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Rapid Acclimatisation to High Altitude by Intermittent Hypoxia Training at Sea-Level: Role of Biochemical Markers

Geetha Suryakumar, Dishari Ghosh, Richa Rathor, Gopinath Bhaumik, Som Nath Singh* and Bhuvnesh Kumar

DRDO-Defence Institute of Physiology and Allied Sciences, Delhi - 110 054, India

**E-mail: cellbiochemistry@gmail.com*

ABSTRACT

Rapid induction of soldiers to high altitude under emergency situation may lead to higher incidence of acute mountain sickness (AMS) and other high altitude illness. Intermittent Hypoxia Training (IHT) at sea level before going to high altitude is an approach for rapid acclimatisation. This approach may be helpful to reduce the occurrence of AMS and leads to better acclimatisation at high altitude in shorter duration. The present study evaluates the role of biochemical markers of acclimatisation after IHT before induction to actual high altitude. The study participants were Indian Army Personnel (n=30) and they were divided into two groups of control (n=16) and IHT exposed (n=14). The intermittent hypoxia training was administered at 12 per cent Oxygen for 4h/day for 4 days at sea level using normobaric hypoxia chamber and within 24 hrs - 48 hrs the subjects were airlifted to Leh, Ladakh, India at 11,700 ft. Preconditioning with IHT may be beneficial in maintaining antioxidant levels and ameliorate oxidative stress at high altitude. The hypoxia responsive proteins like Hemeoxygenase -1 (HO-1) and Vascular endothelial growth factor (VEGF) and the cytoprotective stress proteins, which facilitate the acclimatisation, may also get benefited by IHT exposure.

Keywords: High altitude; Acclimatisation; Intermittent Hypoxia training; Oxidative stress.

1. INTRODUCTION

High altitude is associated with several environmental challenges to natives and sojourners alike. Several environmental stressors such as low temperature, high wind velocity, ionizing radiations and hypobaric hypoxia at high altitude leads to a decrease in physical and mental performance[1-2]. Rapid ascent to altitude without proper acclimatisation in army troops may cause detrimental effects on health and also compromised operational capabilities work performance. Proper acclimatisation can resolve the problems and reduces the severity of illness. Intermittent hypoxic training (IHT) is an approach for faster acclimatisation which can decrease the incidence and severity of high altitude illness. IHT is exposure to normobaric or hypobaric hypoxia at sea level for the purpose of pre-acclimatisation/pre-conditioning to high altitude. Recent studies have reported the use of IHT in sports medicine to enhance physical performance and to induce pre-acclimatisation without any pharmacological interventions[3]. Exposure to a moderate hypoxic episodes have been shown to elicit the beneficial effects by activating the adaptive responses in our body and protects against hypertension, myocardial injuries, heart arrhythmia and bronchial asthma[4, 5]. Our own earlier study reported that IHT have a preconditioning effect for acclimatisation to hypobaric hypoxia prevalent at high altitude[6-8].

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Recent study by Kumar *et al.*[9] demonstrated the better maintenance of redox homeostasis in carotid body by regulating the expression of the HIFα isoforms and modulating the antioxidant enzyme levels in IH trained rats as compared to control. Oxidative stress has been implicated as one of the major reason for high altitude induced illness. It is well known that hypobaric hypoxia at high altitude leads to enhanced ROS generation and several studies have observed increase in oxidative damage markers in response to hypoxia[10-11]. Several physiological processes such as mitochondrial electron transport chain, nitric oxide synthetase and xanthine oxidase get triggered at high altitude that leads to increased oxidative stress[12-14]. Recent studies proposed that enhanced altered hypoxia sensing, rather than hypoxia response mechanism, as the reason for better adaptation in natives[15]. Mammalian cells detect the decrease in oxygen concentrations to activate a variety of responses allowing better adaptation. One such response is stabilisation of the protein hypoxia-inducible factor (HIF-1), which regulates the expression of genes mediating adaptive responses[16]. This includes a prominent role for more than 100 hypoxia-sensitive genes, like erythropoietin (EPO), heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), glucose transporter (GLUT-1), VEGF, stress proteins and others. Stimulation of these proteins through hypoxic preconditioning has been established to play a part in protecting from tissue damage[17-19].

HO-1 induction represents an important redox sensor and an antioxidant defence. In the presence of HO-1, heme is

degraded to carbon monoxide, bilirubin, and iron. All these products have major biological functions; bilirubin, is a potent free radical scavenger showing potent antioxidant activity.[20].

VEGF is one of the most studied HIF-1 alpha targets and promotes angiogenesis[21-22]. VEGF, a homodimeric 34 kDa - 42 kDa, heparin-binding glycoprotein which has potent vascular permeability, angiogenic, and mitogenic activities specifically for endothelial cells. The enhanced expression of VEGF is induced by hypoxia, phorbol ester and TNF-α. Van Patot *et. al.*,[23] reported increased VEGF levels in the plasma of the subjects who developed AMS during ascent to high altitude. On the other hand, soldiers who did not have high altitude sickness had greater plasma soluble VEGF receptor-1 (sFlt-1) before and after ascent to high altitude. Enhanced vascular leakage has been implicated in AMS and HACE.

Heat shock proteins (HSPs) have been shown to protect the cells from various stressful conditions like heat, toxins, infections, free radicals etc. Heat shock proteins are generally responsible for preventing damage to proteins in response to stress. Based on their molecular weight, heat shock proteins can be divided into subfamilies:

- the large hsps of 100-110 kD,
- the hsp 90 family,
- the hsp 60 family,
- hsp 40 family and
- small hsps of 18-40kD.

HSP60 and HSP70 possess a key function in the folding, unfolding and translocation of polypeptides. Beside this, they are also responsible for assembly and disassembly of oligomeric protein complex[24].

Our recent study by Bhaumik *et. al.*,[6] have established that the incidence of AMS was significantly reduced by the administration of IHT during acute ascent to 3500 m altitude in Indian soldiers. IHT also enhanced the VO2 at high altitude which may be due to enhanced ventilation and also increased the level of Erythropoietin, a key hormone involved in high altitude acclimatisation[6].

However, our knowledge in response to IHT on biochemical markers of adaptation (HO-1, VEGF and heat shock proteins) on soldiers was unexplored. The present study was planned to explore the efficacy of biochemical markers of adaption during and after Intermittent Hypoxia Training (IHT).

2. METHODOLOGY

We have conducted the present study with 30 Indian male Army personnel (Control n=16 and IHT exposed n=14) and the volunteers were permanent residents of sea level and not exposed to high altitude areas within 6 months of experiment, with no previous history of cardiac diseases and non-smokers. IHT protocol was followed as published earlier[6]. This study was conducted with the approval of the Institutional Human Ethics Committee. A written informed consent was obtained from all the volunteers prior to conducting the experiments. The sea level data was conducted at our Institute at Delhi (barometric pressure 740 mm Hg). Temperature of 20 $\pm$5 ^{o}C with a relative humidity range of 40 per cent - 50 per cent was maintained in the laboratory. IHT protocol was administered to the subjects for 4 consecutive days. The intermittent hypoxia training was administered at 12 per cent Oxygen for 4h/day for 4 days at sea level using normobaric hypoxia chamber and within 24- 48 hrs the subjects were airlifted to Leh, Ladakh, India at 11,700 ft. At Leh the ambient temperature of the laboratory was maintained at 20 $\pm$5 ^{o}C with a relative humidity range of 40 per cent - 50 per cent

3. SAMPLE COLLECTION

Venous blood samples were collected in fasting condition in the morning at sea level and high altitude at day 1, day 3 and day 6. Plasma was separated by centrifugation at 1000g for 15 minutes. Just after collection, blood (0.25 ml) was mixed with equal volume of meta phosphoric acid (10 %) which was further processed to analyse glutathione levels using fluorimetric method of Hissin and Hilf[25]. The plasma was separated with the remaining portion of blood by centrifugation at 1000g for 15 minutes. All the samples were kept at -20°C during transport back to laboratory and stored at -80°C until assay of the parameters.

Total Antioxidant Status was quantified in terms of ABTS (2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) using commercially available kits (Randox Laboratories Ltd., UK). Uric acid, glutathione reductase, glutathione peroxidase were measured using Randox kit. Hydroperoxides were measured by FOX 1 (Ferrous ion oxidation with Xylenol Orange) assay[26]. Then HSP70, HSP60, HO-1 and VEGF (Krishgen Biosystems, USA and Cloud-Clone Corp., USA) were determined in plasma using commercially available ELISA Kits following manufacturer's instruction.

4. STATISTICAL ANALYSIS

All the data were represented as mean±SD. Two-way analysis of variance with unpaired *t*–test was carried out to compare the biochemical responses of the groups. Probability values of <0.05 were considered as statistically significant. All analysis was conducted using GraphPad Prism ver 7.00 software (GraphPad, CA, USA).

5. RESULTS

5.1 Effect of IHT on the Antioxidant Status of Soldiers Inducted to High Altitude

Reduced glutathione (GSH) were found decreased at high altitude in comparison with basal values at sea level up to day 3 at HA. However, in case of IHT exposed participants, levels were maintained on day 4 of exposure. Minor decrease was observed following hypoxic exposure at sea level and day 1 at HA. There was not much change in oxidised glutathione (GSSG) levels but ratio of GSH/ GSSG was better maintained in IHT group. There was significant decrease in total antioxidant level in case of control group on day 7 at HA, whereas in IHT group no significant change was noted (Table 1).

Marked oxidative stress was observed by increased levels of hydro-peroxides in case of control group. The rise in hydroperoxides was less in case of IHT group. Levels of glutathione peroxidase and glutathione reductase also indicate positive changes with IHT intervention. The rise of uric acid, which is a feature of HA exposure was less in case of

Table1. Effect of IHT exposure on glutathione levels, related antioxidants & oxidative stress markers at HA

Group	Sea Level	Sea level [Post –IHT]	HA Day 1	HA Day 4	HA Day 7
Reduced Glutathione (GSH),µ mol/ L					
Control	1106 ± 51	---	935 ± 86	843± 99*	1007±100
IHT	1116 ± 73	973 ± 150	928 ± 102	1018 ± 102#	1058 ± 116
Oxidized glutathione (GSSG), µ mol/ L					
Control	120 ± 9	---	119 ± 12	128± 18	118±16
IHT	120 ± 10	115 ± 17	121 ± 15	122 ± 15	118 ± 19
GSH/GSSG					
Control	9.2 ± 0.6	---	7.9 ± 0.7*	6.6± 0.8*	8.4± 0.6
IHT	9.3 ± 0.5	8.5 ± 0.8	7.7 ± 0.5*	8.4 ± 0.6#	9.0 ± 0.7
Total Antioxidant (m mol/l)					
Control	1.7 ± 0.06	---	1.8 ± 0.04	1.3± 0.05	0.6±0.4*
IHT	1.7 ± 0.05	1.8 ± 0.05	1.7 ± 0.02	1.3 ± 0.04	1.7 ± 0.04#
Uric Acid (µ mol/ L)					
Control	301 ± 6.7	---	390 ± 8.6*	411± 9.9*	545±16*
IHT	310 ± 7.3	350 ± 15	300 ± 10#	285 ± 14#	305 ± 11#
Hydroperoxides (FOX-1) µ mol/ ml					
Control	520 ± 61	---	688 ± 124*	708± 169*	597±150
IHT	508 ± 53	568 ± 98	542 ± 87#	546 ± 86#	511 ± 53
Glutathione peroxidase (U/L)					
Control	5466 ± 1569	---	5500 ±1455	5675± 1165	6061±1981*
IHT	5460 ± 1500	5600 ± 1105	5450 ± 1002	5455 ± 1055	5480 ± 1400
Glutathione Reductase (µ mol/ ml/min)					
Control	0.35 ± 0.01	---	0.29 ± 0.03*	0.11± 0.01*	0.27±0.04*
IHT	0.33 ± 0.02	0.25 ± 0.03	0.29 ± 0.01	0.30 ± 0.05#	0.36 ± 0.02

All values are Mean±SD; *p<0.05 in comparison with SL & #p<0.05 in comparison with control.

IHT exposure group. Possible reason of these changes may be adaptive response due to hypoxic exposure at sea level (Table 1).

5.2 Effect of IHT on hemoxygenase and VEGF Levels

Ascent to high altitude enhances HO-1 level in plasma at day 3 (Baseline compared to day 3 and day 6). However, the increase of HO-1 was higher in IHT subjects as compared to control subjects (Fig. 1a). VEGF levels were increased in control as well as IHT group (Baseline compared to day 3). But, the increment of VEGF was found higher in IHT subjects as compared to control subject (Fig. 1b).

5.3 Effect of IHT on HSP 60 and HSP 70 Levels

HSP60 levels increased in control as well as IHT group (Fig. 1c). Contrary to HSP60, circulatory levels of HSP 70 were non-significantly decreased in control as well as IHT group (Fig. 1d). The increase of stress protein was higher in the IHT group as compared to control subject.

6. DISCUSSION

The present study evaluates the effect of IHT on biochemical markers of acclimatisation. The results indicate that IHT enhanced the antioxidant status and the levels of hypoxia responsive proteins such as Hemoxygenase, VEGF and cytoprotective heat shock proteins as compared to control subjects. These molecules may help the subjects to better acclimatise at high altitude.

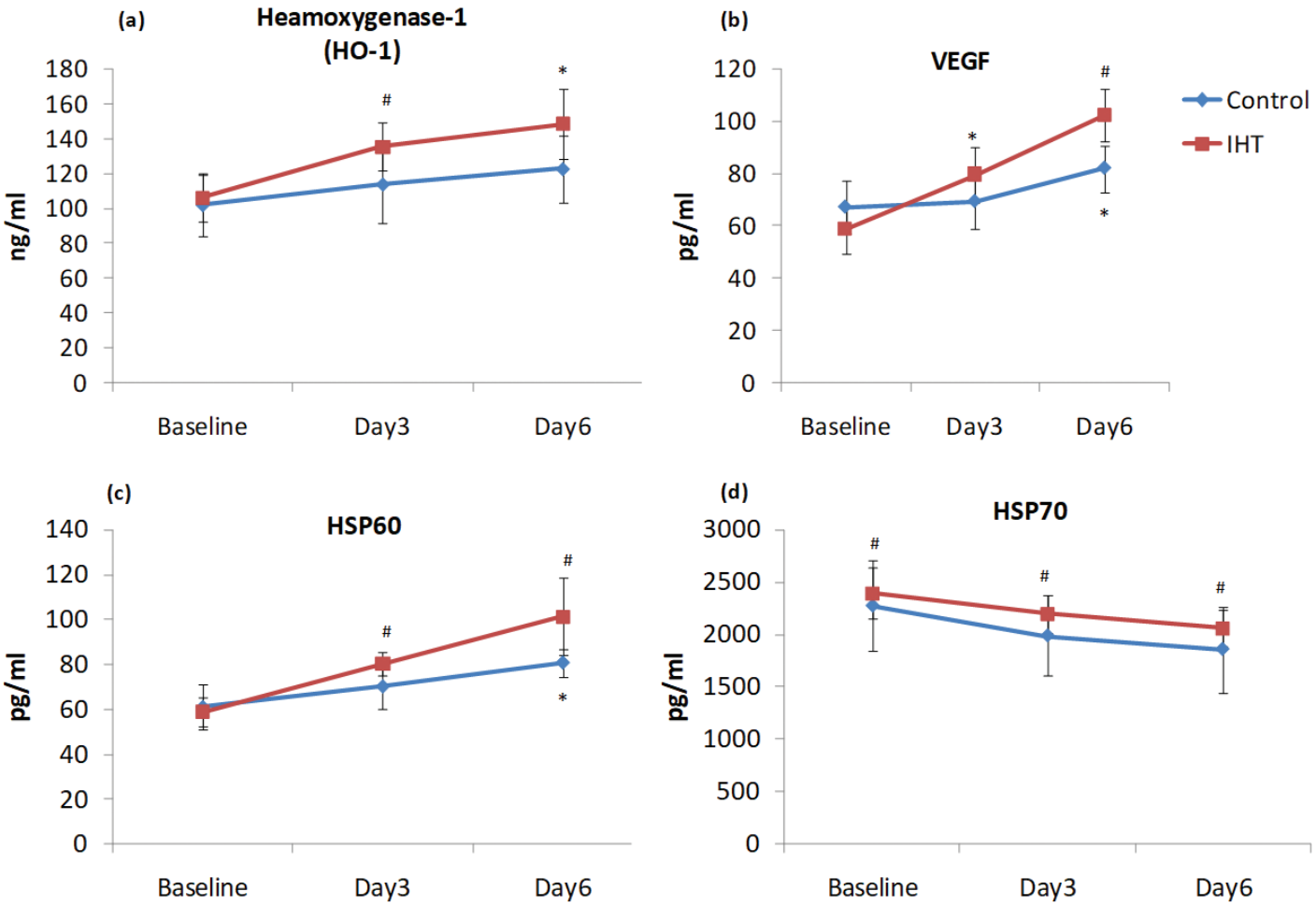

Figure 1. Effect of IHT on HIF-1α regulated proteins (a) plasma heamoxyeanase-1 (HO-1) (b) plasma VEGF (c) plasma HSP70 (d) plasma HSP60. All values are Mean±SD; *p<0.05 in comparison with SL & #p<0.05 in comparison with control.

It is well known that exposure to high altitude leads to enhanced generation of reactive oxygen and nitrogen species (RONS), and oxidative damage to lipids, proteins, and DNA. Decrease in oxygen availability at high altitude affects the mitochondrial respiration, leading to partial reduction of oxygen and enhancing free radical production[12]. Several studies have showed an increase in oxidative stress markers during stay at high altitude[11,27].

Our results show that IH exposure decreased the oxidative stress markers and enhanced the level of antioxidants which facilitates faster acclimatisation to high altitude. Our earlier study Jain *et. al.*[28] highlights that an enhanced tolerance to hypoxia stress is contributed by better cellular antioxidant defense, enhanced level of antioxidant enzymes, increased expression of HIF alpha regulated genes as well as heat shock proteins.

It is an interesting finding that prior exposure with IHT may help in maintaining antioxidant levels and prevent oxidative stress on subsequent exposure to HA. However, it is reported that high altitude natives have better antioxidant status in comparison to acclimatised lowlanders. In detail, high altitude natives have higher superoxide dismutase activity and significantly lower levels of catalase, glutathione peroxidase and glutathione reductase activities in comparison to lowlanders after 1 month stay at altitude. Further, the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) is more in case of high altitude natives of Ladakh[29]. However, they were found to be more prone to exercise induced oxidative damage in comparison to acclimatised low landers[30]. To understand the antioxidant status of high altitude natives, it is hypothesised that post-IHT exposure not only lead to an increment in antioxidant status, but also could provide a better adaptability towards high altitude exposure. Ergo, more research in this direction is required.

HO-1 is induced in response to cellular stress which degrades heme into carbon monoxide, biliverdin, and iron. Further biliverdin reductase converts Biliverdin into bilirubin[31]. Numerous studies have reported that HO-1 derived carbon monoxide and bilirubin causes vasorelaxant/vasodilant effect[32-35]. Vasodilation is widening of blood vessels leading to relaxation of smooth muscle cells and an increase in blood flow[36]. Additionally, arterial dilation resultant into a decrease in arterial blood pressure and heart rate[37]. Kruger *et. al..*,[38] also reported that inducers of HO-1 gene expression activated HO-1 that leads to increased level of CO and bilirubin which restore eNOS, decreased blood pressure, and normalised

kidney function. Therefore, increased HO-1 production in IHT subjects as compare to control subjects could be responsible for decrease in blood pressure and heart rate after IHT at day 4. Hence increase HO-1 observed in IHT group may facilitate antioxidant defense under hypoxia and hence better adaptation.

Hypoxia is a potent stimulus of angiogenesis, a process whereby neovascularisation arises from existing blood vessels. This complex process of angiogenesis begins by increasing production of VEGF[39-40]. VEGF is also known as vascular permeability factor[41-42] and prime regulator of angiogenesis[43]. Recent studies have shown that the expression of VEGF can be induced by hypoxia[44-45], a condition that causes tumor necrosis and stimulates angiogenesis[46-47]. Vilar *et. al.*[48] hypothesised that hypoxia may trigger angiogenesis that result into reduction of blood pressure. In this process, VEGF is playing an important role and in our study VEGF is also increasing more in IHT subjects in comparison to control subjects. Therefore this increment of VEGF could be playing an interesting role in decrement of blood pressure in IHT subjects.

Human HSP60 is well known as the 60 kDa chaperonin (Cpn 60) and mostly present inside the mitochondria. HSP60, an essential chaperonin, presents a critical role in cell survival and mitochondria protection during stress conditions[49]. Under physiological conditions, HSP60 offers a significant role in protein homeostasis inside the mitochondria and prevent cell from apoptosis[50-51]. There is upregulation of HSP60, observed during stress condition which executes a significant role in folding/refolding of proteins inside the mitochondria[52]. Beside maintain proteostasis, HSP60 fulfils other functions too inside the mitochondria, embraces ROS sensor and scavenger to protect cells against oxidative stress[53].

Hypobaric hypoxia condition leads to accumulation of ROS and oxidative stress[54] which further produce oxidative protein and altered proteostasis[55-56]. The oxidative protein modification leads to ER stress condition which resultant into activation of HSP60 and HSP70 to rectify the misfolded proteins[57-59]. Jain *et. al.*[28] reported the protective function of HSP60 against hypobaric hypoxia. Several studies have reported a positive correlation between heat shock proteins and protection against myocardial, hepatic and muscle damage[60-61].

Li *et. al.*[62] reported the adaptive response of HSP70 in SD rats under high-altitude hypoxia environments. Additionally, HSP70 is known for anti-apoptotic, anti-necrotic or chaperonic function[63]. It is also reported that HSP70 showed cytoprotective effect via modulating cellular redox status, reduced intracellular ROS and participate in immunoregulatory mechanism[64-65].

7. LIMITATIONS

The present study is a pilot study to evaluate the effect of IHT on the biochemical markers responsible for rapid acclimatisation to high altitude. The study was carried on limited number of Indian Male Army participants with similar age group, and life style in terms of diet, exercise and work load etc. Further it is necessary to evaluate these results in large number of subjects.

8. CONCLUSION

Prior exposure with IHT may help in maintaining antioxidant levels and prevent oxidative stress on subsequent exposure to HA. IHT also helps in better maintaining the hypoxia responsive proteins like HO-1 and VEGF and the cytoprotective stress proteins (HSP60 and HSP70) which facilitate the acclimatisation process.

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CONTRIBUTORS

Dr Geetha Suryakumar, Sc 'F', obtained her MSc and PhD (Biochemistry).
In the current study, she perfomed the study, data collection, analysis and finally edited the manuscript.

Dr Dishari Ghosh, Sc 'E' (PhD in physiology) and in the current study, she performed field duties for collection of blood samples.

Dr Richa Rathor, Sc 'E', received her MSc and PhD (Biochemistry) and performed the experiment, data analysis and data interpretation and wrote the draft manuscript.

Mr Gopinath Bhaumik, Sc 'F' (M.Sc. in human physiology) and in the current study, he supervised the project.

Dr Som Nath Singh, Sc 'F' and head Nutrition & Biochemistry Department and in the current study, he collected data, analysed the data, finally reviewed the manuscript.

Dr Bhuvnesh Kumar, Sc 'G', Director, DRDO-Defence Institute of Physiology and Allied Sciences, Delhi obtained his PhD (Veterinary Medicine) and in the current study he provided critical feedback and reviewed the final manuscript.