U/L respectively and serum biochemicals (albumin (Alb), aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), urea and creatinine), the mean values of which were 2.13±0.27 mg/dl, 13.870.61 IU/L, 6.03± 0.43 IU/L, 50.17±0.95 mg/dl and 0.88±0.044 mg/dl respectively. The levels of glutathione peroxidase and superoxide dismutase decreased significantly ($p \leq 0.05$) in the infected sheep as compared to that in healthy ones. By contrast, catalase activity was non-significantly decreased in the infected group compared to control one. On the basis of the altered levels of some antioxidant trace elements, serum biomarkers of oxidative stress (enzymatic antioxidant activities) and serum biochemicals (Alb, AST, ALT, urea and creatinine), it was concluded that the animals affected with these environmental factors and microbes developed oxidative stress. The findings suggested the relevance of periodic assessment of oxidative status in ruminants for healthier management through supplementation of these trace elements to the ration and combating bacterial infections.

Key words: Serum · Oxidative stress · Trace element · Respiratory diseases · Sheep

INTRODUCTION

Sheep and goats constitute the majority of animal wealth in the desert and play an important role in maintaining human life. Deserts are characterized by scarcity of food and water. This is further complicated particularly during the long dry season in the North Western Coastal desert of Egypt. Animals living in these regions are also exposed to other stress factors such as summer heat and cold during winter nights [1, 2], consequently due to these adverse environmental factors the animal is easily infected with microbes causing severe diseases especially respiratory. Recent reviews have focused on the role of trace minerals and vitamins in immune function and disease resistance in ruminants [3]. A stressful condition leads to the excessive production of free radicals, which results in oxidative stress, an imbalance in the oxidant/antioxidant system. Generation of free radicals is an integral feature of normal cellular functions. In contrast, excessive generation and/or

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inadequate removal of free radicals results in destructive and irreversible damage to the cell [4]. Disease challenge is the most important stress, immune cells themselves produce free radicals as a weapon to kill pathogens. Therefore, disease challenge is associated with increased free radical production which can damage cellular machinery if antioxidant enzymes are not sufficiently active [5].

Research in oxidative stress has been associated in various pathological processes in veterinary medicine [6]. Respiratory diseases are considered one of the most important pathological processes especially under desert conditions due to its stressful effect on animals. Superoxide, hydrogen peroxide, hydroxyl radical and nitric oxide are thought to contribute to these processes. Not only distressful environmental factors and host defense are responsible for oxidative stress on animal but also a number of microorganisms, including a number of Mycoplasma spp., which generate extracellular O_2 and H_2O_2 [7]. The extent of oxidant-mediated cytotoxicity observed at sites of inflammation is dependent on the balance between host and microorganism-derived prooxidant and antioxidant forces. When the prooxidants increase than antioxidant forces, the cytotoxicity will result on the infected tissue. Consequently clinical manifestations such as acute respiratory disease and lung destruction appear [8].

The antioxidant system, the first line of defense against free radicals includes a number of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) [9,10]. Trace minerals that have been identified as important for normal immune function and disease resistance include zinc, iron, copper, manganese and selenium, only when these metals are delivered in the diet in sufficient amounts can the animal body synthesize these antioxidant enzymes. In contrast, deficiency of those elements causes oxidative stress and damage to biological molecules and membranes and formation of free radicals. Because they are toxic to cells, the body has developed a sophisticated antioxidant system that depends on antioxidant nutrients [11]. More importantly, there is a range of antioxidant enzymes that can be synthesized in the body, but require the presence of diet-derived co-factors for example, selenium in the form of selenocysteine is an essential part of a family of enzymes called glutathione peroxidases. Zn, Cu and Mn are integral parts of another antioxidant enzyme family called superoxide dismutases (SOD), while Fe is an essential part of the antioxidant enzyme catalase (CAT) [12].

MATERIALS AND METHODS

This study was carried out at North Western Coast Barki sheep flock which belonging to Desert Researcher Center, Ministry of Agriculture and Land Reclamation, Egypt, in order to investigate plasma concentrations of some oxidative stress parameters such as antioxidant enzymes (SOD, GPX and CAT) and its relation with serum concentrations of antioxidant trace elements Cu, Fe, Mn, Se and Zn. After clinical examinations thirty Barki sheep (Ewes) 2-4 years old, were selected and divided into two equal groups, the first group was healthy as control while the second one suffered from respiratory signs.

The antioxidant trace elements Cu, Fe, Mn, Se and Zn were analyzed in animal drinking water, ration and serum by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) and summarized in Table (1) for water and ration. Analysis was carried out using microwave digestion technique (in a closed Teflon vessel under high temperature and pressure control) as reported by Littlejohn *et al.,* [13].

Sheep flock of the present work were subjected to clinical examinations which include recording the symptoms of respiratory distress in the diseased sheep were typically manifested by moist painful harsh cough, rhinitis and congested mucous membranes. Serous or mucoid nasal discharges, increased respiratory and pulse rates in addition to elevated rectal temperature (over 41ºC) were predominant signs in almost all cases. Auscultation revealed pleuritic friction rub in early stage or muffled sounds in the late and more sever stages.

Bacteriological examinations were done on nasal swabs from two groups to investigate the role of isolated microbes in oxidative stress. Samples were collected and transported immediately to the lab. in ice box. For bacterial isolation other than Mycoplasma; nasal swabs under aseptic condition were inoculated into nutrient broth and incubated at 37 °C for 24 hour and then subcultured onto the following media; 5 % sheep blood agar and MacConkey agar at 37 °C for 24-48 hour. The produced colonies were identified according to Quinn *et al.,* [14].

Table 1: Trace elements concentration (ppm) in water and ration in the study area

	Mineral				
Samples	Cu	Fe	Mn	Se	Zn
Water	0.02	0.211	0.0661	0.0008	0.1304
Ration	3.31	447.3	31.68	0.179	38.28
Dietary. Req.	5-6	40	25	0.03-0.2	25-40

Dietary requirements by sheep according to Grace and Clark [41].

For Mycoplasma isolation, purification and identification; collected samples were processed under aseptic condition by using Hayflick's medium for recovering Mycoplasmas, followed by purification of the isolates according to Sabry and Ahmed [15], differentiation between Mycoplasma and Acholeplasma isolates using digitonin sensitivity [16]. Biochemical characterization using glucose fermentation, arginine hydrolysis tests [17] and film and spot formation test [18] were carried out. Serotyping of Mycoplasma isolates was carried out by growth inhibition test (GIT) according to Clyde [19].

Blood samples of about 8-10 ml were allowed to flow gently from the Jugular vein of two groups into a clean dry labeled heparinized test tube. Heparinized blood was centrifuged at 3000 rpm for 15 min. at 4 °C. Pipette off plasma without disturbing the white buffy layer. Plasma was kept and stored at-80 °C before investigation. Biochemical analysis Alb, AST, ALT, urea, creatinine and CAT were determined by using Diamond kits. Catalase (U/L) was measured by colorimetric techniques using a commercially available kit (Bio-diagnostic., Egypt) according to Aebi [20].

For determination of SOD enzyme, 0.5 ml of heparinized whole blood was centrifuged for 10 min. at 4000 rpm at 4 °C and then plasma aspirated off. Then red blood cells washed four times with 3 ml of 0.9 % saline (NaCl) solution, centrifuged for 10 min. at 4000 rpm after each wash. The washed centrifuged erythrocytes should then be made up to 2.0 ml with cold redistilled water. Mixed and left to stand at +4 °C for 15 min. and stored at-80 °C until analysis. The lysate is diluted with distilled water (50 fold), so the % inhibition falls between 30% and 60%. SOD measured calorimetrically using a commercially available kit (Bio-diagnostic., Egypt) according to the method described by Nishikimi *et al.,*[21] and expressed as IU/L.

For determination of GPX enzyme, the red blood cells collected by centrifugation of heparinized whole blood (4000 rpm x 10 min.) then plasma drawn off. Erythrocytes washed once (one time) with 10 volumes of cold saline. The red blood cell pellets lysed by adding 4 volumes of cold deionized water to the estimated pellets volume, then the red cell stroma was removed by centrifuging (4000 rpm x 10 min. at 4 °C). The resulting clarified supernatant was collected and stored at-80 °C until assay. GPX measured calorimetrically using a commercially available kit (Bio-diagnostic., Egypt) according to Paglia and Valentine [22] and expressed as IU/L.

Data on content of antioxidant enzymes SOD, GPX and CAT, biochemical analysis and minerals were subjected to the General Linear Model (GLM) procedure of SAS Statistical package [23].

RESULTS AND DISCUSSION

Oxidative stress is an active field of research in ruminant medicine and has been implicated in numerous disease processes. Although the study of oxidative stress is a relatively young field of research in ruminant medicine, the understanding of the role of oxidants and antioxidants in physiological and pathological conditions is rapidly increasing [24]. One of the most common approaches to assessing mineral status of animals involves determination of their concentrations in samples such as whole blood or serum. Serum is the preferred sample for determining. Serum concentrations are reasonably good indicators of nutritional status essential minerals, serum mineral reference ranges are available for major domestic ruminant species [25, 26].

The altered concentrations of trace minerals (ppm) in water and ration samples in the study area are presented in Table 1. These results indicate that there are adequate concentrations of Fe in feed commonly consumed by animals. The trace elements most likely to be deficient for grazing livestock are Cu, I, Co, Se and Zn. Iron is generally not required unless animals are losing blood due to parasites; Mn deficiency is restricted to few regions of the world [27].

Concerning to the microbes causing respiratory diseases, the isolated bacteria were *Mycoplasma ovipneumoniae, Pasteurella multocida, Klebseilla pneumoniae, Staphylococcus aureus,* Shigella spp. and *E. coli* (Table, 2 and 3) which agrees with the finding of Abdou, [28], Nono, [29] and Darwish, [30]. The results revealed the presence of many bacterial species as single isolates or in mixed forms [31, 32 and 33]. A wide variety of bacteria are found in the upper respiratory tract, most of the infectious agents are normal inhabitants of the nasopharynx and these bacteria, after growing in the nose and throat, extend downwards and produce multiple bacterial infections under the stress factors causing respiratory diseases [34, 35].

It was clear that microbial infection is associated with higher oxidative stress and alteration of antioxidant status in diseased group which caused damage of some macromolecules including proteins, lipids and nucleic acids leading to tissue damages and dysfunction which

Table 2: The prevalence of bacterial isolation from nasal swabs of apparently healthy and diseased sheep

Bacterial isolate	Apparently healthy	Diseased	Total	Percent (%)
Mycoplasma ovipneumoniae	-	2	2	5.4
Klebseilla pneumoniae	4	6	10	27.03
Staphylococcus aureus	4	5	9	24.32
Pasteurella multocida.	1	2	3	8.11
Shigella spp.	3	4	7	18.92
E. coli	3	3	6	16.22
Total	15	22	37	100

Table 3: The prevalence of mixed bacterial isolation from nasal swabs of diseased sheep

Mixed Bacterial isolation	No	Percent (%)
Mycoplasma ovipneumoniae + *Pasteurella multocida*	1	14.29
Klebseilla pneumoniae + *Staphylococcus aureus*	1	14.29
Shigella spp. + *Klebseilla pneumoniae*	2	28.56
E. coli + *Staphylococcus aureus*	3	42.86
Total	7	100

appeared as clinical signs of increased respiratory and pulse rates in addition to other respiratory signs. There is no correlation between specific microbe and specific antioxidant status as the different isolates associated with nearly the same results of oxidative parameters which agree with Miller and Britigan, [8] who reported that the extent of oxidant-mediated cytotoxicity observed at sites of inflammation is dependent on the balance between host and microorganism-derived prooxidant and antioxidant forces.

Two isolates of *Mycoplasma ovipneumoniae* were only recovered from diseased sheep which is almost the same result obtained by Haribabu *et al.,* [36]. The role of *Mycoplasma ovipneumoniae* in oxidative stress was demonstrated by Niang *et al.,* [37] through infected tracheal organ cultures with different isolates of *Mycoplasma ovipneumoniae* which showed significantly decreased ciliary activity with marked differences between isolates in the onset and severity of the effects which correlated with their ability to produce extra cellular hydrogen peroxide and this oxidative damage induces coughing syndrome which is characteristic signs of *Mycoplasma ovipneumoniae* infection.

Klebseilla pneumoniae was the most dominant isolates (27.3 %) which agrees with Nono, [29] and Darwish, [30]. *Klebseilla pneumoniae* is characterized by presence of metal ions (a virulence factor) which is highly crucial for its survival when facing the stress of peroxide oxidation [38].

The results revealed a relation between *Pasteurella multocida* which is considered the main pathogen responsible for respiratory infection in small ruminants and oxidative stress which agrees with Roy *et al.,* [39] who reported that the oxygen radical formation in whole blood cells was found to be significantly elevated after *Pasteurella multocida* infection. Although the other organisms that isolated in this study; *Staphylococcus aureus,* Shigella spp. and *E. coli* are normally rapidly killed by oxidants, but they can overcome this defense mechanism by secreting either toxins to kill the phagocyte before they can be killed by it (leukocidin from *Staphylococcus aureus*) or antioxidant enzymes (e.g. catalase from *Staphylococcus aureus*, Shigella spp. and *E. coli*) [8]. By this way, accelerating oxidant production occurs in the organism leading to oxidative stress which was clear in this study.

The mineral concentrations in serum for diseased and healthy sheep are presented in Table 4. Cu, Fe, Mn, Se and Zn concentrations varied between the two groups. Mean concentration of Cu for diseased group was significantly decreased than the healthy group ($P \leq 0.005$) that may be because when copper binds to albumin forming a complex albumin-copper that is the active toxic fraction, then it rapidly accumulates within red cells that cause oxidative damage and intravascular haemolysis [40]. In addition, there were decreased in Fe, Mn, Se and Zn concentrations but not significantly. In general, serum Cu, Fe, Mn and Zn concentrations were within published reference ranges for domestic sheep (Table 4) but Selenium concentrations falling into a deficient range. The importance of Se in the diet of small ruminant is especially well documented based on its ability to reduce the incidence and severity of disease during times of heightened oxidative stress [41, 42, 43].

Table 4: Means (± SE) of trace elements concentration (ppm) in serum samples of two groups, diseased and healthy sheep at North Western Coast, Egypt.

	Mineral				
Samples	Cu	Fe	Mn	Se	Zn
Diseased	1.624[a]±0.07	4.944[a] ±0.53	44.08[a] ±4.55	0.076[a] ±0.007	2.74[a] ±0.50
Healthy	1.369[b]±0.07	5.034[a] ±0.5341	32.767[a] ±4.55	0.063[a] ±0.007	2.415[a] ±0.50
Ref. values (1)	0.70-2.00	1.66-2.22	20-35	0.08-0.50	0.8-1.2
(2)	0.75-1.7	0.9-2.7	------	0.12-0.35	0.55-1.2

Reference values according to Puls [57] & Herdt and Hoff [25].
In the same column, the different litters refer to a significant difference at p=0.05.

Table 5: Means (± SE) of superoxide dismutase (SOD), glutathione peroxidase (GPx) , catalase (CAT), albumin (Alb), ALT , AST, Urea and creatinine in diseased and healthy sheep at North Western.

	Parameter							
Groups	SOD(U/L)	GPx(U/L)	CAT(U/L)	Alb. (mg/dl)	ALT(IU/L)	AST(IU/L)	Urea (mg/dl)	Creat. (mg/dl)
Diseased (n=15)	225.67[A] (±15.34)	145.33 [A] (±15.52)	713.50 [A] (±34.92)	2.13 [A] (±0.27)	7.70 A (±0.43)	16.27 [B] (±0.61)	50.17 [A] (±0.95)	0.88 [A] (±0.04)
Healthy (n=15)	280.67[B] (±15.34)	207.17 [B] (±15.52)	786.83 [A] (±34.92)	3.82 [B] (±0.27)	6.03 [B] (±0.43)	13.86 [A] (±0.61)	47.50 [A] (±0.95)	0.71 [B] (±0.04)

In the same column, the different litters refer to a significant difference at $p \leq 0.05$.

Differences in trace element concentrations between two groups are consistent with the many factors that can affect regulation of mineral elements, such as water, forage and soil components, rumen micro flora, interactions among the various trace elements, breed or genetic effects, physiological state, parasites, toxicant exposure and immune interactions [44]. In living organisms, aerobic metabolism produces toxic reactive oxygen species (ROS). Life can thus be seen as a balance between metabolic rate and a cell's ability to detoxify ROS, this understanding has led to intense public interest and increased consumption of dietary antioxidants [45].

Among many dietary factors, minerals have special importance in the maintenance of rapid and efficient growth, reproduction and immuno-competence in animal production. This concept is based on understanding the contribution of minerals in reducing the detrimental effects of free radicals and toxic metabolites on immune processes in the animal's body. It is generally considered that uncontrolled oxidation reactions caused by various stressors can not only lead to poor animal performance, but may impair the animal's immune status and disease resistance [46,47].

In Table 5, values of serum biomarkers are expressed as mean and mean of standard error (mean ± SEM) of the measured oxidative stress parameters in healthy and diseased sheep. Values with different superscriptions (A and B) in the same column differ significantly (P<0.05). In general, infected animals had lower values of antioxidant parameters. The data indicated that superoxide dismutase 225.67±15.34 and 280.67±15.34 IU/L and glutathione peroxidase 145.33±15.52 and 207.17±15.52 IU/L for diseased and healthy sheep groups respectively, GPX is a selenium dependent antioxidant enzyme but SOD thought to be a copper storage protein [48]. The activity of these two enzyme decreased significantly ($p \leq 0.05$) in diseased sheep as compared to healthy ones. By contrast, CAT activity was non-significantly higher in the infected group compared to control group, their activity recorded 713.50±34.92 and 786.83 ±34.92 IU/L respectively. These results might be due to the high content of (Fe) in water and ration. Moreover, repeated exposure to high concentration of oxygen or presence of iron renders erythrocytes highly susceptible to peroxidative damage [49].

In this study on sheep respiratory infections, our results indicate that infection with these bacteria led to notable changes in measured oxidative stress indices which agree with Beck *et al.,* [50] who mentioned that an altered status of serum antioxidants in diseased animals indicated development of oxidative stress in these animals. Antioxidant mechanisms may become insufficient to prevent oxidative damage completely. Consequently, oxidative stress develops, which is implicated as a pathogenic factor in a number of infections.

As shown in Table 5, altered biochemical concentrations of Alb, AST, ALT and creatinine in diseased sheep were different significantly ($p \leq 0.05$) as compared to healthy ones. By contrast, urea activity was no different significantly ($p \leq 0.05$) in diseased sheep as compared to healthy ones. Biochemical determination of serum constituents can provide valuable information as relating to nutrition, sex, age and physiological status of the animal [51]. Mean concentration values of Alb from diseased and healthy sheep groups were 2.13 ±0.27 mg/dl and 3.82±0.27 mg/dl respectively, concentration in the diseased Sheep decreased significantly ($P \leq 0.05$) than healthy one, these values agree with the observations of Al-Fartosi, *et al.,* [52], [53]. Albumin is the major

extracellular source of thiols, which are scavengers of free radicals allowing albumin to function as an antioxidant [54], this interpretation could explain the decreased of albumin concentration for the diseased group. The serum activity mean values of AST from diseased and healthy sheep groups were 16.27 ±0.61 IU/L and 13.86 ± 0.61 IU/L respectively, activity in the diseased Sheep increased significantly ($P \leq 0.05$) than healthy one. Biochemical analyses of blood serum are very useful to get insight in the metabolic and health status of animals. During diagnostic procedure it is very useful to compare the values obtained from ill animals with normal values in healthy animal [55].

Means ± SEM of ALT activity for diseased and healthy group were 7.70 ±0.43 IU/L and 6.03±0.43 IU/L respectively, in the diseased sheep it increased significantly ($P \leq 0.05$) than the healthy. These results agree with Değer *et al.,* [56] who recorded that ALT and AST activity increased significantly in infected sheep comparison with the control group and demonstrated that changes in the antioxidant abilities of the liver and in the phospholipid structure of the cell membrane were accompanied by rising activities of ALT and AST as markers of liver damage. In addition, changes in serum urea concentration of diseased and healthy sheep were 50.17±0.95 mg/dl and 47.50±0.95 mg/dl, respectively, the results indicated no significant differences in serum urea concentration of healthy and diseased sheep. According to creatinine concentrations, there were 0.88 ±0.04 mg/dl and 0.71±0.04 mg/dl for diseased and healthy sheep it increased significantly ($P \leq 0.05$) for the diseased than the healthy group.

These results show that the levels of some antioxidant trace elements in the desert environment have showed lower values than limited levels. Significant reductions of GPx, SOD activity in diseased animals compared to control healthy group under desert conditions. Also the results clearly demonstrate the occurrence of an oxidative stress due to microbes causing respiratory diseases leading to deficiency of enzymatic antioxidant.

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