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Oxidative Stress and Histopathological Evaluation of Rat Lung Tissue during Hypobaric Hypoxia

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Abstract

Hypobaric hypoxia (HH) is a condition associated with low partial pressure of oxygen in the atmosphere. This lowering of oxygen content in atmosphere leads to decreased circulating oxygen in the humans residing in these regions. This lowering of oxygen does not cause any serious complications in residents due to adaptation. On the other hand these extreme environments are challenging for high altitude (HA) sojourners as they develop range of illness from i.e. from mild altitude sickness to fatal diseases. Several studies have been carried on altitude environment to focus physiological complications and adaptation. Serum-based clinical studies are comparatively easy due to availability of human volunteers. Tissue-based studies are still challenging as the development of animal model is the only approach. Herein we report lung tissue based oxidative stress markers level in conjunction with histopathological findings in rodent model. Male Wistar rats were exposed to 338 mm Hg pressure, simulating an altitude of 15,000ft in decompression chamber. Animals were used exposed for different durations i.e. 2 h, 4h, 8h, 12h, 16h, 24 h and 48 h (n=9/group). Partial pressure of oxygen (PO_2) and carbon dioxide (PCO_2) in arterial blood of animals were checked to confirm induction of hypoxia. Decreased value of PO_2 and PCO_2 during different exposure groups confirmed successful induction of hypoxia in animals. Altered valve of superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), lipid peroxidation (LPO) and reduced glutathione (GSH) in lung tissue of these groups confirmed occurrence of oxidative stress. Lung histopathological analysis of hypoxic groups provided evidence of lung injury on prolonged exposure. Short term hypoxic exposure (2h-4h) did not showed any major effect on lung architecture but with increased duration of exposure (8h-16h) deformities in lung parenchyma were observed, leading to development edema during 24h-48h of exposure. Alteration in oxidative stress markers during 12h-16h can be correlated with common altitude sickness which on prolong exposure (24h-48h) can turn into serious complication like pulmonary edema.

Keywords: Hypobaric hypoxia; Hypoxia chamber; PO_2; PCO_2; Histopathological analysis; Oxidative stress

Abbreviations: HH: Hypobaric hypoxia; PO_2: Partial pressure of oxygen; PCO_2: Partial pressure of carbon dioxide; HAPE: high altitude pulmonary edema

Introduction

In the field of high altitude (HA) medicine, the term HA refers elevations over 1500 m (4921 ft), and is commonly divided into three regions: high altitude (4,921 ft–11,483 ft), very high altitude (11,483 ft-18,045 ft) and extreme altitude (18,045 ft–29,035 ft) [1]. Environmental conditions at these elevations become increasingly inhospitable with progressive ascent. These environmental conditions include diminished ambient oxygen pressure, decreased temperature, lower humidity, and increased ultraviolet radiation [2]. Hypoxia arising due to these conditions is collectively know n as hypobaric hypoxia (HH) [3]. High altitude stresses also have medical consequences commonly termed as altitude related illnesses. High altitude environments provide scientists a natural laboratory to study the genetic and physiological aspects of HH in endemic highlanders and species [4,5]. High altitude sojourners travel to altitudes for several reasons; if not acclimatized, suffer altitude sickness. High altitude disorders are also challenging to for our army people who are employed at HA regions.

Hypobaric hypoxia affects different organs like kidney, brain, liver, and lung [6,7]. Among these organs lungs are the first interface between oxygen in the environment and the metabolic machinery of the body [8-10]. Gases exchange takes place among these two at the alveolar-capillary membrane which is necessary to achieve an adequate supply of oxygen to the tissues and for the elimination of carbon dioxide [11]. The ability of alveolar epithelial cells to cope with low oxygen tensions is crucial to maintain the structural and functional integrity of the alveolar epithelium. Alveolar epithelial cells appear to be remarkably tolerant to oxygen deprivation as they are able to maintain adequate cellular ATP content during prolonged hypoxic exposure [12] and hence considered as an elegant gatekeeper between environmental hypoxia and physical performance at high altitude [8,13].

Hypoxia is a life-threatening stress that must be dealt with at both cellular and systemic levels [12]. In the lung, hypoxia is the natural consequence of many respiratory diseases resulting from inadequate alveolar ventilation, as observed in chronic obstructive lung disease or in pulmonary edema due to heart failure or acute lung injury and above all HA [14]. Although lungs are considered to be tolerant to hypoxia there are precedents of lung malfunction under low oxygen pressure conditions.

A unique consequence of HA is formation of reactive oxygen species (ROS). Formation of ROS under excessive oxygen is a natural phenomenon [15,16]. The formation of ROS in the hypoxic context has been a subject of wide ranging debate [14,17]. Logic tells us that there

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is a need of oxygen for the generation of ROS and oxidative damage; likewise, it would be unlikely that oxidative damage occurs when this element is less available [18]. Currently, this paradigm has been changed by identifying hypoxia as the generator of ROS and oxidative damage for systems as well as specific organs. This generation of ROS triggers several enzymatic as well as non-enzymatic antioxidants present in the tissues [19,20]. In lungs important antioxidant enzymatic system includes SOD, CAT and GR [21] and non-enzymatic part includes malonaldehyde (MDA) and reduced glutathione (GSH) especially in case of hypoxia [6,22]. GSH is an abundant antioxidant in lung epithelial cells and is the key molecule to modulate the development of inflammatory oxidative pulmonary injury [23]. Oxidative stress to lipids (lipidperoxidation) is one of the most studied phenomena regarding oxidative stress by hypoxia [24]. A significant increase in thiobarbituric acid reactive substances (TBARS) in rats exposed to 12% O2 for 48 h was found [25]. Hence evaluation of above antioxidant system makes an important part of hypoxia study. It has also been reported that ROS formation increases in both ischemia and reperfusion (we are referring hypoxic ischemia i.e. blood flow to cells and organs is not sufficient to maintain their normal function, combined with a lowered oxygen concentration in arterial blood) [26]. Hypoxic Ischemic Encephalopathy (HIE) is one of the major brain disorder occur in infant when an infant's brain doesn't receive enough oxygen and blood [27]. Although restoration of blood flow to an ischemic organ is essential to prevent irreversible tissue injury, reperfusion may result in a local and systemic inflammatory response that may augment tissue injury. The pathogenesis of ischemia reperfusion injury begins with a hypoxic insult to the vascular endothelium which further demonstrates protective responses [28]. Although oxidative damage associated with hypoxia on the organism has been the subject of several studies [29-31] the knowledge of the effects on lung tissue is relatively poor, particularly in humans. The latter is probably because of the difficulty in obtaining samples of this organ. The condition remains the same for histopathological changes also. Evidence of oxidative damage by hypoxia, in the lungs, is consistent; however, the mechanisms about how it is produced are still being discussed.

At present tissue based HA studies have limitations dues to availability of sample. It is quite difficult to obtain human tissue sample especially in HA studies. The best solution to this limitation is development of animal models. To date, animals like rabbit, sheep, horse have been used in HA studies but rodent model is the most widely used animal model. Due to its small size handling becomes comparatively easy. It has relatively short life span which permits the observation of different cellular changes and above all reproducibility of results is possible in quite short time [32,33]. Hypobaric hypoxic experiments are performed in especial chambers (simulation/decompression chamber) and rats can be easily placed in large number at one time due to small in size. For the above reasons we have used Wistar rats as our experimental animal.

Materials and Methods

Animals

Male Wistar rats (150-180g, 8week old) were procured from central animal house of Jamia Hamdard, New Delhi. They were housed in polypropylene cages in groups of nine rats per cage under standard conditions (25 ± 2°C, 50 ± 10% relative humidity with a 12-h light/dark cycle). The animals were given free access to standard laboratory feed (Amrut Laboratory, rat and mice feed, Navmaharashtra Chakan Oil Mills Ltd., Pune, India) and water ad libitum. All the experiments were performed in accordance with the Institutional Animal Ethics Committee constituted as per directions of the Committee for the Purpose of Control and Supervision of Experimental Animals (173/CPCSEA) under the Ministry of Animal Welfare Division, Government of India, New Delhi.

Chemicals and reagents

Oxidized glutathione (GSSG), NADPH, thiobarbituric acid (TBA), hydrogen peroxide, trichloroacetic acid (TCA), EDTA, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and Tris buffer were purchased from Merck, Ltd. and Himedia, India. All other chemicals and reagents were of analytical grade.

Experimental protocol

Male Wistar rats (8 weeks old; 150-180gms) were randomly divided into eight groups with 9 animals in each group. Experimental animals were placed in simulation chamber (barometric pressure 338 mm Hg, equivalent to a height of 15,000 ft). The airflow into the chamber was maintained at 8 l/min. The control animals were exposed to normoxia-barometric pressure, 740 mm Hg and temperature, 25°C. The experimental design and exposure protocol is shown in Table 1.

Arterial blood gases (ABG) analysis

On the day of experiment, immediately after removing the rats from the simulation chamber, rats were then anesthetized with sodium pentobarbitone; (50 mg/kg i.p) and blood was quickly collected by direct puncture and sampling from the left ventricle using heparinised syringes. All blood samples for arterial blood gas analysis were immediately analysed by blood gas analyzer (combi line analyzer system).

Tissue preparation

Rats were then euthanized and their lungs were excised immediately and perfused with ice-cold saline. To prevent auto-oxidation or *ex vivo* oxidation of the tissue, homogenization was carried out at 4°C in lysis buffer containing 7M urea, 2M thiourea, 4% CHAPS, 20mM DTT, 40mM Tris HCl (pH 7.4) and 1mM PMSF. The homogenate was then centrifused at 8000 ×g for 20 min and supernatant was collected and stored at -80°C for protein analysis. For histopathological studies portion of tissue were post-fixed in buffered formalin (10 %) for 24 h. Following fixation, sections (3-4 mm in thickness) of these tissues were dehydrated with absolute alcohol, embedded in fresh paraffin and allowed to cool. At least four crosssections (5 µm thick) from each tissue were cut on a microtome and stained with haematoxylin and eosin (H & E). The tissue sections were then washed twice (2 min each) with xylene and later mounted with DPX mountant. The slides were observed for histopathological changes and microphotographs were taken using an Olympus BX50 microscope system (Olympus, Japan).

Group	Duration of exposure
Control	No hypoxic exposure
Group I; 2 hr. exposure	Animals were given HH exposure for 2 hour
Group I; 4 hr. exposure	Animals were given HH exposure for 4 hour
Group III; 8 hr. exposure	Animals were given HH exposure for 8 hour
Group IV; 12 hr. exposure	Animals were given HH exposure for 12 hour
Group V; 16 hr. exposure	Animals were given HH exposure for 16 hour
Group VI; 24 hr. exposure	Animals were given HH exposure for 24 hour
Group VII; 48 hr. exposure	Animals were given HH exposure for 48 hour

Table 1: Experimental animals group on the basis of duration of exposure to hypoxia.

Biochemical estimations

Lung tissue was homogenized in a chilled phosphate buffer (0.1 M, pH 7.4) using a Polytron homogenizer (tissue homogenate=10%w/v). This homogenate was used for the estimation of LPO. The homogenate was further centrifuged at 800 ×g for 5 min at 4°C to separate the nuclear debris. The supernatant was further centrifuged at 10,000 ×g for 20 min at 4°C to get PMS (pos-mitochondrial supernatant), which was used for various biochemical assays.

Assay for catalase: Catalase (CAT) activity was assayed by the method of Claiborne [34]. Briefly, the assay mixture consisted of 0.05 M phosphate buffer (pH 7.0), 0.019 M hydrogen peroxide (H_2O_2) and 0.05 ml PMS in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. CAT activity was expressed as nmoles H_2O_2 consumed/min/ mg protein.

Assay for glutathione peroxidase: Glutathione peroxidase (GPX) activity was estimated using coupled enzyme assay with glutathione reductase (GR). As a result of GPX activity, the glutathione disulphide is produced, which is immediately reduced by GR thereby maintaining a constant level of GSH in a reaction system. The assay takes advantage of concomitant oxidation of NADPH by GR, which was measured at 340 nm [35,36]. Specific activity of enzyme was measured according to the procedure described by Mohandas et al. [37]. The reaction mixture consisted of 1.53 mL phosphate buffer (0.05 M, pH 7.0), 0.1 mL of ethylene diaminetetra acetic acid (1 mM), 0.1 mL of sodium azide (1 mM), 0.1 mL of glutathione (1 mM), 0.1 mL of NADPH (0.2 mM), 0.01 mL of hydrogen peroxide (0.25 mM) and 0.02 mL of PMS in a final volume of 3 mL. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nmole NADPH oxidized/ min/mg protein

Assay for superoxide dismutase: SOD has an important role in catalyzing the conversion of superoxide to H_2O_2 and O_2. Superoxide dismutase activity was determined for its ability to inhibit the auto-oxidation of pyrogallol according to the method of Nandi and Chatterjee [38]. Tissue homogenate was prepared in ice-cold 0.25 M sucrose solution containing 0·5% Triton X-100. The crude homogenate was centrifuged at 34,800 *g* for 30 min and the supernatant was used. The reaction mixture (1 mL) consisted of 50 mM Tris (hydroxymethyl) aminomethane (pH 8.2), 1 mM diethylenetriamine pentaacetic acid, and 50 µL of homogenate. The reaction was initiated by the addition of pyrogallol (final concentration of 0.2 mM), and the absorbance was measured kinetically at 420 nm (25°C) for 3 min. SOD activity was expressed as unit per milligram protein.

Assay for malonaldehyde: Thiobarbituric acid reactive substances (TBARS), measure of the end product of lipid peroxidation. It was determined by the method as described by Wright et al. [39]. A reaction mixture in a total volume of 2.0 mL contained 1.8 mL of phosphate buffer (0.1 M, pH 7.4), 0.2 mL lung homogenate. The reaction mixture was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1.0 mL TCA (10 % w/v). Following the addition of 1.0 mL TBA (0.67 % w/v, prepared in warm distilled water), all the tubes were placed in a boiling water bath for 20 min. In the end, the tubes were cooled on ice and centrifuged at 2,500 ×g for 10 min, the supernatant containing the thiobarbituric acid reactive substances (TBARS) formed in each sample was assessed by measuring the optical density at 535 nm. The results were expressed as nmoles of MDA formed/ mg protein using a molar extinction coefficient of 1.56 × 105/M/cm.

Assay for reduced glutathione: Reduced glutathione (GSH) in the lung was determined by the method of Jollow et al. [40]. One mL of PMS was precipitated with 1 mL of sulphosalicylic acid (4%). The samples were kept at 4°C for 1 h, centrifuged at 1,200 ×g for 20 min at 4°C and the supernatant was separated. The assay mixture in a total volume of 3.0 mL contained 0.1 mL of above supernatant, 2.7 mL phosphate buffer (0.1 M, pH 7.4) and 0.2 mL 100 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The acid and GSH react to generate yellow colored complex of 5-thio-2-nitrobenzoic acid. Therefore, GSH concentration can be determined by measuring absorbance of yellow colour complex at 412 nm. The activity was calculated using GSH as standard and expressed as mmol conjugate glutathione/g tissue.

Histopathological examinations

For histological examinations, lung sections from different groups were stained with hematoxylin and eosin (H&E). Lung were removed quickly and postfixed in buffered formalin (10 %) for 24 hr. After fixation was completed, slices (3-4 mm) of these tissues were dehydrated and embedded in paraffin. At least four cross sections were taken from each tissue in 5 µm thickness and stained with H&E. Following two washings with xylene (2 min each), tissue sections were mounted with DPX mountant. The slides were observed for histopathological changes, and microphotographs were taken using an Olympus BX50 microscope system (Olympus, Japan).

Statistical analysis

The data are expressed as mean ± standard deviation (n=9). Analyses are done using the GraphPad InStat 3 software. Comparisons of the means of sera of control and treated rats were made by repeated measures ANOVA followed by Tukey test, with, *P<0.05, **P<0.01 and ***P<0.001 as limits of significance.

Results

Arterial blood gas analysis and behavioral changes of hypoxic Vs control rats

Arterial blood gases is a collective term applied to three separate measurements i.e. pH, PO_2, and PCO_2 generally made together to evaluate acid-base status, ventilation, and arterial oxygenation. Oxygen and carbon dioxide are the most important respiratory gases, and their partial pressures in arterial blood reflect the overall adequacy of gas exchange (Trulock; Arteral blood gases: Clinical methods) [41]. Arterial PO_2 and PCO_2 were found lowered in animals upon exposure, showing that the animals were hypoxic. The values have been shown in Figure 1.

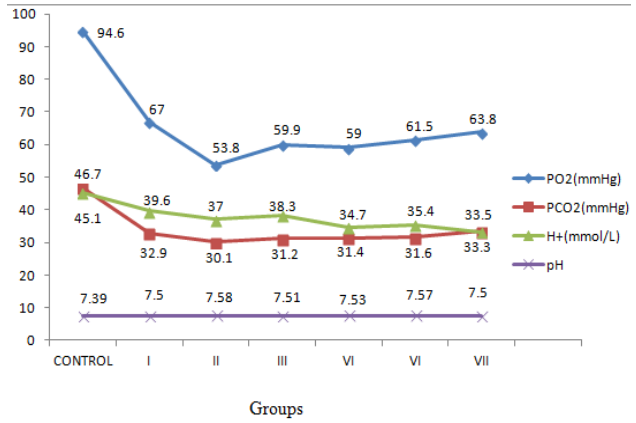

Figure 1: Arterial blood gas analysis of control and hypoxic rats.

The animals from both the control and hypoxic groups were examined regularly for any behavioral changes. The following observations were made there after:

Control - No signs of lethargicity (-)

Group I-II Animals were haphazardly moving within the cage

(Suffocating appearance)

Group III-IV Little sign of lethargicity (+)

Group V-VI - Increased sign of lethargicity (++)

Group VII Completely lethargic (+++)

(We consider lethargicity level (as sign fatigue which is one of the apparent behavioral change observed during assent to high altitude) on the basis of active movement of rats within the cages in hypoxia chamber). High altitude exposure impairs physical activities, sleep patterns, mood, and cognitive functions [42,43] and therefore we considered the above changes in animals as behavioral response to hypoxia.

No remarkable weight loss was observed in animals as they were provided with food and water in the chamber throughout the exposure.

Biochemical estimation

Generation of ROS during hypoxia is a proven fact and hence antioxidants measurement is an important parameter to evaluate hypoxic condition. Antioxidants can be classified according to their nature as enzymatic or nonenzymatic antioxidants. In lungs, we have evaluated CAT, GPX, and SOD as enzymatic reducers; LPO and GSH as noenzymatic reducers. These have been considered as major enzymatic and no enzymatic reducers in lungs [44]. The values of CAT, GPX, SOD, LPO and GSH in lung tissue of control and hypoxic rats showed that there was significant difference in oxidative stress markers between control and hypoxic animals after certain period of exposure.

Enzymatic reducers

Catalase, glutathione peroxidase and superoxide dismutase: CAT and GPX activities were also found to be altered in hypoxic groups (Figure 2 and Figure 3 respectively). The CAT activity in initial exposure groups (gr.I-IV) showed non-significant difference in comparison to control. A significant increase by 4.4% ($P < 0.01^{**}$), 7% ($P < 0.001^{***}$), 9.1% ($P < 0.001^{***}$) was observed in gr.V, gr.VI and gr.VII respectively as compared to control. The GPX activity was also found to be altered in hypoxic rats. Significant increase of 5% (P<0.01**), 7% (P<0.001***), 10% (P<0.001***), 12% (P<0.001***) in gr.IV, V, VI and VII respectively as compared to control. SOD activity was found significantly decreased in hypoxic groups (Figure 4). A significant decrease of 12.5% (P<0.001***), 18.5% (P<0.001***), 21.2% (P<0.001***), 25% (P<0.001***) were observed in gr.IV-VII respectively in comparison to control.

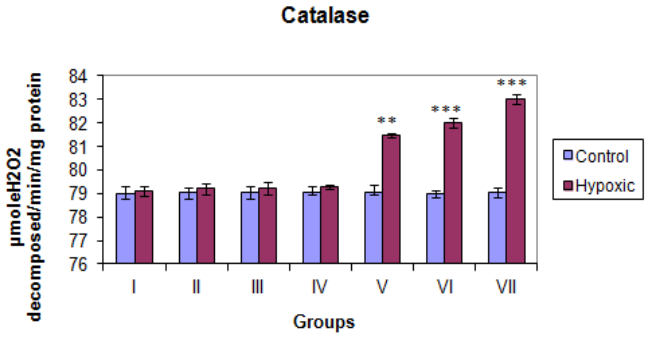

Figure 2: Levels of catalase activity (nmole H_2O_2 decompose/ min/ mg protein) measured in the lung tissue of control and hypoxic groups reduced. Values are expressed as mean+SD, n=9; and are significantly different (***P<0.001).

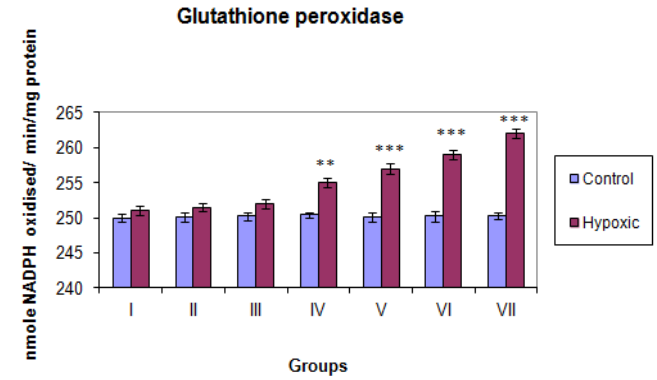

Figure 3: Glutathione peroxidase levels (nmole NADPH oxidized/min/mg protein) measured in the lung tissue of control and hypoxic groups reduced. Values are expressed as mean+SD, n=9; and are significantly different (***P<0.001).

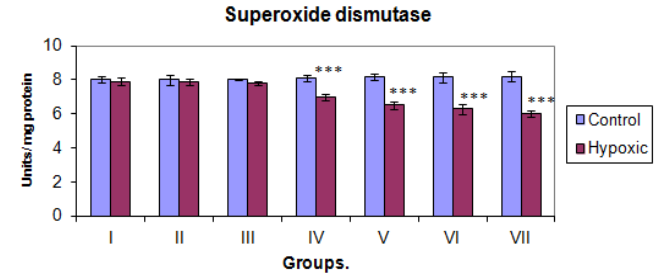

Figure 4: Levels of Superoxide dismutase activity (unit SOD/mg protein) measured in the lung tissue of control and hypoxic groups reduced. Values are expressed as mean+SD, n=9; and are significantly different (***P<0.001).

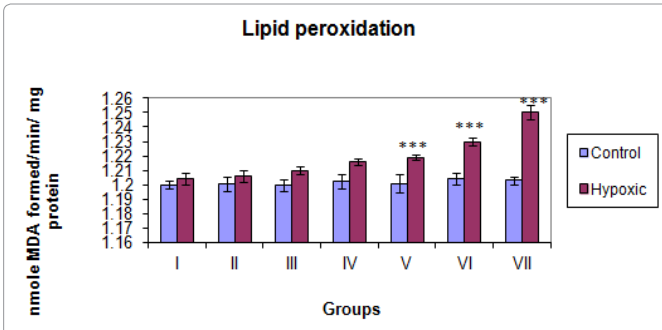

Figure 5: Levels of MDA measured in the lung tissue of control and hypoxic groups reduced Lipid peroxidation expressed as nmole of MDA formed/ mg protein. Values are expressed as mean+SD, n=9; and are significantly different (***P<0.001).

Non-enzymatic reducers

Lipid peroxidation and reduced glutathione: Changes in lipid peroxidation in lung at different time interval are shown in Figures 5 and 6. Initially there was no significant change in MDA level found in gr.I-

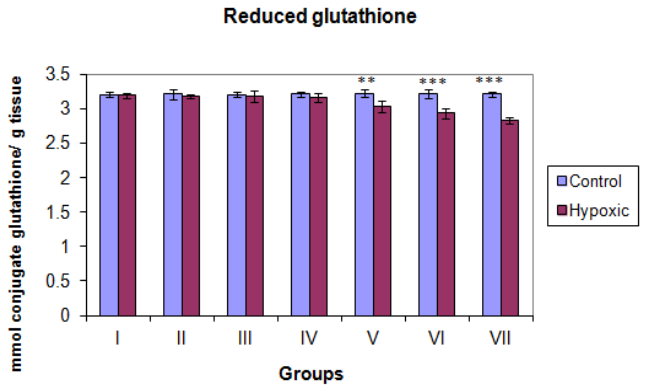

Figure 6: Levels of reduced glutathione measured in the lung tissue of control and hypoxic groups reduced glutathione expressed as mmol conjugate glutathione/ g tissue. Values are expressed as mean+SD, n=9; and are significantly different (***P<0.001).

gr.IV (P>0.05). The MDA level was found to be increased significantly by 9.16% (P<0.001***), 14.16% (P<0.001***) and 18.9% (P<0.001***) in gr.V, VI and VII respectively as compared to control. The effect of hypoxia on the levels of non-enzymatic antioxidant GSH in lung tissue is shown in fig.6. GSH activity was found to be lowered in hypoxic rats. A significant decrease of 6.25 % (P<0.01**), 8.43 % (P<0.001***) and 11.83 % (P<0.001***) was found in gr.V, VI and VII respectively when compared to control group.

Histopathological observation: Pulmonary edema can occur in pathological situations that are directly or indirectly linked with alveolar hypoxia. High-altitude pulmonary edem (HAPE) can be solely attributed to alveolar hypoxia. It occurs after rapid ascent to high altitude [45]. The histological examination of control lung sections showed normal architecture of lung parenchyma (Figure 7a) Lung section of gr.I and gr.II animals (Figure 7b, Figure 7c respectively) showed no major change in lung parenchyma. With increased duration of exposure in gr. III and V lung section (Figure 7d, Figure 7e respectively) showed moderate reduction in alveolar space area,

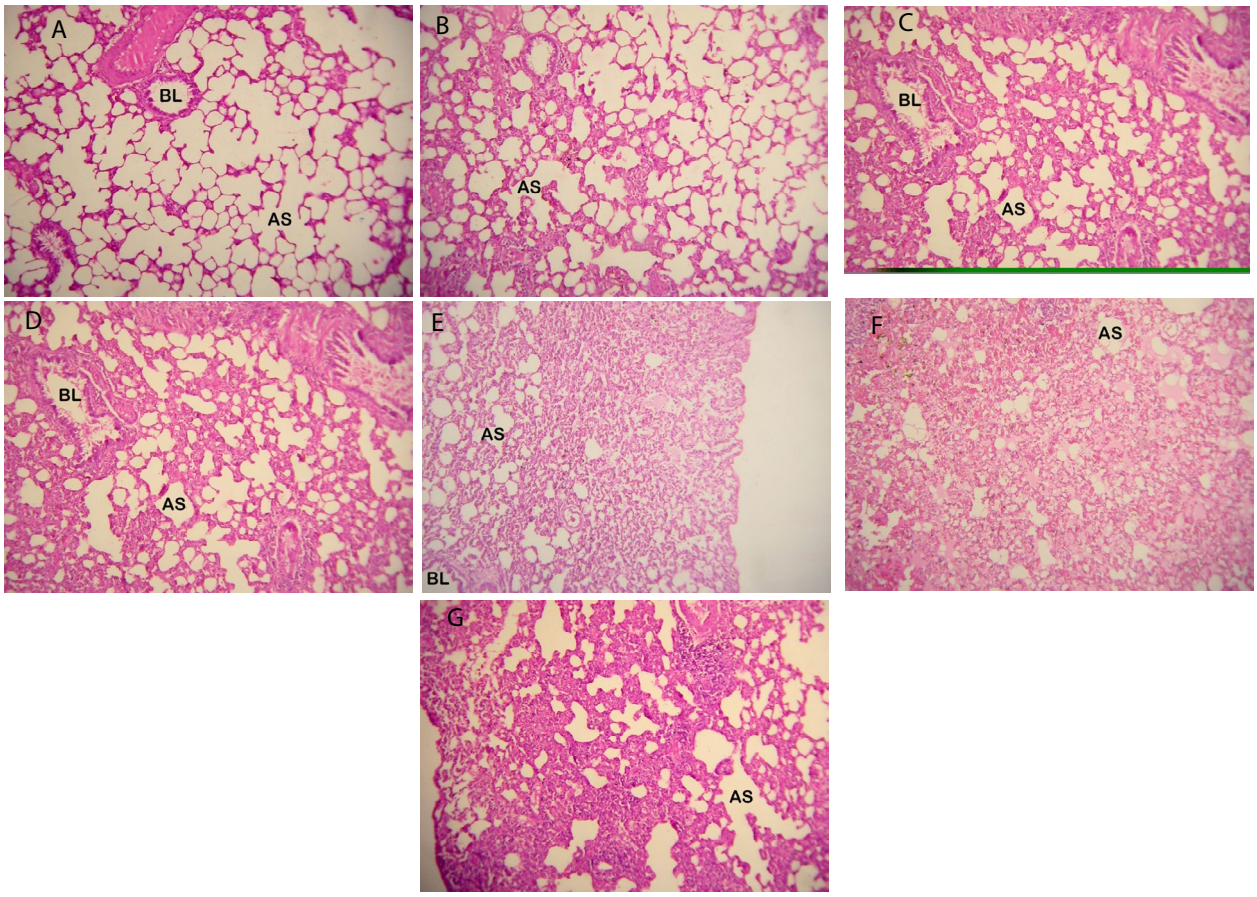

Figure 7 Photomicrographs showing histopathological changes in lung tissue:

a: Control Lung: Low power photomicrograph of section of lung from control group showing normal lung parenchyma. (HE x 100); **b:** Low power photomicrograph of section of lung from animal exposed to hypoxia for **2 hour** showing normal lung parenchyma. BL = Bronchial Lumen, AS = Alveolar Space. (HE x 100). **c:** Low power photomicrograph of section of lung from animal exposed to hypoxia for **4 hour** showing only mild thickening of alveolar spaces with marginal reduction in alveolar space area, mild thickened inter-alveolar septae. Alveolar spaces are almost normal in size (HE x 100) **d:** Low power photomicrograph of section of lung from animal exposed to hypoxia for **8 hour** showing thickening of alveolar spaces with moderate reduction in alveolar space area, moderately thickened inter-alveolar septae and smaller alveolar spaces (HE x 100); **e:** Low power photomicrograph of section of lung from animal exposed to hypoxia for **16 hour** showing thickening of alveolar spaces with moderate reduction in alveolar space area (Some of the alveolar spaces show traces of edema fluid), moderately thickened inter-alveolar septae (HE x 100); **f:** Low power photomicrograph of section of lung from animal exposed to hypoxia for **24 hour** showing widespread changes of pulmonary edema alveolar spaces filled with pale eosinophilic edema fluid, the inter-alveolar septae are mildly thickened (HE x 100); **g:** Low power photomicrograph of section of lung from animal exposed to hypoxia for **48 hour** showing, pronounced thickening of alveolar spaces with resultant reduction in alveolar space area, and severely thickened inter-alveolar septae (HE x 100).

moderately thickened inter-alveolar septae and smaller alveolar spaces. Group VI (Figure 7e) animals showed changes major pathological changes with accumulation of fluid (edema), which persisted with advancement (pronounced thickening of alveolar spaces with resultant reduction in alveolar space area, and severely thickened inter-alveolar septae) in gr. VII (Figure 7f).

Discussion

The first physiological adjustment that occurs in response to sudden exposure to hypoxia is hyperventilation (abnormally increased pulmonary ventilation) [46]. It is initiated by hypoxic stimulation of peripheral arterial chemoreceptors (carotid and aortic bodies) and central integration of chemosensory inputs in brain stem via medullary respiratory center [47]. Hyperventilation leads to markable change in arterial blood gas tension [46,48,49]. Reported ABG values at altitude supports fall in both PO_2 and PCO_2 [50]. The reduction in PCO_2 is mainly due decrease in the O_2 dissociation curve, resulting in decreased alveolar ventilation [51]. This results in respiratory alkalosis (acid-base disturbance initiated by a reduction in PCO_2) which is a common problem often found in sojourners and in many respiratory disease [52,53]. We also report lowered ABG values in our study.

Logic tells us that there is a need of O_2 presence for the generation of ROS and oxidative damage; likewise, it would be unlikely that oxidative damage occurs when this element is less available [54]. The formation of ROS in the hypoxic context has been a subject of wide-ranging debate in both theory and reported results. Currently, this paradigm has been changed by identifying hypoxia as a generator of ROS and oxidative damage for systems as well for specific organs [55]. The complete description of the ROS generation at a mitochondrial level has been recently reviewed [56]. Evidence of oxidative damage by hypoxia, in the lungs, is consistent; however, the mechanisms about how it is produced are still being discussed. The inflammatory process is one of the factors involved in the increase of ROS generation and tissue oxidative damage. Alveolar hypoxia corresponds to a proven stimulus that triggers inflammation that is first localized and later it becomes systemic and the alveolar macrophages seem to play essential role in this process [57].

Catalase and Glutathione peroxidase have demonstrated a great variety of results from non variation to increases and decreases during hypoxia. CAT and GPX share the function of controlling the concentrations of H_2O_2 and are the most outstanding cellular mechanisms for the neutralization of ROS [58]. Whenever the concentrations of H_2O_2 are in a high level, CAT becomes more effective than GPX and vice versa [59]. Nevertheless, GPX is capable of removing organic peroxides, such as those derived from lipid peroxidation [60]. To control the concentrations of H_2O_2, the enzymatic reaction of GPX uses GSH, which acts as a reducer. Both CAT and GPX activity was found increased significantly in our study. Slight increase in GPX activity in lung homogenate has been reported in rats placed into a simulated environment at 5,500 m [6]. Similar increases of GPX in lungs of rats subjected to 12 h. of ischemic preservation in cold solution at 4°C [61]. On the contrary, another finding reports decrease in GPX activity in lung homogenate of rabbits exposed to simulated height of 27, 000ft for 3 h [62]. Increased GPX activity with increased CAT activity in the lungs during exposure to hypobaric hypoxia has been reported [6]. Superoxide dismutase is another enzyme in antioxidant defense that eliminates superoxide radicals to form H_2O_2 and hence protect the cells from the toxic effects induced by free radicals [63]. We have found significant decrease in SOD activity in hypoxic rats. Decreased activity of SOD has been reported in aging, disease and in hypoxia. Decrease in Mn-SOD activity has also been reported in rat lung tissue exposed to hypobaric hypoxia [6]. It has been observed that free radicals attack proteins, mainly enzymes [64]. Hence, it is assumed that the decrease in activities of SOD in our present study might be due to the increased free radical attack, i.e. due to elevated lipid peroxidation products (MDA) in lung tissue during hypoxia.

In our study, we found increased TBARS concentration (Figure 4) in gr. IV-VII significantly indicating increased level of oxidative stress during hypoxic exposure. An increase in TBARs (thiobarbituric acid reactive substances) and conjugated dienes of lung homogenates in rats exposed to 6% O_2 during 120 min [65]. Similar increase MDA level during hypobaric hypoxia has also been reported by [6]. Increased MDA level in lung homogenates was also reported in rats exposed to hypobaric hypoxia in hypobaric chamber for 5 h at 29,500ft. GSH is another abundant antioxidant in lung epithelial cells and in ELF (epithelial lining fluid) is the key to modulate the development of inflammatory-oxidative pulmonary injury [66]. Reduction of H_2O_2 and lipid hydroperoxides is carried out together with GPX or peroxiredoxin. In both cases, GSH is converted in GSSG and quickly reduced by glutathione reductase and NADPH. The decreased GSH level may be due to increased level of lipid oxidation products which may be associated with the less availability of NADPH required for the activity of glutathione reductase (GR) to transform oxidized glutathione to GSH [67] due to increased production of ROS at a rate exceeding the ability to regenerate GSH.

The biochemical findings were further correlated with histopathological observation of the control and hypoxic animals lung (Figure 7). With the onset exposure, (grI-III) there was no major lung architecture change, but with increased exposure time lung sections were showing alveolar thickening, severely thickened inter-alveolar septae with marked reduction of alveolar space area and accumulation of fluid confirming full blown pulmonary edema (gr VI-VII). Report shows almost one mountain trekker or climber out of two develops several symptoms of high altitude illness after a rapid ascent to an altitude above 4000 m [68]. Pulmonary edema and alveolar thickening in lung tissue of rats exposed to low oxygen content has been reported [69,70]. In case of humans symptoms of HAPE can be present within 2-5 days after arrival at HA [71]. Chest radiographs and CT-scans of early HAPE show a patchy, peripheral distribution of edema.

Conclusion

In our study we have focused overall changes taking place in lungs over short term (2h-4h) to long term (16-48h) HH. In conclusion, our findings support self compensatory ability of lung during low oxygen condition over short term (none of the rats showed any major change in either in antioxidants system or lung pathology). During 12hr to 16hr antioxidant system showed significant change but mild pathological change in lung. On prolonged exposure (16h-48h) lungs undergo major pathological changes (edema) with significant change in antioxidant system. Again we observed that rats responds HH in very short time (16h-24h), hence rodent model can be the best approach to study high altitude related illness at tissue level.

Conflict of Interest

The authors declare that they have no conflict of interest.

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