African Journal of Biotechnology Vol. 11(41), pp. 9835-9843, 22 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3669
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Lack of ameliorative effect of Vitamins E and C supplements to oxidative stress and erythrocytes deterioration after exhaustive exercise at high altitude in native rats

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Accepted 23 February, 2012

The purpose of this study was first, to determine the effects of acute forced swimming to exhaustion, by native rats at high altitude, on some hematological and biochemical parameters and on lipid peroxidation and cell anti-oxidant system in red blood cells of these rats. Secondly, to investigate any protective effect conferred by Vitamins E and C pre-co treatment. Young male Wistar rats weighing 150 to 160 g (aged three to four months) were used in this study. All rats were from the same lineage and were born in the high altitude environment (2,800 m), and were from the 10th generation whose parents lived in the high altitude area for six months. The rats were divided into two groups: non-stressed group (n = 6) used as the control, and stressed group (n = 12). The non-stressed group was given normal saline intraperitoneally (i.p.) and was not exercised. The stressed group was divided into two subgroups, each consisting of six rats, classified as stressed group 1 (given normal saline) and stressed group 2 (given Vitamins E and C supplements). All rats in the stressed group were exposed to acute forced swimming exercise to exhaustion. Blood samples were collected at the end of the experimental procedure from all groups for routine hematological analysis and for determination of serum total bilirubin and iron. Red blood cell (RBC) levels of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) were also determined. Acute forced exhaustive swimming caused significant decreases in erythrocyte count, hemoglobin (Hb) concentration, and packed cell volume (PCV) with no changes in mean corpuscular volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) but with significant increases in the levels of serum total bilirubin and iron. Exhaustive exercise also caused a significant increase in TBARS level, and significant decreases in the activities of SOD and CAT and in GSH level. With the exception of CAT, pre-treatment with Vitamins E and C supplements did not produce any significant improvement in the levels of these hematological and biochemical parameters. In conclusion, single forced exhaustive swimming exercise at high altitude resulted in elevation of oxidative stress and deterioration of erythrocyte structure (hemolysis) in native rats which were not ameliorated by antioxidant Vitamins E and C co-treatment.

Key words: Exercise, oxidative stress, Vitamins E, C and erythrocytes.

INTRODUCTION

Stress may be defined as any condition which overwhelms the maintenance of homeostasis such that the body can no longer respond adequately to physiological, mental, emotional or physical demands. The stress resulting from physical exercise induces a variety of physiological responses which are ultimately beneficial to the body. However, it is known that exhaustive exercise, especially when it occurs sporadically, may lead to loss of

the beneficial effects of physical activity and in some cases, to structural damage or inflammatory reactions within muscles and other organs of the body. This damage is due, at least in part, to the production of reactive oxygen species (ROS) (Mackinnon, 2000). Oxidative stress is a general term used to describe the steady state level of oxidative damage in a cell, tissue or organ caused by an imbalance between the production of ROS and the biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage (Sies, 1986). To prevent oxidative stress, there is an elaborate antioxidant defense system in cells consisting of enzymatic antioxidants, such as catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and numerous non-enzymatic antioxidants, including GSH, Vitamins C, E and Q, carotenoids, and uric acid (Vertuani et al., 2004). Oxidative stress results when the antioxidant defenses are overwhelmed by pro-oxidant forces and ROS are not adequately removed (Sies, 1986).

Lipid peroxidation is a major event induced by oxidative stress. Free radicals generate a cascade of reactions which induce lipid peroxidation (Kovacheva and Ribarov, 1995) leading to a range of enzymatically damaging consequences (Kovacheva and Ribarov, 1995) including membrane disorganization. Thus, lipid peroxidation is considered a serious consequence of free radical toxicity which may even cause cellular death (Urso and Clarkson, 2003). Forced swimming in small laboratory animals has been widely used for studying the capacity of the organism to adapt to the physiological stress of heavy exercise (Tan et al., 1992). Swimming has got a number of advantages over other types of exercise such as treadmill running. The amount of work done during swimming exercise is far greater than that done during treadmill running of identical duration (Nagaraja and Jeganathan, 1999). The forced swimming stress developed by Porsolt et al. (1977) has now become a widely accepted model for studying physical exercise stress in animals and a good model to induce oxidative stress in these animals (Tan et al., 1992). Under normal conditions, cells continuously produce free radicals and ROS as part of metabolic processes.

During heavy physical exercise, oxygen utilization may increase 10 to 15 folds (Ji, 1999) and free radicals may be produced in excess of the body's natural defense leading to oxidative stress in muscles and other tissues. Similar to anaerobic physical exercise, residence at high altitude may result in oxidative damage to macromolecules (Bailey et al., 2000a, b). A considerable body of literature exists documenting an increased production of oxidative stress indicators in breath, blood, urine and tissues of laboratory rats in response to short or long term hypoxia (Bailey et al., 2000a, b; Arteel et al., 1999). Similar results have been found in humans exposed to hypoxia (Schmidt et al., 2002). Although the percent oxygen in the atmosphere remains relatively unchanged as elevation is gained at altitude, the reduction in barometric pressure causes an obligatory decrease in the partial pressure of atmospheric oxygen (PO_2) leading to a decrease in arterial blood oxygen content (PaO_2) and resultant tissue hypoxia (Arteel et al., 1999). Most subjects can tolerate mild oxidative stress and often respond to it by synthesizing extra antioxidant defenses. However, severe oxidative stress can overwhelm antioxidant defenses and cause cell damage, even cell death.

Measurement of breath pentane shows that oxidative stress is present during work at moderate altitude as well as at high altitude (Bailey et al., 2000b; Arteel et al., 1999), and that some part of this oxidative stress may be attenuated with antioxidant supplement. Studies of antioxidant supplementation during exercise at sea level abound, however, only a few such studies at high altitude exist (Schmidt et al., 2002). In general, these studies have shown that antioxidant supplements reduce oxidative stress indicators in blood, urine and breath at altitude (Schmidt et al., 2002). Studies of antioxidant supplementation and oxidative stress generated by acute exercise for indigenes of high altitude are lacking. Erythrocytes are probably the cells most exposed to peroxidative damage by free radicals. During their relatively short lifespan, during which no protein synthesis occurs, these cells are adversely affected by free radicals (Sumikawa et al., 1993). The effect of acute heavy exercise on free radical production and the antioxidant system in rats at sea level has been intensively investigated (Neubauer, 2001), but very little is known about such effects on erythrocytes at high altitude. The present study was undertaken to determine the effects of acute maximal exercise on red blood cells (RBCs) and on their antioxidant system in indigenous rats at high altitude (2,800 m) following antioxidant supplementation.

MATERIALS AND METHODS

Area of the study

The study was carried out in Abha city in Southwestern Saudi Arabia between the 1st and 20th of December, 2010. Abha, the capital of Aseer Province (population, 352,303) is located in the Aseer Mountains at an altitude of 2,800 to 3,150 m above sea level. It has the lowest mean annual temperature of any city in southern Saudi Arabia, a low atmospheric pressure and a relatively high annual rainfall, with most of the rain falling in winter and spring. Relevant geographical information about the city (Central Department of Statistic and Information, 2004) is shown in Table 1.

Experimental animals

Young male wistar rats weighing from 150 to 160 g (aged three to four months) were supplied from the Animal House of the College of Medicine, King Khalid University, Abha. All rats were from the same lineage and were born in the high altitude environment (Abha region) and were from the 10 generation whose parents lived in the high altitude area for six months. The rats were housed in plastic

Table 1. Environmental data on high altitude area of the study (Abha).

Data	Value
Altitude (meters)	2800-3150
Barometric pressure (mm Hg)	550-590
Atmospheric O2 tension (mm Hg)	110-120
Relative humidity (%)	20-30
Summer temperature (shade) (°C)	16-28
Winter temperature (shade) (°C)	5-15

cages (six rats/cage) at regulated temperature (22°C) and humidity (55%) with 12 h light/12 h dark cycles. Water and standard pellet diet were supplied *ad libitum*. All rats were subjected to overnight fasting prior to the start of the experimental procedures. All studies were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Experimental groups

Rats were divided into two groups: the non-stressed (control) group (n = 6) and the stressed group (n = 12). The control group was given normal saline i.p. but not exercised. The stressed group was further divided into two subgroups each consisting of six rats. Stressed group 2 was given single i.p. injection of Vitamin E (25 mg/kg) (Lee et al., 2009) and Vitamin C (20 mg/kg), orally (Owu et al., 2006) 1 h before the start of the experimental procedure. Stressed group 1 was given normal saline i.p. and served as control for stressed group 2.

Stress protocol

After treatment, the experimental procedure was started at 9:00 a.m. All rats in the two stressed subgroups were exposed to acute forced swimming to exhaustion (about 2.5 h) as shown by the inability of rats to continue swimming and start sinking. The swimming pool was a glass tank (length 100 cm; width 40 cm; depth 60 cm) filled with tap water at 36°C to a depth of 30 cm. A maximum of two rats were made to swim together during each stress session.

Blood collection

Blood was collected directly by cardiac puncture into ethylenediaminetetraacetic acid (EDTA) tubes for routine hematological analysis. A second blood fraction was collected without anticoagulant and centrifuged at 5000 rpm for 10 min for determination of serum iron content and total bilirubin using special commercially available kits. Some EDTA blood was used for the preparation of hemolysate for determination of reduced GSH level and SOD and CAT activities. Packed cell volume (PCV), hemoglobin concentration (Hb conc.), total RBC count and other red cell indices including MCV, MCH and MCHC were determined using the Automated Hematological Analyzer (Sysmex- SE 9500, Japan).

Preparation of hemolysate

Blood samples in EDTA tubes were centrifuged at 3000 rpm for 5 min to remove the buffy coat. The packed cells obtained at the bottom were washed twice with phosphate buffered saline (0.9% NaCl in 0.01 M phosphate buffer, pH 7.4). A known amount of erythrocytes was lysed with hypotonic phosphate buffer. The hemolysate was obtained after removing the cell debris by centrifugation at 3000 rpm for 5 min and was used for determination of TBARS, SOD, CAT, reduced GSH activities.

Estimation of the level of lipid peroxidation

Lipid peroxidation was assayed by measurement of TBARS, using the method described by Ohkawa et al. (1979). In brief, the reaction mixture contained 0.1 mL of homolysate, 0.2 mL of sodium dodecyl sulfate, 1.5 mL of acetic acid and 1.5 mL of an aqueous solution of tert-butyl alcohol (TBA). The pH of 20% acetic acid was pre-adjusted with 1 M NaOH to 3.5. The mixture was made up to 4 mL with distilled water and heated to 95°C for 1 h in a water bath. After cooling, 1 mL of distilled water and 5 mL of a mixture of n-butanol and pyridine (15:1) were added and the mixture was shaken vigorously on a vortex mixer. The mixture was centrifuged and absorbance of the upper organic layer was read at 532 nm. The values were expressed as mM/100 g of tissue.

Measurements activities of SOD, CAT and reduced GSH level in hemolysate

SOD activity in red cell hemolysate was measured using commercial kits (Randox laboratories Limited UK). The activity was expressed as U/mL. One unit of SOD activity is the amount which causes a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) under the conditions of the assay. CAT activity was determined by using a commercial kit (Biovision K773-100). CAT activity was expressed as U/ml. One unit of CAT activity is the amount of CAT which decomposes 1.0 µmol of H_2O_2 per min at pH 4.5 and 25°C). Reduced GSH level was determined by a commercial kit (Randox laboratories Limited UK) and expressed as mmol/dL.

Statistical analysis

Data are expressed as Mean ± standard deviation (SD). One way analysis of variance (ANOVA) test (SPSS version 16) was used to determine the difference between groups. Statistical significance was set at $p<0.05$.

RESULTS

Changes in total erythrocyte count, Hb conc. PCV and other blood indices are presented in Table 2. The present

Table 2. Mean values of some haematological parameters of the different experimental groups of rats.

Parameter	Non stressed (control) group	Ex group	Ex + Vitamins E and C
RBCs (x103)	12.0±17	10.67±1.18*	10.74±0.94*
Hb (g/dl)	20.8±0.654	17.35±0.572*	18.4±1.82*
PCV (%)	62.6±0.64	50.9±1.04*	51.4±0.91*
MCV (fL)	51.8±2.16	51.3±0.883	50.4±0.811
MCH (pg)	17.5±1.51	17.6±0.739	17.6±0.315
MCHC (g/dL)	34.1±0.488	34.7±1.24	34.1±0.677`

Values are given as Mean±SD for groups of six rats each. Analysis was carried out using one way ANOVA; *statistically different when compared to control group. RBCs, Red blood cells; Hb, hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; Ex, exercise groups.

Table 3. Mean values of serum iron and total bilirubin levels of the different experimental groups of rats.

Parameter	Non stressed (control) group	Ex group	Ex + Vitamins E and C
Iron (µg/dL)	133±24.7	278±17*	269±13.1*
Total bilirubin (mg/dL)	0.362±0.077	0.793±0.0486*	0.737±0.05*

Values are given as Mean±SD for groups of six rats each. Analysis was carried out using one way ANOVA; Ex, exercise group; *statistically different when compared to control group.

study reveals that forced swimming to exhaustion at high altitude by native rats (stressed group 1) caused significant decreases in erythrocyte count, Hb conc. and PCV, with no changes in the MCV, MCH and MCHC in comparison to the non-stressed (control) group. Levels of these hematological parameters did not show significant changes in stressed group 2 pre-treated with single injections of Vitamins E and C when compared to stressed group 1 as indicated by ANOVA test. Levels of serum iron and total bilirubin are contained in Table 3 and show significant increases in the levels of both biochemical parameters in stressed group 1 when compared to the non-stressed (control) group. In comparison to stressed group 1, the levels of these parameters remained high and were not significantly different from the corresponding values in stressed group 2. Data for lipid peroxidation as shown by serum levels of TBARS, and activities of reduced GSH, SOD and CAT are shown in Figures 2, 3, 4 and 5, respectively. The data shows significant decreases in GSH level and in the activities of SOD and CAT with a significantly increased level of TBARS in stressed group 1 when compared to the non-stressed (control) group. The ANOVA test revealed that treatment with Vitamins E and C did not cause any significant change in these biochemical parameters except for CAT, which significantly increased after vitamin supplements relative to stressed group 1 values (Figure 1).

DISCUSSION

The effect of acute heavy exercise on free radical production and the protective effect of natural antioxidant in the rats at sea level has been intensively investigated (Neubauer, 2001), but very little is known about such effects at high altitude and under hypoxia conditions. Further, most of the studies carried out on different organ functions at different altitudes utilized high altitude laboratories or hypobaric chambers (Coates et al., 1979; Welsh et al., 1993), though, no study before now has investigated this effect in high altitude native rats. This observation prompts this study to thoroughly investigate this effect in the RBCs using native rats under high altitude conditions in a natural hypoxic environment provided by the high altitude locations of the laboratories used for this study. In this study, non-exercised rats (control rats) had expectedly high levels of red cell count, Hb conc. and hematocrit. The low oxygen partial pressure of inspired air at high altitude induces several adjustments in both humans and animals aimed at improving oxygen delivery to tissues (Leon-Velarde et al., 2000). Hypoxia raises erythropoietin levels (Eckardt et al., 1981) which elicits the increase in Hb conc. and hematocrit (Ferretti et al., 1990) seen in the control group. Acute forced swimming exercise to exhaustion resulted in increased rate of hemolysis as shown by decreased red cell count, Hb conc., and hematocrit probably due to the fact that oxidant mediated mechanisms are insensitive to short-term antioxidant (Vitamins E and C) supplements.

Other evidence of accelerated hemolysis is shown in Table 3. The concentration of serum iron was significantly increased immediately after the swimming exercise, no doubt due to the acute phase red cell destruction. Serum total bilirubin also increased following exercise due to

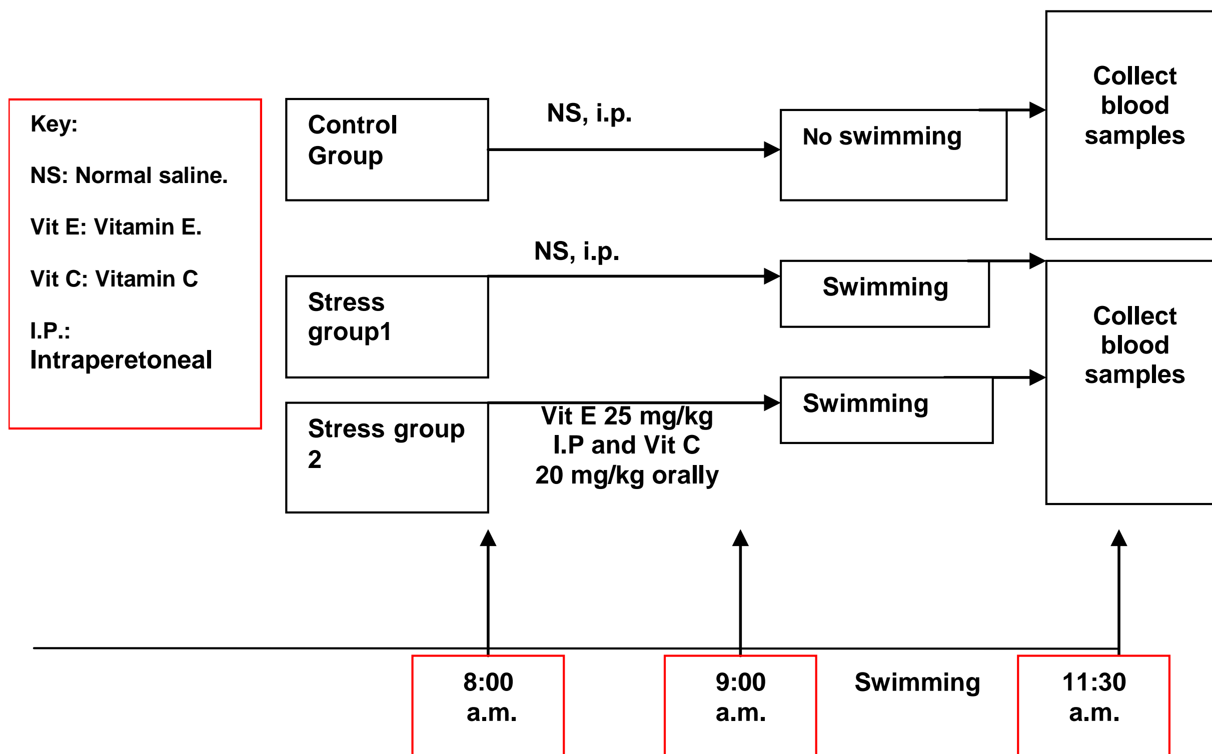

Figure 1. Experimental procedure.

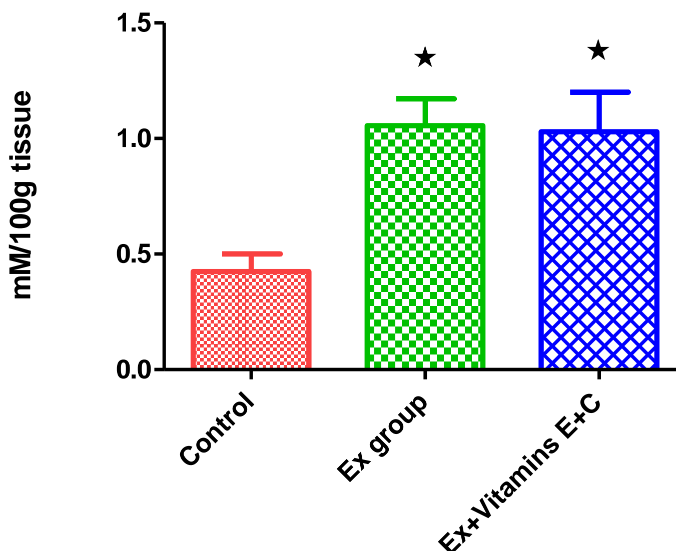

Figure 2. Levels of TBARS in the erythrocytes of all experimental groups of rats. Values are given as Mean±SD for groups of six rats each. Analysis was carried out using one way ANOVA. Ex, Exercise group; *statistically different when compared to control group. TBARS, thiobarbituric acid reactive substances.

increased hemolysis. On the other hand, levels of MCV, MCH and MCHC were unaffected by exercise confirming that the observed transient anemia was caused by reduced red cell numbers and hemoglobin levels rather than reduced red cell size (Halliwell, 1991). TBARS was assayed to evaluate lipid peroxidation in tissues of low

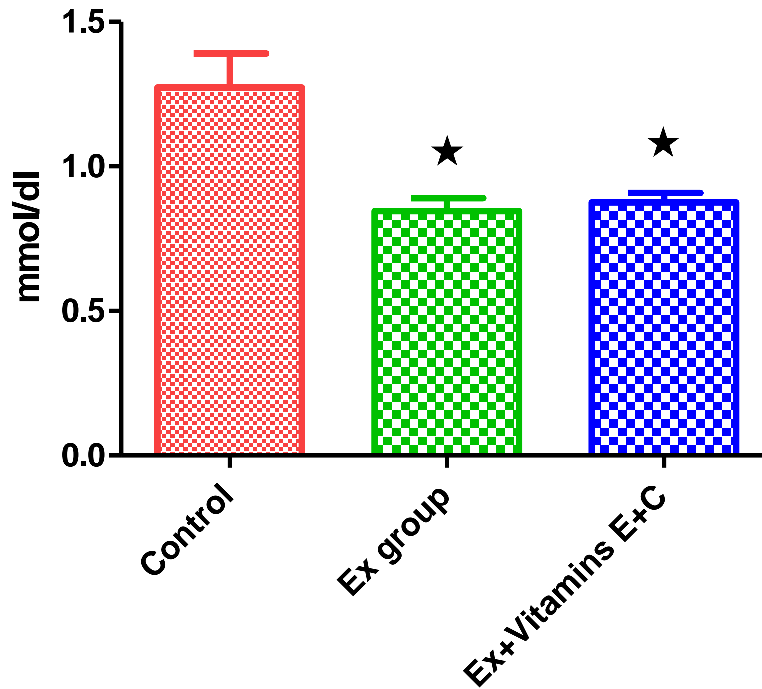

Figure 3. Levels of reduced GSH in the erythrocytes of all experimental groups of rats. Values are given as Mean±SD for groups of six rats each. Values analysis by one way ANOVA. Ex, Exercise group; *statistically different when compared to control group.

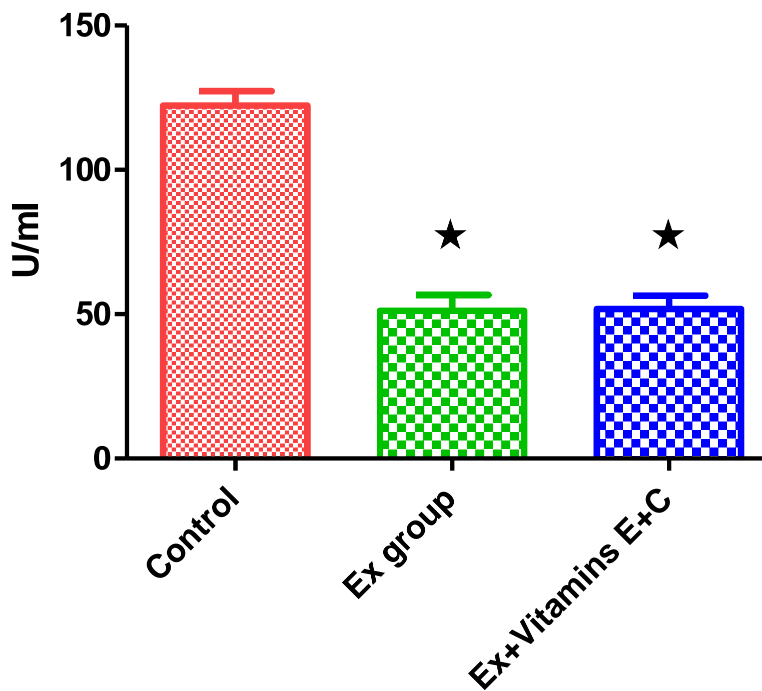

Figure 4. Levels of SOD in the erythrocytes of all experimental groups of rats. Values are given as Mean±SD for groups of six rats each. Analysis was carried out using one way ANOVA. Ex, Exercise group; *statistically different when compared to control group. One unit of SOD is that which cause a 50% inhibition of the rate reduction of INT under the conditions of the assay. SOD, superoxide dismutase.

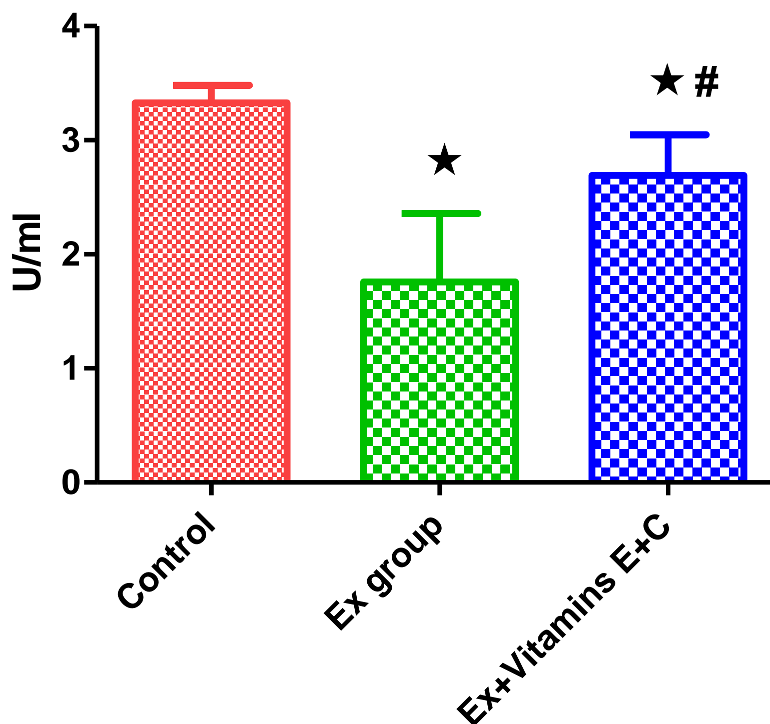

Figure 5. Levels of CAT in the erythrocytes of all experimental groups of rats. Values are given as Mean±SD for groups of six rats each. Analysis was carried out using one way ANOVA. Ex, Exercise group;*statistically different when compared to control group; #significantly different when compared to Ex group. One unit of CAT is the amount of CAT decomposes 1.0 µmol of H_2O_2 per min at pH 4.5 at 25°C. CAT, catalase.

and high altitude rats. Although its specificity has been questioned (Latini et al., 2003), this particular assay is widely used for *in vivo* and *in vitro* measurements (Latini et al., 2003) and is accepted as an empirical window for the examination of the complex process of lipid peroxidation (Davydov and Shvets, 2001). This study's data show that forced exhaustive swimming caused a significant increase in TBARS level in RBC hemolysates of high altitude native rats when compared to the non-stressed (control) group. This finding suggests increased lipid peroxidation and higher rate of generation of free radicals in the RBCs.

The increase in TBARS level was accompanied by significant decreases in both enzymatic (SOD and CAT) and non-enzymatic antioxidant (GSH) systems in RBC hemolysates of high altitude rats after acute forced exhaustive swimming stress. The GSH peroxidase system consists of several components, one of which is GSH (Maher, 2005). GSH serves as a cofactor for GSH transferase, which helps to remove certain drugs and chemicals, as well as other reactive molecules, from cells (Maher, 2005). Moreover, GSH can interact directly with certain ROS, such as hydroxyl radicals, to detoxify them. GSH is probably the most important antioxidant present in cells (Wu and Cederbaum, 2003). Therefore, the significant decrease in GSH level after exercise is a strong evidence for increased generation of free radicals. SOD together with enzymes which catalyze the decomposition of hydrogen peroxide such as CAT reduce the damage done to body cells by oxygen free radicals. SOD catalyzes the rapid removal of superoxide radicals and in the process generates hydrogen peroxide (H_2O_2) while CAT catalyzes the decomposition of H_2O_2 to water and oxygen thus preventing accumulation of toxic levels of H_2O_2.

In this study, forced, exhaustive swimming at highaltitude induced free radicals resulting in decreased activities of SOD and CAT. This decrease in antioxidant enzyme activity may result from their use against generated free radicals and their inhibition by free radical species (Hodgson and Fribdowich, 1985). Hydrogen peroxide is known to inhibit SOD activity by reducing Cu^{2+} to Cu^{+} (Akkus, 1995). Recent studies have also shown that decreased SOD and CAT activities in cells could result from inactivation of these enzymes by interaction with oxygen radicals (Yuksel and Asma, 2006) in liver and lung tissues of high altitude native rats. There are a number of potential sources of ROS under this study's experimental conditions. Hypoxia generates a type of stress called 'reductive stress', a term often used to

describe hypoxia induced change in redox potential in mitochondria (Kehrer and Land, 1994). Work at high altitude is unique because of the influence of reduced oxygen tension on the redox status of working muscle cells. Although, at first glance, it might be assumed that reduced free radical formation would occur due to the reduced oxygen pressure for cellular respiration, in fact, the opposite influence on oxidative stress occurs as shown in this study. During work under the reduced barometric pressure of high altitude, less oxygen is available to terminally accept electrons from oxidative phosphorylation to form water and reducing equivalents accumulate in the mitochondria (Kehrer and Land, 1994).

Under these conditions, ROS may be formed by the auto-oxidation of mitochondrial complexes, such as the ubiquinone/ubiquinol redox coupled with subsequent direct reduction of oxygen to superoxide and the hydroxyl radical (Kehrer and Land, 1994). Another source of ROS under this study's experimental conditions is the xanthine/ xanthine oxidase system. Exercise utilizes large quantities of adenosine-5'-triphosphate (ATP) for muscular contraction producing adenosine diphosphate (ADP) and adenosine monophosphate (AMP), which are normally used to regenerate ATP through oxidative phosphorylation. The combined effects of high rates of ATP utilization and lack of oxygen for regeneration of ATP during exercise lead to accumulation of xanthine and hypoxanthine (Hellsten, 1994). Xanthine is converted to uric acid by xanthine oxidase giving rise to superoxide in a stepwise process. The ensuing accumulation of calcium (due to lack of ATP to pump calcium from the lumen of the mitochondria to the cytoplasm) triggers a calcium-activated protease, which in turn activates xanthine oxidase, leading to superoxide production. This reaction is expected to occur more in metabolically compromised muscles such as those working under hypoxic conditions where ATP is rapidly depleted (Hellsten, 1994).

Some investigators have suggested that the oxidative stress produced by the xanthine/xanthine oxidase system may also contribute to impaired physiologic function in tissues other than muscles, such as the lung (Hoshikawa et al., 2001). Several studies have demonstrated that exercise induced oxidant stress occurring in various tissues of experimental animals and humans may be prevented by antioxidant interventions (Ramel et al., 2004). Vitamin E (α-tocopherol) and Vitamin C (iascorbic acid) are thought to exert protective effects by reducing or preventing oxidative damage. Lipid soluble Vitamin E prevents lipid peroxidation chain reactions in cellular membranes by interfering with the propagation of lipid radicals. Vitamin C interacts directly with free radicals, thus preventing oxidative damage (Beyer, 1994). Due to their different subcellular locations, a combination of Vitamins E and C has been shown to have a better antioxidant effect than either of the two vitamins alone (Rinne et al., 2000), hence both vitamins were used in this study. In this study, a combination of Vitamins E and C supplements did not protect RBCs of high altitude native rats from oxidative damage produced by exhaustive swimming. In comparison to stressed group 1 given normal saline and forced to swim, the Vitamins E and C administered group showed no significant improvement in hematological parameters, serum iron and bilirubin and in the levels of SOD and GSH. There was a moderate improvement in CAT in stressed group 2 rats, but this did offer protection from oxidative damage. These results suggest the inability of these vitamins to ameliorate the antioxidant defense system of the RBCs at these doses, and this may be due to the low concentration of these vitamins absorbed by the RBCs.

Further work using higher doses is highly recommended to be tested in the future. The disappearance of the effect of these vitamins on the antioxidant defense system of the RBCs could be due to decreased absorption of these vitamins by the RBCs at this altitude or due to a maximum uptake of these vitamins by other peripheral tissues such as the liver and lungs at these concentrations used. In a separate study (not published yet), it was discovered that the use of these vitamins at the same concentration with those used in this study ameliorated the oxidative damage in lung and livers of same rats undergoing similar swimming exercise on the same altitude, suggesting maximum absorption of these vitamins by the liver and the lungs rather than the RBCs. One weakness in this study is that the levels of these vitamins were not measured in the peripheral tissues to prove such suggestion. At this time, reference can be made to previous findings by Knight and Roberts (1985) who demonstrated that administration of alpha-tocopherol resulted in high uptake by the liver, whereas administration of alpha-tocopheryl acetate produced the greatest uptake in the lung. So, low uptake by blood may explain the lack of protection by vitamin E of blood tissue seen in this study. Further studies using higher doses of these vitamins may confirm or refute this hypothesis.

Abbreviations

RBC, Red blood cell; **TBARS,** thiobarbituric acid reactive substances; **SOD,** superoxide dismutase; **CAT,** catalase; **GSH,** reduced glutathione; **Hb,** hemoglobin; **PCV,** packed cell volume; **MCV,** mean corpuscular volume; **MCH,** mean cell hemoglobin; **MCHC,** mean cell hemoglobin concentration.

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