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Original article

Preconditioning effect of cobalt chloride supplementation on hypoxia induced oxidative stress in male albino rats

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ABSTRACT

The present study was designed to understand the preconditioning effect of cobalt chloride (CoCl2), a known hypoxia mimetic, on promoting acclimation to hypobaric hypoxia in male Sprague Dawley rats. Rats were exposed to hypoxia (7619 m) for 1,2,3 and 5 days with and without prior supplementation of cobalt chloride (12.5 mg cobalt/kg bw for 7 days). After hypoxic exposure, rats were sacrificed and various oxidative stress parameters were studied in the blood/plasma. Further protein expression studies were carried out in the heart. The results showed significant increase in lipid peroxidation and protein oxidation and decrease in reduced glutathione (GSH) in the plasma of animals exposed to hypoxia without cobalt supplementation as compared to control group. Prior administration of cobalt appreciably attenuated oxidation of lipids and proteins. There was increase in antioxidant enzyme superoxide dismutase (SOD) on exposure to hypoxia, whereas SOD levels were maintained in cobalt supplemented groups. Expressions of hypoxia inducible factor (HIF-1), hemoxygenase (HO-1) and metallothionein (MT) were higher in the cobalt supplemented group as compared to control group. Therefore, cobalt preconditioning appears to decrease oxidative stress by alleviating lipid peroxidation and maintaining higher HO-1 and MT levels leading to faster acclimation to hypobaric hypoxia.

1. Introduction

High altitude regions have adverse environmental conditions such as low oxygen partial pressure and extreme cold. In addition to these, there are other factors like high wind velocity, ultraviolet radiation, etc. Hypoxia and cold either alone or in combination cause many deleterious effects at the cellular and systemic level which lead to high altitude ailments such as acute mountain sickness (AMS), high altitude pulmonary edema (HAPE) and high altitude cerebral edema (HACE), that develop in unacclimatized persons shortly after ascent to high altitude [1]. The occurrence of high altitude illnesses depends primarily on the rate of ascent, the altitude attained and the individual person's susceptibility. Symptoms include headache, fatigue, nausea, gastrointestinal upset, sleeplessness, etc. All these changes might be due to oxidative stress which is enhanced by hypoxia because of the excessive production of free radicals [2–4]. Reactive oxygen species (ROS) are natural and physiological modulators of cellular redox millieu and thereby signaling and controlling factors of a wide range of known and unknown pathological and physiological processes and a necessary consequence of aerobic metabolism [5].

Under normal circumstances, ROS is generated in small amounts during mitochondrial electron transport, phagocytosis and drug metabolism, but are effectively neutralized by a host of antioxidant molecules constituting the antioxidant defense system. During limited oxygen supply such as at high altitude, less oxygen is available to terminally accept electrons from oxidative phosphorylation. As a consequence, there is accumulation of reducing equivalents in the mitochondria. This reducing environment favours incomplete reduction of oxygen to highly reactive superoxide, peroxide and hydroxyl radicals [6]. Free radicals have been implicated in various degenerative diseases such as ageing [7], atherosclerosis [8], carcinogenesis [9] and neurodegenerative diseases [10].

Our earlier studies, carried out in animals [2], and other researchers have reported induction of oxidative stress by hypobaric hypoxia [11,12]. Several studies revealed that hypoxia preconditioning protects brain, heart and kidney from various types of injury including ischemia, seizures, edema [13,14]. Hypoxia preconditioning has clinical usefulness and can be mimicked by cobalt chloride [15,16]. Cobalt chloride has been used in treating anemia in infants and pregnant women especially associated with chronic renal disease and refractory anemia [17–20]. Chemical preconditioning by cobalt chloride has lot of advantages over physical preconditioning as it reduces the period of acclimatization at high altitude and large number of people can be preconditioned together, compared to physical preconditioning in simulation chambers [21]. Cobalt chloride stimulates a signal cas-

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cade with cytochrome b as receptor and hydrogen peroxide as second messenger for erythropoietin production in normoxia [22], and increases oxygen delivery by increasing erythropoiesis. Cobalt was also shown to be cytoprotective against terbutylhydroperoxide induced oxidative stress in HepG2 cells [23]. Matsumato et al. [24] reported induction of reno-protective genes in rats when cobalt chloride was given with drinking water for 13 days. Similarly, improved cardiac contractile function was observed in rats administered with water containing 0.01% $CoCl_2$ for 6–7 weeks [15].

Further, evaluation of cobalt chloride induced reduction in oxidative stress in high altitude ailments could also enhance the possibility of use of cobalt chloride in other illnesses involving oxygen deprivation. Most of the studies on the hypoxia induced oxidative stress are carried out on hypoxia re-oxygenation, and there is paucity of data available on oxidative stress induced by hypobaric hypoxia and preconditioning with cobalt in blood and heart of rat. Heart is the most vital organ of the body distributing oxygen rich blood to all parts of the body and a constant supply of oxygen is indispensable for cardiac viability and function. Oxygen is a major determinant of myocardial gene expression and as myocardial oxygen levels decrease under certain conditions; viz pulmonary diseases, ischemia and high altitude, gene expression patterns in the heart are significantly altered [25]. Oxygen dependant gene expression is governed by several mechanisms including regulation by hypoxia inducible transcription factor (HIF-1α) [26]. It regulates the transcription of an extensive repertoire of genes, viz those involved in angiogenesis, vascular remodeling, erythropoiesis, metabolism, inflammation, etc. It is a redox sensitive protein that is constitutively transcribed and translated in the heart and most tissues but under normoxic conditions, it undergoes prolyl hydroxylation by the action of specific cellular prolyl hydroxylases. When oxygen is less abundant (hypoxia), prolyl hydroxylases are inactivated, resulting in accumulation of HIF1-α, nuclear translocation and binding to HIF-1β to form the transcriptionally active HIF-1 complex that binds to the hypoxia response element (HRE) present in different hypoxia responsive genes, thus influencing their transcription. [27].

Cobalt, a known hypoxia mimetic, stabilizes HIF-1α by antagonizing Fe2+, which is an essential cofactor along with oxygen for prolyl hydroxylases that degrade HIF-1α under normoxia [28]. Rat (*Rattus rattus*) has proved to be a model system to study the physiological and molecular changes under hypoxia in lung and other tissues [29] and their mechanism of acclimatization was found to be similar to that of human beings [30,31]. We have earlier reported the protective effect of cobalt chloride on hypobaric hypoxia induced oxidative stress in rat brain and lungs [32,33].

In the present study, we report the preconditioning efficacy of cobalt chloride administration in prevention of hypobaric hypoxia induced oxidative stress in blood and heart of rat.

2. Material and methods

Male Sprague Dawley albino rats weighing 150–175 g were used for the study. The animals were maintained in the Institute's Animal House at (24 ± 2 °C) with a 12:12 hour light/dark cycle. Relative humidity was maintained at 40–50%. The animals were fed standard pelletized diet (Lipton India Ltd) with free access to water. The experimental protocol was carried out in accordance with the guidelines of the Ethics Committee of this Institute and was in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals, India (CPCSEA). Efforts were made to minimize animal suffering and number of animals used for experimental purpose. The animals were maintained under the surveillance of a qualified veterinarian from the Institute.

3. Cobalt chloride treatment and hypoxic exposure

Male albino rats were divided into two major groups: control and experimental, which were further subdivided into five groups of eight rats each. Dose response studies were carried out earlier in our laboratory [21] and the optimum dose was found to be 12.5 mg cobalt/kg body weight. Experimental animals were, therefore, supplemented with $CoC1_2.6H_2O$, orally at a dose of 12.5 mg cobalt/kg body weight for seven days prior to exposure to hypoxia using a gastric canulla. Remaining five groups were fed with normal saline. Seven days after supplementation of cobalt chloride/saline, different groups of animals were exposed to a simulated altitude of 7,619 m in a decompression chamber (Decibel Instruments Delhi, India) set at a temperature of 28 ± 2 °C for 0, 1, 2, 3, and 5 days, respectively. We exposed rats to hypobaric hypoxia at a higher altitude, because smaller animals have higher capillary density in tissues, making them more resistant to hypoxia than man. Relative humidity was maintained at 45–50%. The rate of ascent was 300 meters/min and air flow was maintained at the rate of 2 l/min. The rats were taken out of the chamber once after every 24 h for 15 min to replenish food and water. After hypoxic exposure the animals were sacrificed under ketamine chlorhydrate (80 mg/kg) induced anesthesia. Blood was drawn by cardiac puncture into heparinised tubes. Parameters to be estimated in the blood were determined immediately. Remaining blood was centrifuged at 3000 rpm for 20 minutes and the plasma was processed immediately or kept at −80 °C till further analysis. Heart was perfused with PBS, dried, weighed and stored at −80 °C for protein expression studies.

4. Hematological analysis

Hemoglobin was analysed in heparinised blood by Blood Cell Counter (MS-4, Desing Laboratories, France).

5. Biochemical analysis

5.1. Lipid peroxidation

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) formed by thiobarbiturate (TBA) reaction as thiobarbituric acid reactive substances (TBARS) using 1,1,3,3 tetraethoxypropane as standard [34]. Levels of lipid peroxides was expressed as nmol MDA/ml plasma.

5.2. Protein oxidation

Protein oxidation was measured by determining the carbonyl groups after derivitization of proteins with dinitrophenyl hydrazine (DNPH) [35]. Briefly, 500 μl of plasma was incubated with 500 μl of 10 mM DNPH/2 M HCl for 60 minutes at 50 °C. Protein was then precipitated using 20% TCA and the untreated DNPH was removed by centrifugation at 14,000 g for 10 minutes. The pellet was washed three times with cold ethyl acetate:alcohol (1:1) dissolved in 500 μl of 1 mol/L NaOH and the absorbance was measured at 450 nm.

5.3. Glutathione-S transferase (GST) and lactate

GST was determined using a protocol described by [36]. Briefly 100 μl of plasma was mixed with 2.790 ml of 0.1 potassium phosphate buffer (pH 6.5) and 100 μl of GSH (100 mM). Reaction was initiated by adding 10 μl of 1-chloro 2, 4-dinitrobenzene 40 mg/ml in ethanol and the increase in optical density is recorded at 340 nm for a period of 5 minutes. L-lactate was estimated in plasma using Randox kits, following the manufacturer's instructions.