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ANTIOXIDANT DEFENSE ENZYMES ACTIVITY IN HYPOBARIC HYPOXIA SUSCEPTIBLE AND TOLERANT SPRAGUE DAWLEY RATS

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ABSTRACT

Antioxidant enzymes are known defense molecules, which eliminate reactive oxygen species (ROS) and free radicals that damage useful enzymes in the cell under various stress conditions. Significant efforts have been made to explore these antioxidant enzymes as a potential candidate for the diagnosis of certain diseases. Hypobaric hypoxia is a condition of oxidative stress leading to various diseases through the involvement of free radicals. In the present study, we have analyzed the expression of antioxidant enzymes in the plasma of male albino rats (185 ± 10 g) that were segregated into hypobaric-hypoxia susceptible (HHS) and tolerant (HHT) on the basis of their 'gasping time'. After one week of normalization, both HHS and HHT rats were exposed to 30,000 ft for 1 h and sacrificed to collect the blood sample. Catalase (CAT) and peroxidase showed 3.9 and 2 fold increased enzymatic activity, respectively in HHT rats as compared to HHS rats. There was no significant difference in superoxide dismutase (SOD) activity in HHT when compared with HHS rats. Differential activity of CAT and peroxidase in HHS and HHT rats under hypoxia stress was also confirmed by zymography. Thus differential activity of these antioxidant enzymes in HHS and HHT may have a regulatory role under hypobaric hypoxic conditions.

Keywords: Antioxidant enzymes, Catalase, Peroxidase, Hypobaric hypoxia, SOD.

INTRODUCTION

Hypobaric hypoxia leads to oxidative stress condition to the body as there is decreased available oxygen at high altitude [1] leading to slow metabolic processes. Hypobaric hypoxia at tissue level is known to be involved in molecular signaling resulting into acute mountain sickness (AMS), high altitude pulmonary edema (HAPE) and high altitude cerebral edema (HACE) [2-4]. Under oxidative stress, ferrous ion in hemoglobin forms ferrylhemoglobin (Fe^{4+}) [5]. Ferrylhemoglobin, being a strong oxidizing agent, is believed to mediate the peroxidation of macro-biomolecules, *viz.*, lipids, proteins, carbohydrates and nucleic acids [6, 7]. Oxidative stress and oxygen- derived free radicals leads to hypoxic injury or reoxygenation injury [8, 9]. Increased free radical production in body mediates tissue injury in many diseases [10]. Oxygen derived free radicals modify metabolically important proteins such as Na^+ / Ca^+ exchanger and sodium pump [11, 12]. Some intercellular defense enzymes (antioxidant enzymes) include glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) are known to counteract the effects of oxygen-derived free radicals [13]. SOD, catalyses the conversion of O_2^- into hydrogen peroxide (H_2O_2) and are called first line of antioxidant defense while GPx and CAT catalyze the final conversion of H_2O_2 into H_2O [13].

Hypobaric hypoxia exposed tissues are known to

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generate reactive oxygen species (ROS) which further leads to the expression of antioxidant enzymes at different level of body organization [1]. Difference in the expression of metabolites (mainly proteins and enzymes) may lead to further tolerance and susceptibility of an individual. These differential expressions could pave the way towards discovery of some biomarkers to help finding the population with adaptive immunity at high altitude. In the present work, we have studied the activity of antioxidant enzymes in HHS and HHT rats exposed to hypobaric hypoxia.

MATERIALS AND METHODS

Experimental animal

Sprague Dawley adult male albino rats were used as experimental animal for the present study. Inbred rats were maintained at DIPAS, DRDO under hygienic conditions at 24 ± 2ºC with a natural light-dark cycle (12 h:12 h). All animal related experiments were performed at DIPAS, DRDO. Rats weighing between 185 ± 10 g were used and had free access to standard pelletized diet and sterile tap water throughout pre and post-treatment. All animal procedures were approved by the Institute's Animal Ethical Committee (DIPAS, DRDO, Delhi) and were in the compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Minimum numbers of animals were used for experiment and efforts were made to reduce their pain.

Segregation and selection of hypoxia susceptible and tolerant rats

Sprague Dawley rats were screened for susceptibility and tolerance by exposing the randomly selected adult male rats to 32,000 ft (9,754 m) altitude equivalent to barometric pressure = 205.8 mm of Hg at 32ºC in hypoxic chamber with manually controlled air pressure, altitude, temperature and the barometric pressure. The hypoxia tolerance of rats was determined by measuring 'gasping time' [14]. The 32,000 ft (9,754 m) altitude equivalent was achieved at a rate of 3,000 ft per min (914 m min^{-1}) with an increase in air pressure of maximum 2 L min^{-1}. As soon as gasping was observed in an animal, the barometric pressure was released and the altitude was decreased gradually to the sea level at a rate of 3,000 ft per min. Based on their gasping time animals were segregated into hypoxia susceptible (gasping time of 0-8 min) and hypoxia tolerant (gasping time more than 27 min). Both hypoxia susceptible and tolerant groups of animals were kept at normal hygienic conditions at 24 ± 2ºC for a week for acclimatization.

Collection and estimation of protein from blood plasma

The animals were normalized for a week and then given exposure to an altitude equivalent of 30,000 ft (barometric pressure = 225.6 mm of Hg) at 24ºC for 1 h [15]. The rats were anesthetized using ketamine (80mg kg^{-1} body weight, *i.p.*) and xylazine (20 mg kg^{-1} body weight, *i.p.*) and sacrificed to collect blood sample. Plasma was separated by centrifugation of blood sample at 4,000 rpm for 5 min at 4ºC. Total amount of proteins present in plasma sample from HHS and HHT group of animals were estimated by using Bradford [16] method. Catalase, peroxidase and superoxide dismutase activities were determined in the plasma of HHS and HHT rats in triplicate.

ANTIOXIDANT ENZYMES ASSAY

Catalase assay in HHS and HHT samples

Catalase activity was determined in the plasma of HHS and HHT samples following the method suggested by Luck [17] after modification. Initial A_{240} against 50 mM phosphate buffer, pH 7.0 was set to 0.50 using 10 mM H_2O_2 in same buffer. Catalase activity was observed by adding 20 µg (20 µg plasma protein) in 980 µl mixture of 50 mM potassium phosphate buffer, pH 7.0 and 10 mM H_2O_2 having O.D of 0.5. A decrease in absorbance was recorded for 10 min.

Peroxidase assay in HHS and HHT samples

Peroxidase activity was determined in both HHS and HHT groups by using guaiacol as substrate at room temperature. The rate of formation of guaiacol dehydrogenation product is a measure of the peroxidase activity and can be assayed by spectrophotometry at 436 nm after modification of protocol of Leon et al [18]. A reaction mixture of 1 ml consisting of 0.97 ml assay buffer (9.55 ml of 0.1 M potassium phosphate buffer, pH 7 mixed with 150 µl of 0.02 M guaiacol) and 20 µl (20 µg plasma protein) was taken. Reaction was started by adding 10 µl of 10 mM H_2O_2 solution and the change in absorption (A_{436}) was recorded.

Superoxide dismutase (SOD) assay in HHS and HHT samples

Superoxide dismutase activity in HHS and HHT samples was assayed photo-chemically using methionine, riboflavin and nitro blue tetrazolium (NBT) in glass test tubes of uniform thickness and colour [19]. The reaction mixture consisted of 1.3 µM riboflavin, 13 mM methionine, 63 µM NBT 0.05 M sodium carbonate (pH 10.2) and 20µl (20µg) of plasma protein. Final volume was raised to 3 ml by adding distilled water and the solution was illuminated in white light. Spectrophotometric reading was taken at A_{560} and identical set of enzyme that was not illuminated served as blank.

Zymography of Catalase

Equal amount of blood plasma protein from HHS and HHT groups was loaded onto a 10% (wv^{-1}) non-denaturing polyacrylamide gel and separated at a constant current of 30 mA. The gel was washed twice each for 10

min with double distilled water and for another 10 min with 15 mM H_2O_2 solution. After treatment, the gel was stained with a solution containing 2% (wv^{-1}) ferric chloride and 2% (wv^{1}) potassium ferric cyanide till the clear zone appears in contrasting dark background [20].

Zymography of Peroxidase

In gel activity of peroxidase in HHS and HHT samples was determined by loading equal amount of proteins on non-denaturing gel (10%) and was resolved in tris-glycine buffer at constant current of 30 mA for 45 min. After separation, the gel was incubated in 0.2 M sodium acetate buffer (pH 5.0) containing 1.3 mM benzidine and 1.3 mM fresh H_2O_2 solution [21]. Benzidine was dissolved in acetate buffer by heating and continuous stirring. Bands of peroxidase were visible after an incubation of 30 min.

Fig 1. Activity of antioxidant enzymes; CAT (a), Peroxidase (b) and SOD (c) in plasma of HHS and HHT

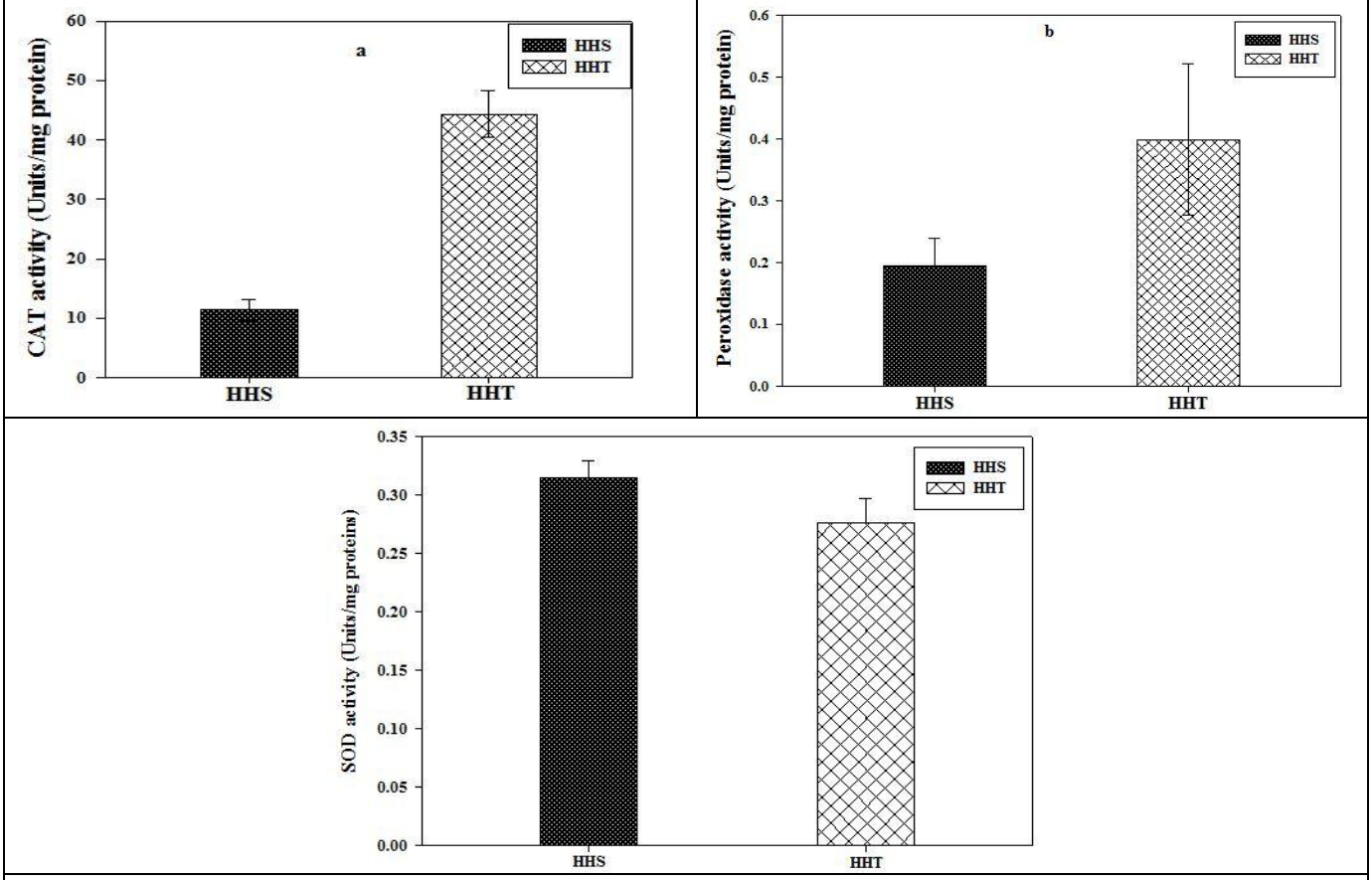
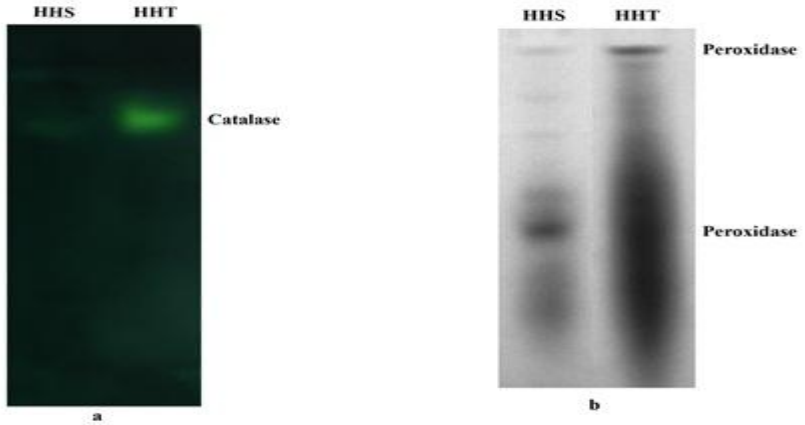


Fig 2. Zymograms of hypobaric hypoxia mediated antioxidant enzymes; CAT (a), and Peroxidase (b) in blood plasma of HHS and HHT rats



RESULTS AND DISCUSSION

Oxidative stress conditions arise by a disturbance in the pro-oxidant and antioxidant ratio, which potentially damages the cell [22]. At the same instant, reactive oxygen species (ROS) possibly damages proteins, lipids, nucleic acids under oxidative stress conditions [23]. The most abundant ROS generated during oxidative stress is superoxide radical ($O_2^{\cdot-}$), which is mostly produced during electron transport in the mitochondria and sometimes as a result of decomposition of oxyhemoglobin [24, 25]. To combat such oxidative stress mediated events, polymorphic forms of free radical scavenging enzymes such as CAT, glutathione peroxidase, SOD are induced [26-29].

In the present study, male albino Sprague Dawley rats segregated into HHS and HHT were assessed for changes in antioxidant enzyme activity in their plasma under hypobaric hypoxia. The experiment demands altitude at which the animals could experience the stress and exhibit the response at both morphological and molecular level. Rats were exposed to hypobaric hypoxia at a higher altitude because smaller animals have higher capillary density in tissues, making them more resistant to hypoxia than human [30, 31]. Therefore the studies were done at an altitude of 32,000 feet where experimental organism (rats) can experience the stress even though human population cannot resist this altitude.

Plasma of HHS and HHT individuals were assayed for CAT, peroxidase and SOD activity. A 3.9 and 2 fold increase in CAT (HHS= 11.395 enzyme units/mg, HHT= 44.534 enzyme units/mg) and peroxidase activity (HHS= 0.195 enzyme units/mg, HHT= 0.399 enzyme units/mg) respectively were observed in HHT as compared to HHS (Fig. 1a & 1b). However, no significant difference in SOD activity (HHS= 0.315 enzyme units/mg, HHT= 0.276 enzyme units/mg) was seen in HHT as compared to HHS (Fig. 1c). This differential expression of antioxidant enzymes was also observed on zymogram (Fig. 2a and 2b).

Hypoxia stress leads to several adaptive changes at molecular level thereby opening a possibility to develop biomarkers for the identification of hypoxia tolerance and development of strategy to impart hypoxia tolerance to susceptible individuals. Difference between distinct individual have been shown based on the gasping time to impart tolerance in rats [32]. Antioxidant enzymes have played a significant role and the level of tissue oxygenation is changed under hypoxia conditions [33-35]. Erythrocytes are one of those cells that are most exposed to oxidative damage by free radicals [1]. HIF-1 is involved in hypoxia mediated physiological changes in mammals [36] and served as an intermediate for the O_2 dependent expression of genes for glycolytic enzymes, erythropoietin, etc. Such HIF-1 dependent genes may serve as basis for the regulation of hypoxia through erythropoiesis, angiogenesis and changes in energy metabolism [37]. Superoxide dismutase (SOD) catalyzes the conversion of superoxide anions to produce H_2O_2 and O_2 in rats [38]. The increased level of H_2O_2 during hypoxia [39] leads to generation of antioxidant enzymes [40]. Elimination of this excess H_2O_2 is done by a combined effort by catalase (CAT) and glutathione peroxidase (GPX) where CAT eliminates the enzymes that produces H_2O_2 while GPX eliminates H_2O_2 produced [41]. Thus higher level expression of CAT and peroxidase in HHT indicates the possibility of involvement of these enzymes in imparting tolerance to HHT. High altitude exposure either disrupts the efficiency of the antioxidant enzyme system [42] or increases its level to combat ROS [13]. Pharmaceutical use of antioxidant enzymes have shown to have beneficial effects thereby preventing the oxidative damage associated with hypobaric hypoxia [42].

The present study showed increased expression of CAT and peroxidase in blood plasma of HHT sample. An earlier study also suggested increased expression of some antioxidant enzymes like Mn-SOD in rat serum and GSH-Px activity in heart and lungs of rat [38], while in some cases activity of SOD during hypoxia stress was found decreased [43]. The increase in the expression of antioxidant enzymes in HHT rats indicated that cellular anti-oxidative system might have triggered to resist oxidative damage.

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CONFLICT OF INTEREST

The authors have no conflict of interest in both research and financial issues.

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