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Adaptations of the antioxidant system in erythrocytes of trained adult rats: Impact of intermittent hypobaric-hypoxia at two altitudes

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Abstract

We have investigated the effects of daily exposure to intermittent hypobaric-hypoxia to two simulated altitudes (5700 m and 6300 m) in adult male rats that had been regularly swim trained in normoxia at sea level prior to exposures. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) along with the oxidative stress (OS) indices, malondialdehyde (MDA) and protein carbonyl content were measured in erythrocytes and their membranes. Hemoglobin increased in the trained animals exposed to 5700 m and in untrained rats exposed to 6300 m. Osmotic fragility in terms of hemolysis increased in altitude exposed animals. SOD increased in those exposed to 6300 m, while CAT increased in trained rats exposed to 5700 m and to 6300 m unlike in untrained rats where CAT increased only at 6300 m. GSH-Px showed varying degrees of elevation in all animals exposed to both altitudes. Erythrocyte membranes showed significant elevations in malondialdehyde (MDA) at 6300 m, while elevated protein carbonyls were noticeable at both altitudes in whole cells and membranes. These results suggest a positively associated elevation in protein oxidation with altitude in trained rats. At 5700 m, animals were less stressed, unlike at 6300 m, as seen from the magnitude of elevations in the OS indices and from the responses of the antioxidant enzymes.

Keywords: Catalase; Erythrocytes; Exercise; Hypobaric-hypoxia; Lipid peroxidation; Oxidative stress; Protein oxidation; Superoxide dismutase

1. Introduction

Erythrocytes are continuously exposed to the action of reactive oxygen species (ROS) due to their peculiar biological function. One of the factors involved is hemoglobin (Hb), which is present in large amounts within the erythrocyte. Hb and its oxidative breakdown products stimulate lipid peroxidation (LP). Due to their high ferrous ion concentration, erythrocytes might be exposed to risks of increased oxidative stress (OS), mainly through the formation of ferryl hemoglobin (Giulivi and Davies, 1990), and in part, through the Fenton reaction of hydrogen peroxide (H_2O_2) with ferrous ion (Fe^{2+}) of Hb, that generates the powerful, hydroxyl radical. Erythrocyte membrane proteins are susceptible to covalent damage, including cross-linking and aggregation by free radical-induced LP products. Red blood cells (RBC) have a competent antioxidant system and any ROS formed are effectively scavenged. The mechanism by which the erythrocyte defends itself against oxidative damage is very efficient and is located in both the cytosol and membrane domains. Although the cytosolic antioxidant

Abbreviations: AL_1 (N), altitude 1 (5700 m) normal; AL_1 (T), altitude 1 (5700 m) trained; AL_2 (N), altitude 2 (6300 m) normal; AL_2 (T), altitude 2 (6300 m) trained; AOS, antioxidant system; CAT, catalase; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; HA, high altitude; Hb, hemoglobin; HBT, hypobarotherapy; imosM, ideal milliosmolar; LP, lipid peroxidation; LP-I(+i), lipid peroxidation-induced with catalase inhibitor; LP-I(−i), lipid peroxidation-induced without catalase inhibitor; MDA, malondialdehyde; OS, oxidative stress; PO_2, partial pressure of oxygen; RBC, red blood cells; ROS, reactive oxygen species; SE-C (N), sedentary control; SOD, superoxide dismutase.

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system (AOS) is relatively complex and includes enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), RBC membranes contain vitamin E as a major lipid-soluble chain-breaking antioxidant and membrane stabilizer that avoids LP through its action on a variety of free radicals.

Many studies have indicated that several parameters of RBC function and integrity are negatively affected by increased oxidative stress (OS). In fact, changes in membrane permeability increase LP (Rohn et al., 1998), oxidation of protein sulfhydryl groups and activation of proteolysis when they are challenged with different free radical generating systems.

High altitude (HA) poses a different kind of threat to the erythrocytes. Acute exposure to HA results in a decreased amount of oxygen available to the body and reduced blood oxygen saturation. The reduced pressure of atmospheric oxygen limits energy generation at exactly the time it is most needed. The reduced partial pressure of oxygen at altitude has several consequences for the oxygen economy of the body. Much greater volume of air must be moved by the lungs to compensate for the effectively reduced barometric pressure at altitude. There is a reduction in the ability of oxygen to diffuse from the air to the blood across the alveolar-capillary membrane, thus accentuating the resultant hypoxemia. However, because the body has compensatory mechanisms to the resulting hypoxia, acclimatization is possible. Hypobaric-hypoxia at high altitude leads to OS (Simon-Schnass, 1996) and depletes the body's antioxidant capacity to withstand OS (Askew, 2002). Hypoxic red cells are particularly susceptible to OS, a phenomenon commonly known as 'Oxygen Paradox' (Goldfarb and Sen, 1994) leading to free radical generation. Hypoxia and resultant reductive stress (alteration of redox potential), even without subsequent reperfusion, can give rise to free radical production due to the accumulation of reducing equivalents within the mitochondrial respiratory components. The direct transfer of these reducing equivalents to molecular oxygen can give rise to superoxide, singlet molecular oxygen, hydroxyl radical, nitric oxide and peroxynitrite. A number of potential sources of ROS that might arise under the conditions of hypoxia are briefly referred as 'reductive stress'. Although at first glance, it might be surmised that less free radical formation might occur due to the reduced oxygen pressure for cellular respiration, in fact, the opposite influence on OS seems to occur.

However, a number of studies have shown that endurance training is capable of boosting physiological antioxidant capacity (Sen, 1995). Although enzymic antioxidants have often been studied, our knowledge on the possible benefits of intermittent hypoxia is fairly limited. The present study is an attempt to evaluate the role of physical training at sea level before ascending to high altitudes to overcome the oxidative stress and to obtain an insight on the possible adaptations to hypobaric-hypoxia induced by vacuum equipment, since intermittent hypoxic hypobarotherapy (HBT) is effectively used in medicine to correct many pathological situations in humans.

The current study was therefore intended to (i) expose rats to intermittent high altitudes (5700 m–6300 m), (ii) assess the interactive effects of physical training at sea level followed by high altitude exposures on the erythrocyte's antioxidant system and (iii) evaluate the changes in erythrocyte lipid peroxidation as a marker of oxidative stress under the above conditions. Although our findings are on rats, which do not live naturally at high altitudes, it gives us a rationale to look into induced intermittent hypobaric-hypoxia since rats mirror the physiological processes in humans.

2. Materials and methods

2.1. Materials

Hemoglobin reagent was obtained from Coral Clinical Systems, Goa, India. Thiobarbituric acid, Triton X-100, epinephrine and bovine serum albumin stock were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other chemicals used were of reagent grade and organic solvents were of spectral grade.

2.2. Animal care and maintenance

Studies were conducted on 4-month-old male albino Wistar rats (*Rattus norvegicus*) obtained from the Central Animal Facility, I.I.Sc, Bangalore. 2–3 rats were housed per cage fitted with stainless steel wire-mesh bottoms, at 28±1 °C and under a daily 12-h light and 12-h dark cycle. Animals were fed with lab chow (Lipton India Ltd., Bangalore) and tap water ad libitum. The present study was approved by the Institutional Animal Ethics Committee (IAEC), Bangalore University, India.

2.3. Experimental design

2.3.1. Altichamber for simulation of hypobaric-hypoxia

The unit consisted of a thick-walled high pressure-resistant glass animal chamber fitted with brass lid and with three brass outlets connected to the other components of the unit via vacuum tubes. In brief, the first outlet was connected to a high pressure vacuum pump with a pressure gauge through a copper tube. The second outlet was connected to a manometer which indicates the barometric pressure, while the third outlet was fitted to an adjustable knob to regulate the entry of air and hence the developed pressure in the chamber. During the simulation, pressure was gradually decreased until a particular pressure was reached. This was achieved by exposing the animal to stepwise reduction in pressure by 30 mm Hg/day until the required pressure, i.e., 370 mm Hg at an altitude of 5700 m

and 340 mm Hg (6300 m) were reached. After exposing the animals for 30′or 90′, the pressure was gradually increased to reach the normal level. The schedule was followed every day and for 9 or 15 days (Table 1).

2.3.2. Sea level exercise training and altitude exposure

An outline of the study is shown in Table 1. It consisted of four major stages. First, adult male rats were allowed to swim for 5 min on the first day and duration of swim was gradually increased until they reached 30 min/day and were swimming with an intensity of 3% of their body mass. We have previously shown that for adult (Asha et al., 2003a) and old rats (Kiran et al., 2004) this period as a pre-training phase is essential for them to be trained. The second stage involving the training period lasted for 2 weeks. During the third stage, each of the two groups of animals, those that remained untrained and exposed to altitude 1 (5700 m, pressure 370 mm Hg, 77.39 mm PO_2), AL_1 (N), and those that were swim trained and then exposed to altitude 1, AL_1 (T), were placed in an experimental hypobaric-hypoxic chamber as described above and exposed to 5700 m for 90 min/day and for a total period of 9 days through a gradual increase in altitude per day. A set of two other groups that were either untrained or trained were exposed to altitude 2 (6300 m, pressure 340 mm Hg, 71.11 mm PO_2), AL_2 (N) and AL_2 (T), with a similar pre-altitude exposure until they reached the final altitude of 6300 m but for a reduced period of 30 min/day and an extended period of 6 days compared to AL_1 animals. The fourth stage involved a step-wise descent until the animals reached sea level. One group of five rats was kept at sea-level and served as sedentary controls, SE-C (N).

2.4. Blood sampling

Animals were lightly anaesthetized with ether and restrained in dorsal recumbancy as described earlier (Asha et al., 2003a). In brief, the syringe needle was inserted just below the xyphoid cartilage and slightly to the left of midline. Blood was carefully aspirated from the heart into EDTA-coated tubes.

Erythrocytes were isolated by centrifugation for 20 min at 1000×*g*. The plasma and buffy coat were removed by aspiration. The cells were washed three times with 310 imosM isotonic phosphate buffer, pH 7.4 and finally suspended in an equal volume of the same buffer. This constituted the erythrocyte suspension.

Table 1
Experimental design

Group	Swim training			Altitude exposure	
	Total period (days)	Duration/ day (min)	Intensity (load carried/% body mass)	Period (days)	Duration/ day (min)
SEC (N)	Cage activity only				
SWT (N)					
AL_1 (T)	15	30	3	9	90
AL_2 (T)				15	30
AL_1 (N)	–	–	–	9	90
AL_2 (N)	–	–	–	15	30

SE-C (N), sedentary normal; SW-T(N), swim trained normal; AL_1 (N), altitude 1; AL_1 (T), altitude 1+trained; AL_2 (N), altitude 2; AL_2 (T), altitude 2+trained. AL_1, 5700 m; AL_2, 6300 m.

2.5. Erythrocyte membrane preparation

Hemolysis was achieved by pipetting 2 ml aliquots of washed erythrocyte suspension in tube containing 28 ml of hypotonic buffer (20 imosM, pH 7.4). The contents were mixed by gentle swirling and then centrifuged at 20,000×*g* for 40 min. The supernatant was decanted carefully and the ghost (membrane) was resuspended by adding same strength buffer to reconstitute the original volume. The ghosts were washed three times subsequent to hemolysis. The membrane pellet was resuspended in the isotonic buffer for the assay (Dodge et al., 1963).

2.6. Hemoglobin (Hb)

Hb was measured by the cyanomethemoglobin method. Whole blood was incubated with Hb reagent for 3 min at room temperature and absorbance was measured at 540 nm (Hemocor-D Kit, Coral Clinical Systems, Goa, India).

2.7. Antioxidant enzymes

2.7.1. Superoxide dismutase (SOD, EC 1.15.1.1)

SOD was measured by the method of Misra and Fridovich (1972). 100 µl of hemolysate was added to 880 µl of carbonate buffer (0.05 M, pH 10.2). 20 µl of epinephrine (30 mM in 0.05% acetic acid) was added to the mixture and measured at 480 nm for 4 min. SOD activity is expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50%, which is equal to 1 unit.

2.7.2. Catalase (CAT, EC 1.11.1.6)

CAT was determined by the method of Aebi (1984). Briefly, 100 µl of the hemolysate with 10 µl absolute alcohol was incubated for 30 min at 0 °C followed by addition of 10 µl Triton X-100. An aliquot of 50 µl was taken up in 1.25 ml of 0.066 M H_2O_2 in phosphate buffer and decrease in absorbance was measured at 240 nm for 60 s in a spectrophotometer. An extinction coefficient of 43.6 M cm^{-1} was used to determine enzyme activity. One unit equals the moles of H_2O_2 degraded/min/mg Hb.

2.7.3. Glutathione peroxidase (GSH-Px, EC.1.11.1.9)

GSH-Px was analyzed by the method of Flohe and Gunzler (1984). 50 µl of 0.1 M phosphate buffer (pH 7.0), 100 µl enzyme sample, 100 µl glutathione reductase (0.24 units) and 100 µl of 10 mM GSH were mixed. The mixture

was pre-incubated for 10 min at 37 °C followed by the addition of 100 μl of 1.5 mM NADPH in 0.1% $NaHCO_3$. The overall reaction was started by adding 100 μl of pre-warmed hydrogen peroxide and the decrease in absorption at 340 nm monitored for 3 min.

2.8. Oxidative stress indices

2.8.1. Lipid peroxidation (LP)

Malondialdehyde (MDA), a product of LP, was determined according to the method of Ohkawa et al. (1979). In brief, the sample was added to 8.1% SDS, vortexed and incubated for 10 min at room temperature. This was followed by the addition of 375 μl of 20% acetic acid and 0.6% thiobarbituric acid, and placed in boiling water bath for 60 min. The samples were allowed to cool and 1.25 ml butanol–pyridine (15:1) was added and centrifuged at 640×*g* for 5 min. Absorbance of the colored layer was measured at 532 nm with 1,1,3,3-tetramethoxy propane as standard. MDA concentration was expressed as nmol/mg protein in the membranes and as nmol/g Hb in whole erythrocytes.

2.8.2. Lipofuscin (LF)

The samples were examined under fluorescence microscope (Olympus IX 70). In the micrographs, LF pigments appeared bluish in color. Cells were imaged using cool-snap PC-controlled camera coupled with microimage software for the quantification of fluorescent changes and expressed in arbitrary units. An individual experiment (*n*) was observed in a field of 13–15 cells on a cover slip. Experiments were repeated on five animals on separate cover slips and results are reported as mean±S.E. Changes were analyzed by one-way ANOVA followed by DMRT and considered significant at $P<0.05$.

2.8.3. Protein oxidation

Carbonyl content was measured as an index of protein oxidation as described by Uchida and Stadtman (1993). In brief, the experimental tube included 0.8 ml of the sample and 0.8 ml of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2N HCl; the control tubes included 0.8 ml of sample and 0.8 ml of 2 N HCl. Both the sets were incubated for 1 h. After incubation, 0.8 ml of 20% trichloroacetic acid was added and the contents were centrifuged at 1900×*g* for 10 min. After washing with ethanol/ethylacetate (1:1 v/v), residues were dissolved in 2 ml of 8 M guanidine hydrochloride prepared in 133 mM Tris solution (pH 7.2) containing 13 mM EDTA and centrifuged at 1900×*g* for 10 min. The optical density of each sample was read at 365 nm against the control. The results were expressed as μmol of 2,4-DNPH incorporated/mg protein based on a molar extinction coefficient of 2.1×10^4 M cm^{-1} for aliphatic hydrazones.

2.8.4. Osmotic fragility

The procedure was a slightly modified method of O'Dell et al. (1987). A 100 μl aliquot of washed erythrocyte suspension was added to tubes with 0.3%, 0.4% and 0.9% buffered salt solution (pH 7.4). Tubes were allowed to stand at RT for 30 min, centrifuged at 1270×*g* for 10 min to pellet the cells and the absorbance of the supernatant measured at 540 nm. Hemolysis in each tube was expressed as percentage taking as 100%, the maximum value of absorbance of the distilled water. 0.9% buffered salt solution was considered as control sample.

2.8.5. Hemolysis

A 5% suspension of washed and packed erythrocytes in buffer (310 imosM) was mixed with the same volume of 8 mM H_2O_2 so that the final mixture consisted of 2.5% erythrocyte and 0.5% H_2O_2. The mixtures were incubated at 37 °C for 2 h. One of the samples was pre-treated with 1 mM sodium azide, an inhibitor of CAT, at 37 °C for 10 min before inducing oxidative stress as above. Hemolysis was determined by measuring released hemoglobin into the supernatant of the induced samples at 540 nm and was expressed on the basis of the maximum absorbance (100%) in the aliquots of erythrocytes completely hemolysed in distilled water (Senturk et al., 2001).

2.8.6. Protein determination

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.9. Statistical analyses

Values are expressed as mean±S.E. Significant changes between the groups were analyzed by ANOVA followed by Duncan's multiple range tests (DMRT).

$P<0.05$ was considered significant.

3. Results

3.1. Hemoglobin

Hb content increased in AL_1 (T) and AL_2 animals (Table 2).

Table 2
Hemoglobin content in rats exposed to high altitude

Groups	Hb (g/dl)
SE-C (N)	10.11 ± 0.59^{A}
SW-T (N)	11.6 ± 0.71^{AB}
AL_1 (N)	13.49 ± 0.97^{BC}
AL_1 (T)	13.37 ± 0.50^{BC}
AL_2 (N)	14.06 ± 1.26^{BC}
AL_2 (T)	14.09 ± 0.99^{C}

Values are expressed as mean±S.E. of five animals per group. Changes between the groups are analyzed by one-way ANOVA followed by DMRT. ABC values between groups are significantly different at $p<0.05$ when not sharing the same letters. SE-C (N), sedentary normal; SW-T (N), swim trained normal; AL_1 (N), altitude 1; AL_1 (T), altitude 1+trained; AL_2 (N), altitude 2; AL_2 (T), altitude 2+trained.

3.2. Antioxidant enzymes

There was an increase in superoxide dismutase (SOD) activity AL_1 (N) and AL_2 animals when compared with control [SE-C (N)]. AL_2 showed increased SOD activity over the other experimental groups (Fig 1a). Catalase (CAT) activity increased in altitude-exposed animals compared to control and by two-fold in AL_2 (T) (Fig. 1b). Glutathione peroxidase (GSH-Px) activity increased in AL_1 (N) and AL_1 (T) with respect to control. AL_2 animals showed elevated levels (three-fold) of enzyme activity with respect to control (Fig. 1c).

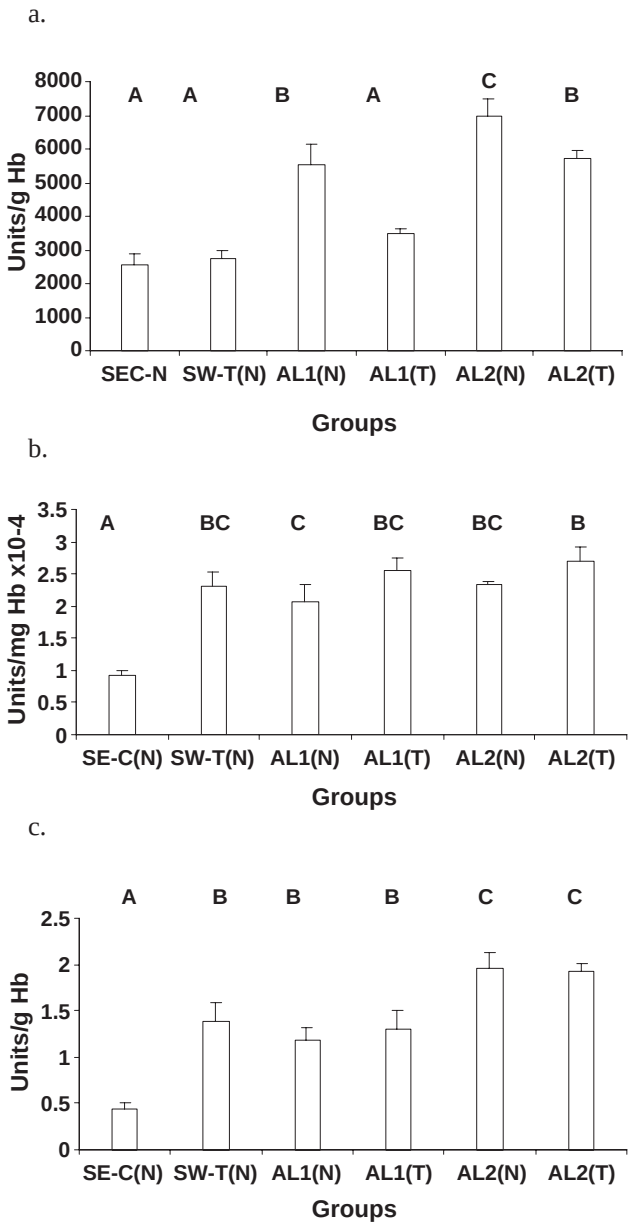

Fig. 1. Antioxidant enzymes in erythrocytes of rats subjected to intermittent hypoxia. (a) Superoxide dismutase (SOD). (b) Catalase (CAT). (c) Glutathione peroxidase (GSH-Px). Values are expressed as mean±S.E. of five animals per group. Significance between groups is analyzed by DMRT and represented in upper case at $p<0.05$. Those not sharing the same letters are significantly different.

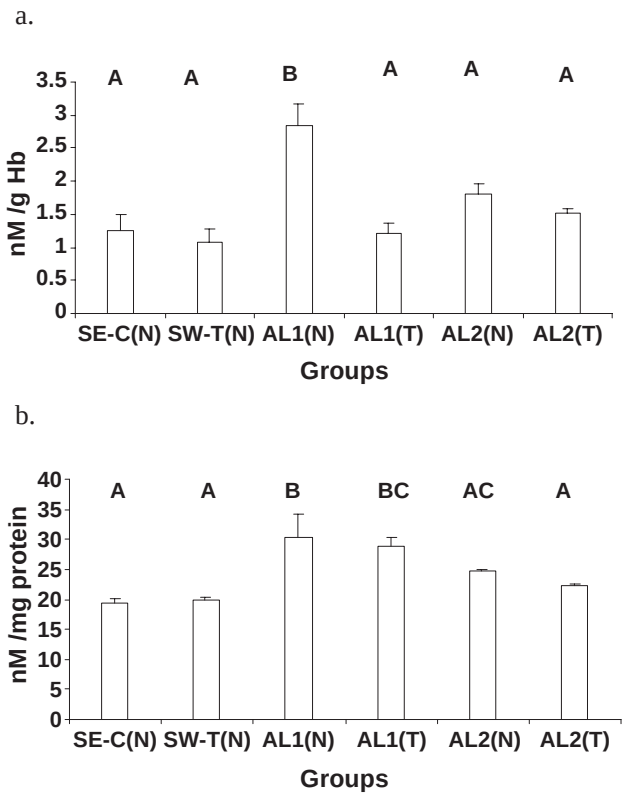

Fig. 2. Lipid peroxidation in erythrocytes of rats subjected to intermittent hypoxia. (a) Lysate. (b) Membrane. LP expressed in terms of MDA. Values are expressed as mean±S.E. of five animals per group. Significance between groups is analyzed by DMRT and represented in upper case at $p<0.05$. Those not sharing the same letters are significantly different.

3.3. Oxidative stress biomarkers

Lipid peroxidation (LP) when measured in terms of MDA concentration showed increase in erythrocytes of AL_1

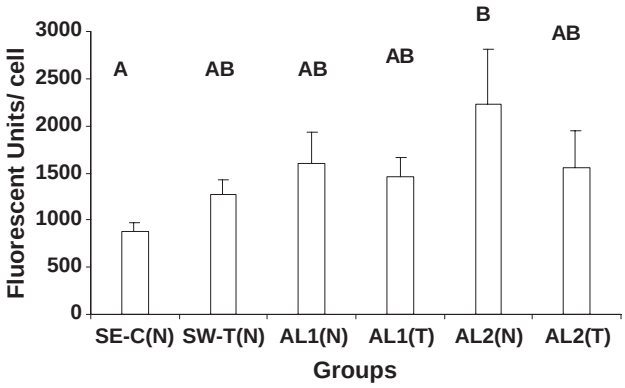

Fig. 3. Lipofuscin in erythrocytes of rats subjected to intermittent hypoxia. Values are expressed as fluorescence units per cell. $n=5$. Changes as analyzed by one-way ANOVA followed by DMRT and represented in upper case at $p<0.05$. Those not sharing the same letters are significantly different.

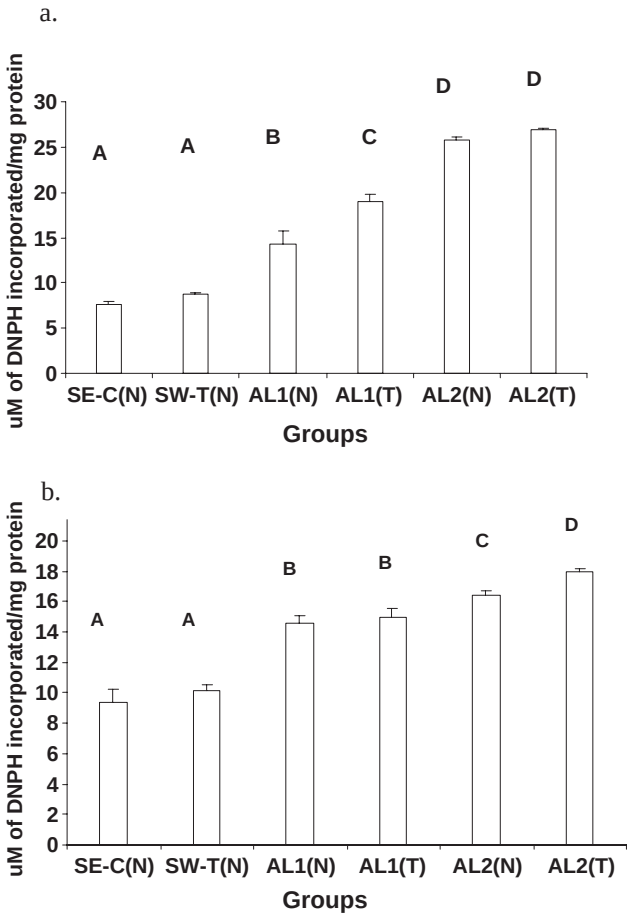

Fig. 4. Protein oxidation in erythrocytes of rats subjected to intermittent hypoxia. (a) Hemolysate. (b) Membrane. It is measured in terms of protein carbonyl content. Values are expressed as mean±S.E. of five animals per group. Significance between groups is analyzed by DMRT and represented in upper case at $p<0.05$. Those not sharing the same letters are significantly different.

(N) compared to control (Fig. 2a). The membranes exhibited elevations in AL_1 (T) animals and AL_2 (N) animals when compared to control (Fig. 2b).

Lipofuscin was identified by the bluish autofluorescence and quantified in terms of fluorescence units/cell, and showed an increment of 152% in AL_2 (N) and 76% in AL_2 (T) when compared to control (Fig. 3).

Oxidative damage to proteins was accompanied by an increase in the carbonyl content in erythrocytes in altitude-exposed animals (Fig. 4a). Membrane carbonyl content in

Table 4
In vitro hemolysis in rats exposed to high altitude

Groups	LP-I(−i)	LP-I(+i)
SE-C (N)	79.1 ± 1.76^{A}	91.74 ± 2.45^{A}
SW-T (N)	80.7 ± 1.56^{AC}	94.91 ± 1.96^{AC}
AL_1 (N)	82.57 ± 4.03^{ABC}	104.17 ± 4.97^{ABC}
AL_1 (T)	87.98 ± 1.69^{B}	104.9 ± 7.02^{B}
AL_2(N)	94.58 ± 2.95^{B}	103.18 ± 4.15^{B}
AL_2 (T)	93.33 ± 1.23^{BC}	97.42 ± 4.74^{BC}

Hemolysis is expressed as %. Values are expressed as mean±S.E. of five animals per group. SE-C (N), sedentary normal; SW-T (N), swim trained normal; AL_1 (N), altitude 1; AL_1 (T), altitude 1+trained; AL_2 (N), altitude 2; AL_2 (T), altitude 2+trained. LP-I(−i) and LP-I(+i) induced lipid peroxidation in the absence and presence of CAT inhibitor, respectively. Changes between the groups are analyzed by two-way ANOVA followed by DMRT. ABC values with different superscripts between groups in each column are significantly different at $p<0.05$. Changes seen between LP-I(−i) and LP-I(+i) are significant.

AL_1 animals showed an increment over the control whereas in the AL_2 animals; there was an elevation over the other experimental groups (Fig. 4b).

Osmotic fragility is measured in terms of % hemolysis. In AL_1 (T) and AL_2 animals, there was increase in 0.3% and 0.4% in comparison to control (Table 3).

Hemolysis was measured as a marker of OS in erythrocytes. AL_1 (T) and AL_2 animals showed increase in hemolysis over the control as well as between LP-I(+i) and LP-I(−i) (Table 4).

4. Discussion

Erythrocytes are perhaps the cells most exposed to peroxidative damage by free radicals. During their relatively short life, in which no protein synthesis occurs, the cells come in close contact with free radicals from various sources (Constantinescu et al., 1993).

Antioxidant defenses in the red cell can temper the negative influence of free radicals and related reactions and keep them in check (Goldfarb, 1993; Packer, 1997). SOD, GSH-Px and CAT form a substantial defense network against oxidative stress (Sen, 1995; Clarkson and Thompson, 2000) imposed by physical activity. We have previously reported age-related elevations in the activities of total SOD, GSH-Px and CAT (Asha et al., 2003b) as well as of Mn-SOD (Kiran et al., 2004) in the heart ventricles of

Table 3
Osmotic fragility in terms of % hemolysis in rats exposed to hypobaric-hypoxia

Concentration % NaCl	SE-C (N)	SW-T (N)	AL_1 (N)	AL_1 (T)	AL_2 (N)	AL_2 (T)
	A	AB	B	C	C	C
0.3[a]	14.06±1.37	20.88±3.06	26.09±3.89	37.69±7.9	37.31±2.19	36.61±2.03
0.4[a]	13.72±1.96	17.06±2.59	23.46±2.32	34.26±6.2	38.58±3.04	35.58±3.71
0.9[b]	10.28±1.87	14.89±2.28	16.65±1.78	27.07±5.7	24.84±1.66	25.68±1.73

Values are mean±S.E. of five animals per group. SE-C (N), sedentary normal; SW-T (N), swim trained normal; AL_1 (N), altitude 1; AL_1 (T), altitude 1+trained; AL_2 (N), altitude 2; AL_2 (T), altitude 2+trained. Significance between the groups in the columns as analyzed by two-way ANOVA followed by DMRT is significant at $p<0.05$ and is represented in upper case. Changes between the concentrations in the rows are represented in lower case.

male rats for overcoming oxidative stress under physiologic situations such as exercise and aging.

Hypoxia can trigger a cascade of signaling events that ultimately lead to adaptation at high altitude. Repeated episodes of hypoxia interspersed with episodes of normoxia results in periods of intermittent hypoxia characterized by free radical production. Usually, 5000 m–5500 m is considered as high altitude (Nakanishi et al., 1995; Simon-Schnass, 1996; Westerterp-Plantenga et al., 1999) and we exposed our animals to a higher altitude of 6300 m, since the responses were insignificant at an altitude of 5500 m. Higher hemoglobin levels were found in samples drawn from AL_1 (T) and AL_2 animal. The increase in the former group may be due to the fact that training can induce shorter life span of erythrocytes, leading to younger cells that emerge with a better antioxidant defense (Smith, 1995; Smith et al., 1995).

Animals trained at moderate intensity (3% of their body mass) and exposed to 5700 m could resist the oxidative stress compared to untrained rats as evident from our results on similar activity of SOD, in contrast to elevated SOD in the altitude-exposed. However, the effect was different in those exposed to 6300 m in that the OS was felt to a greater extent. An increase in SOD activity indicates that hypobaric-hypoxia may be effective in inducing higher SOD activity and hence effective in scavenging free radicals.

The present study revealed elevations in erythrocyte CAT activity in the untrained as well as trained rats exposed to 5700 m and 6300 m. Our findings on the antioxidant enzymes are in accordance with those reported by Robertson et al. (1991). CAT is a major H_2O_2-decomposing enzyme in normal erythrocytes (Gaetani et al., 1996; Mueller et al., 1997). In the present study, parallel experiments on in vitro H_2O_2-induced OS in the CAT-depleted cells also revealed elevated lipid peroxidation and protein damage. Erythrocyte CAT activity showed an increase following training, suggesting the capacity to overcome hypoxia in the trained (Robertson et al., 1991) and possibly also extended to situations such as intermittent hypobaric-hypoxia at high altitudes. Furthermore, a two- to three-fold increase in the GSH-Px activity of animals exposed to 5700 m and 6300 m may indicate a higher oxidative stress in the latter and our results are somewhat similar to that reported by Nakanishi et al. (1995) on elevated GSH-Px activity in the heart and lungs during hypobaric-hypoxia. GSH-Px is an enzyme capable of destroys the end products of ROS generation pathway involving H_2O_2 and organic peroxides (Ji, 1999).

MDA content measured as a suitable marker of LP was of a similar magnitude in erythrocytes of animals exposed at 5700 m as well as 6300 m. Oxidant generation during physical training may influence the adaptive responses and confer protection against OS, and thereby suggesting the magnitude of protective adaptation (Bailey et al., 2001) under hypoxia. An elevated level of LP is a sufficiently reliable factor most often sought after in support of the oxidative stress. Increase in MDA of erythrocyte membranes of altitude-exposed animals in contrast to whole cells suggests the possible sites of unsaturated phospholipids, glycolipids and cholesterol and other organized systems as prominent targets of oxidant attack. These can result in a degenerative process that perturbs the structure and function of the target system (Girotti, 1998). A greater reduction of MDA in membranes from sea level trained and exposed to high altitude of 6300 m as compared to 5700 m may be attributed to a better protection from OS through elevated antioxidant enzymes in the former situation.

LF, one of the products of lipid peroxidation, is undegradable and cannot be removed via exocytosis (Brunk et al., 1992). Brunk and Terman (2002) have proposed a possible role for the involvement of OS in LF formation. In the present study, OS elicited changes in LF only in animals exposed to 6300m, may be attributed to the intermittent rather than a continuous hypobaric-hypoxic condition.

Oxidation of proteins by free radicals plays a major role in many oxidative processes within the cells (Hawkins and Davies, 2001), the magnitude of which is often measured by the carbonyl content (Stadtman and Oliver, 1991; Adams et al., 2001). Erythrocytes of animals exposed intermittently to 6300 m exhibited high carbonyl content indicative of higher protein oxidation under hypoxic stress and hypobarism. But the cells of those exposed to 5700 m showed increase, but to lesser extent with respect to 6300 m exposed animals. There could be a possibility that the lipid-soluble vitamin E acts to reduce the ROS to a certain extent. The scenario was different at 6300 m in that a higher membrane carbonyl content was seen, a situation that may call for supplementation of antioxidants to overcome the additional OS. It is worth noting that membrane proteins are the major targets of post-biosynthetic alterations during erythrocyte intravascular aging, a situation similar to oxidative stress, both processes involve an increased generation of free radicals (Bartosz, 1990; Gallagher et al., 1998).

Osmotic fragility has been studied as a marker of hemolysis in erythrocytes at different salt concentrations. Osmotic stress at 0.3% and 0.4% NaCl imposed hemolysis in animals exposed to hypobaric-hypoxia and thereby signifying an oxidative stress-dependent impairment of erythrocyte stability. Extensive lipid peroxidation in biological membranes causes alterations in fluidity, falls in its membrane potential and increases in its permeability to different ions followed by an eventual rupture (Halliwell and Gutteridge, 1990). Hemolysis caused by radicals can be characterized mainly by two key events: LP and redistribution of oxidized band 3 within the membrane (Sato et al., 1995), studies on which are under progress. Our findings on elevated hemolysis in part may be attributed to these events that were higher in animals exposed to hypobaric-hypoxia. Our findings on in vitro studies in CAT-inactivated erythrocytes have revealed a higher % hemolysis. A positive correlation between H_2O_2-

induced membrane LP and osmotic fragility of erythrocytes suggests the existence of a similar condition as that of in vivo.

In summary, in the present protocol, healthy rats that underwent daily moderate swim training at sea level were exposed to a single bout of intermittent hypobaric-hypoxia daily (30 min) over a 2-week period. The daily imposition of exercise appeared to result in a cumulative oxidative stress in erythrocytes as demonstrated by the stable hydrazone derivatives. MDA, a marker of oxidative damage to lipids, exhibited a direct correlation with protein carbonyls in the stressed erythrocytes, indicating that the free radicals produced during exercise training as well as altitude exposures attack not only the lipids, but also the proteins. In contrast to the subtle changes at 5700 m, hypoxia during hypobaric exposures at 6300 m is a more effective stimulus for triggering metabolic adaptations that may be required for continuous hypoxic exposures lasting for a longer period. The resulting intermittent hypoxic adaptations may be extremely beneficial in terms of the protection that they may offer against pathological conditions and in sports performance, yet another situation wherein one stress acts on another for overcoming free radical injury especially at higher altitudes exceeding 5700 m. Our findings will give an insight into the benefits that may be derived from intermittent hypobaric therapy. Although our results on erythrocytes are fairly interesting, more studies need to be pursued in the direction of suitable antioxidant supplementations at different altitudes besides training at sea level and before any conclusions can be made on the interacting factors that can overcome oxidative stress caused by hypobaric-hypoxia.

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