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Physiological Indications and Gut-Microbial Community in Army Personnel in High- Altitude and Base-Line Environments: A Comparative Study

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Abstract

High altitude (HA) environments contain low levels of oxygen, which often cause nonspecific gastrointestinal (GI) disorders associated with acute mountain sickness (AMS). This study is conducted to investigate the alternation of microbial population and the physiological indices after being exposed to HA. Fecal and blood samples were collected from twelve army personnel in base-level environments in (Delhi, India) after acclimatization for seven days at 3505 m HA (Lah, India). Different bacterial groups, oxygen saturation level (SPO_2), serum hemoglobin (Hb), hematocrit (HCT), blood urea, creatinine and microbial enzymes such as the α-amylase, proteinase, alkaline phosphatase and β-glucuronidase levels were all measured. It was observed that the total aerobic bacteria decreased significantly and the anaerobic and facultative anaerobic increased gradually in the intestines. Strict anaerobes including *Bacteroidetes* sp., *Bifidobacterium* sp., *Lactobacillus* sp. as well as pathogenic bacteria such as the *Salmonella* sp. exhibited positive growth. Various physiological parameters including serum hemoglobin, hematocrit, and urea-creatinine were also significantly changed. The microbial enzyme- activity also increased at HA. This confirms that the exposure to HA can change the intestinal microbial population affecting the microbial enzyme production possibly causing GI dysfunctions as a result of the decreased availability of oxygen.

KeyWords*:* High-altitude (HA); Acute mountain sickness (AMS); Microbial enzymes; Gut-microflora; Oxygen saturation level (SPO_2)**.**

1. Introduction

Hypobaric hypoxia is a characteristic environmental condition in high-altitude areas where people are known to develop varieties of physiological difficulties in such conditions (Hackett and Roach 2001). The stresses are solely dependent on the rise of altitude from sea level [an increase of 1 km above sea level drops 10 kilopascal (kPa) air pressure; with a sea level air pressure of 101.3 kPa (at 15 ℃ and 0% humidity)] (Hackett and Roach 2001). At high altitude, individuals may suffer from several patho-physiological disorders including a change in body weight, hematological changes, gastrointestinal disorders which are collectively called the altitude related sickness (ARS) (Shaov and Wan 2005; Paula and Niebauer 2012). Acute mountain sickness (AMS) is a frequent complication of individuals at high altitudes (Anand *et al.* 2006). One of the important problems in AMS is the gastrointestinal disorders that consist of indigestion, acid formation, flatulence, vomiting, anorexia, and diarrhea (Rook and Brunet 2005).

It is well-known that the normal human gastrointestinal (GI) tract contains vast and diverse groups of microbes in a complex ecosystem consisting of nearly one-hundred trillion (over fifty bacterial phyla; about 500 to 1,000 bacterial species). On the whole, these bacteria are very active, and play a significant role in diseases and human health (Hao and Lee 2004). Any responses of the host inside the state of an exacting range of exogenous factors (stress, temperature, drugs, cancer, etc.) and endogenous factors (inflammatory bowel diseases, peristalsis disorders) can change the GI microenvironment as well as its microbial ecology (Rhee *et al.* 2009). A disproportion of GI microbial ecology affects the gut physiological homeostasis, and may induce GI problems (Ley *et al.* 2012). It was shown that at high altitude (HA), gastrointestinal disorders are common problems for soldiers, veterans, athletes and travellers. Clues behind these disorders have not yet been explored, and there is no

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thorough evidence for variations in the GI microbial flora at high altitude. To analyze the microbial community of the GI tract, generally cultivable and non-cultivable approaches are used. The major drawback of cultivable approaches is that it reflects only a limited number of microbes (30-40%) of the whole microbial community. Now, the restriction of the cultivable process was minimized by the advance of modern molecular techniques (non-cultivable approach). However, the latter are very expensive, and so the cultivable method is still regarded as a classical technique (Lemos *et al.* 2012).

In the present study, quantitative variation of some common bacteria of fecal samples of healthy army male soldiers at a base level environment (Delhi), and at a HA environment (Leh-Ladakh) were examined. Microbial analysis including total aerobes and prominent anaerobes, an indicator strain *Escherichia coli*, Lactic acid bacteria, *Bifidobacterium* sp., *Bacteroidetes* sp. and a pathogenic strain *Salmonella* sp. were studied to investigate the impact of hypobaric hypoxia on the composition of gastrointestinal microbiota and some physiological indices. In relation to the microbial alteration, the activity of some microbial enzymes was also monitored.

2. Materials and Methods

2.1. Subject Selection

Twelve young healthy Indian army male soldiers (base level residents) within the age group of 25 – 30 years were selected for this study; their body mass index (BMI) was approximately 24.55 kg/m^2 (± 0.84). They all were healthy, not under the treatment of any medication, and they were not suffering from any bacterial or viral infections. They consumed army-specific homogenous diets throughout the experiment. The sea level (i.e. Base line or '0' day) study was carried out at Delhi (barometric pressure 740 mm Hg). After recording the physiological parameter and collecting the samples, the subjects were flown to an altitude of 3,505 m at Leh (barometric pressure 483 mm Hg) in the Western Himalayas. The subjects arrived at a 3500 m altitude in the morning and the day of arrival was taken as day one at 3500 m.

2.2. Collection of Fecal Samples

After a brief medical counseling, the fecal samples (~10 g) were collected in a pre-sterilized spatula–container at Delhi, and these were considered as the 'Base Line' (or '0' day sample) samples. Thereafter, the samples were collected on the first, fourth, and the seventh days at Leh, Jammu and Kashmir, India (~ 3500 m) during acclimatization. The samples were transported in a sterilized carrier solution containing peptone, 10 % (w/v); glycerol, 5 % (v/v). pH was adjusted to 7.0±0.2, and the samples were stored at 4 ºC until the analysis.

2.3. Collection of Blood Samples

Blood samples were collected from subjects using 21-Gauge needles (21G) mounted on a 5-mL syringe (Hindustan Syringes and Medical Devices Ltd, Faridabad, India) into heparin-coated sample bottles for analysis. Samples were collected on '0' (base line), during the first, fourth, and seventh day periods.

2.4. Analytical Measurement

2.4.1. Microbial Analysis

The cultivable microflora was enumerated on agar plates on the basis of colony-forming units (CFU). CFU represent the actual number of bacteria present in the fecal samples. These CFU values were converted to their logarithmic value and tallied with the corresponding experimental set of specified conditions. The total aerobic and anaerobic fecal bacteria were enumerated by a standard pour-plate technique in a single-strength trypticase soya agar (HiMedia, India), and reduced Wilkins Chalgren agar (supplemented with sodium succinate, hemin, vancomycin, menadione, oleandomycin phosphate polymyxin B and nalidixic acid) respectively. The Anaerobic condition was maintained in CO_2 incubator filled with 10% of CO_2 and H_2 gases (Adak *et al*., 2013). *Escherichia coli*, *Bacteroidetes sp.*, total Lactic acid bacteria, *Bifidobacterium sp.*, and *Salmonella sp.* were cultured on Mac-Conkey, bacteroides bile esculin agar (supplemented with gentamicin 100 mg/L),De Man, Rogosa and Sharpe agar (MRS), bifidobacterium and Brilliant green agar modified (HiMedia, India) respectively (Wehr and Frank 2004; Maity *et al*., 2012).

2.4.2. Somatic Index and Haematological Parameter

The individual body weight, body temperature, and heart rate were recorded at the beginning (Base Line) and throughout the experiment on the first, fourth, and seven days. A finger pulse Oximeter probe (Model MU 300, China) was set on the right index finger to measure the pulse and oxygen saturation level (SPO_2). The blood samples were used for analyzing the hematological parameters including hemoglobin (Hb) by the standard kit method (Merck, Japan) and hematocrit (HCT).

2.5. Blood Uremia Profile

2.5.1. Biochemical Estimation of Blood Urea

The plasma fraction was separated after the centrifugation of the blood samples at 3,000 rpm for five minutes. Plasma urea levels were measured by the commercially available standard blood urea kit (Merck, Japan) following the standard protocol for photometric determination of urea according to the glutamate dehydrogenase method (Burtis and Ashwood 1999).

2.5.2. Biochemical Estimation of Blood Creatinine

Plasma creatinine levels were measured by the commercially available standard creatinine kit (Merck, Japan) following the standard protocol for photometric determination of creatinine according to the Jaffe kinetic method without deproteinization (Sabbagh *et al.* 1988).

2.6. Fecal Enzyme Activity

The fecal samples were centrifuged at 10,000 rpm for fifteen minutes at 4 ºC. for the enzymatic analysis. After centrifugation, the supernatant was collected and used in the enzyme assay. For the determination of α-amylase activity, the dinitro-salicyclic acid method was used (Miller 1989). Proteinase, alkaline phosphatase and β-glucuronidase activities were assayed by the following protocols of Brock *et al.* (1982), Yotton and Savage (1976) and Kent *et al.* (1972) with slight modifications. Total protein in the feces was determined by Lowry *et al.*

(1951). The enzyme activities were expressed as specific activity (U/mg of protein).

2.7. Statistical Analysis

The collected data are presented as the arithmetic mean of three replicas (mean ± SE). The variations in microbial count hematological parameters were examined by one-way ANOVA. The alteration in the bacterial quantity was tested by Bonferroni for post hoc testing. Significant variation was accepted at the level of 5 %, i.e. $p<0.05$.

3. Results

A large number of aerobic bacteria were present in the fecal samples at normobaric conditions. But it was reduced significantly ($p<0.05$) after the seventh day of acclimatization at Leh. The quantity of total anaerobes was 9.10 ($\log_{10}^{CFU/g}$) on the 0^{th} day at base line (Delhi) and increased significantly to 11.15 ($\log_{10}^{CFU/g}$) after the seventh day of acclimatization. The ratio of total anaerobe to aerobic bacteria was 10^3 in Dehli; it had increased to 10^7 on the seventh day at Leh. The *E. coli* content was 6.9 ($\log_{10}^{CFU/g}$) at the base level, it was expended after the seventh days acclimatization of hypoxic condition and the changes of the above population were statistically significant, with $p < 0.05$. The quantity of strict anaerobic such as *Bacteroides* sp. and *Bifidobacterium* sp. was increased after the seventh day (Table 1). The total lactic acid bacterial population was increased though it showed no significant ($p<0.05$) change up to the seventh day of acclimatization. Selected pathogen such as Salmonella sp. was increased at the seventh day of acclimatization higher than the base line population. The body weight of the experiment group of the army personnel (AP) was decreased at the end of seventh day of acclimatization compared with their initial body weight; though it was not changed significantly ($p < 0.05$) (Table 2). Initially the body temperature (Table 2) and SPO_2 decreased (Table 2), during acclimatization it was further increased towards the normal. Heart rate (Table 2), the level of hemoglobin (Hb) and hematocrit (HCT) were significantly increased during acclimatization (Table 3).

The alteration of urea and creatinine levels were significant in group AP, it was noticed that during acclimatization (AP group) urea and creatinine levels were increased gradually and on the seventh day of hypoxic exposure they returned to the initial base line values (Table 3).

The microbial enzyme activity was also used for the evidence of the alternation of GI micro-environment during HA. The base levels of alkaline phosphatase, proteinase, β-glucuronidase and α-amylase activity increased respectively % in their specific activity after seven days of acclimatization (Table 4).

Table 1. Alteration of microbial population in human feces (Army Personnel - AP) on different days during acclimatization at high altitude (3500m).

MicrobialParameters	Hypobaric hypoxic exposure duration (in days)			
	'0' (Base Line)	1	4	7
Total aerobes	6.13 ± 0.554^{a}	6.30 ± 0.561^{a}	4.77 ± 0.548^{b}	3.94 ± 0.560^{c}
Total anaerobes	9.1 ± 0.581^{b}	9.10 ± 0.583^{b}	11.10 ± 0.584^{a}	11.15 ± 0.585^{a}
Escherichia coli	6.9 ± 0.482^{c}	6.97 ± 0.480^{c}	7.98 ± 0.483^{b}	8.95 ± 0.481^{a}
Bacteroidetes sp.	7.03 ± 0.275^{c}	7.20 ± 0.271^{c}	7.78 ± 0.700^{b}	8.23 ± 0.277^{a}
Total Lactic Acid Bacteria	6.3 ± 0.312^{b}	6.34 ± 0.310^{b}	7.35 ± 0.313^{a}	7.45 ± 0.314^{a}
Bifidobacterium sp.	5.21 ± 0.450^{b}	5.38 ± 0.451^{b}	6.54 ± 0.453^{a}	7.08 ± 0.452^{a}
Salmonella sp.	2.26 ± 0.474^{c}	2.33 ± 0.470^{c}	3.52 ± 0.471^{b}	4.21 ± 0.472^{a}

*Microbial population density was expressed (mean of $\log_{10}$ CFU/g ± SD). Letters (a, b, c) in superscript form in the row are significantly different at $p<0.05$.

Table 2. Changes physiological parameter on different days of acclimatization to 3500m.

	Changes of physical parameter			
	Base Line	HA Day 1	HA Day 4	HA Day 7
Body Weight (kg)	69.5 ± 2.58	69 ± 2.67	66 ± 2.69	66 ± 2.65
Heart Rate (pulse/min)	64.83 ± 3.49	81.5 ± 7.46	84.33 ± 6.20	81.66 ± 6.41
SPO2 (%)	98.83 ± 0.23	91.66 ± 1.07	97.16 ± 0.43	97.83 ± 0.92
Temperature (ºF)	98.41 ± 0.21	96.3 ± 0.80	96.96 ± 0.62	97.06 ± 0.63

* Data are expressed as Mean ± SE.

Table 3. Changes of blood parameter of Army Personnel (AP) on different days of acclimatization and its alteration after seven days at 3500m.

Blood parameters	Groups	Hypobaric hypoxic exposure duration (in days)			
		'0' (Base Line)	1	4	7
Hb (g/dl)	AP	14.16 ± 0.52^{c}	16.1 ± 0.31^{a}	15.76 ± 0.48^{b}	15.84 ± 0.52^{b}
Hematocrit (HCT)	AP	50.8 ± 1.93^{a}	51.4 ± 1.71^{a}	48.4 ± 1.19^{b}	48 ± 1.41^{b}
Urea (mg/dl)	AP	33.16 ± 1.43^{c}	37.83 ± 3.37^{b}	43 ± 3.22^{a}	34.5 ± 3.21^{c}
Creatinine(mg/dl)	AP	0.83 ± 0.05^{c}	0.95 ± 0.08^{a}	1.06 ± 0.08^{a}	0.87 ± 0.04^{b}

*Data are expressed as Mean ± SE.Letters (a, b, c) in superscript form in the row are significantly different each other at $p<0.05$.

Table 4. Changes in the enzyme profiles on different days of acclimatization.

Enzyme activity	Hypobaric hypoxic exposure duration (Day)				
	'0' (Base Line)	1	2	4	7
A-amylase	200.22 ± 4.45^{d}	210.34 ± 4.38^{c}	222.22 ± 4.11^{b}	241.44 ± 5.05^{a}	244.57 ± 4.21^{a}
Proteinase	3.83 ± 0.06^{d}	4.05 ± 0.05^{c}	4.47 ± 0.02^{b}	5.82 ± 0.08^{a}	5.90 ± 0.1^{a}
β-glucuronidase	5.75 ± 0.06^{e}	6.04 ± 0.04^{d}	6.63 ± 0.03^{c}	7.82 ± 0.02^{b}	8.05 ± 0.05^{a}
Alkaline phosphatase	4.43 ± 0.01^{e}	5.12 ± 0.02^{d}	6.17 ± 0.02^{c}	6.67 ± 0.04^{b}	7.18 ± 0.1^{a}

* Data are expressed as Mean ± SE. Letters (a, b, c, d, e) in a row are significantly different each other at $p<0.05$.

4. Discussion

The population of microbes present in the microenvironment of the gastrointestinal tract performs several important and essential activities in the host body. Little disturbances in the balanced gastrointestinal environment result in the alteration of the whole ecosystem with resultant physiological changes (Rhee *et al.,* 2009). Acute exposure to high altitude, the individual suffers from AMS due to the decrease in inspired PO_2, while traveling from sea level. Acclimatization to high altitude decreases the tissue oxygen delivery, which causes microcirculatory dysfunctions and cellular dyslexia including indigestion, acid gas formation, bowel motility, permeability etc. This dyslexia in the gastrointestinal track (GI) mucosa leads to metabolic dysfunctions that finally have an effect on the largest number of GI symbionts.

The results of this study showed that the total aerobes of the fecal samples decreased significantly during acclimatization of army personnel at high altitude, and the total anaerobes increased after seven days of HA acclimatization. This is likely related to the higher anaerobic state of intestinal epithelia, and the alteration of GI mucosal microenvironment was the major factor causing the modulation of specific bacterial subpopulations.

It has been established that the *E. coli* population was generally 10^6 times higher than the total aerobes in feces. The total aerobes, facultative anaerobes (*E. coli*) and total anaerobes are present in the ratio of $4.36:1:4.03\times10^5$ in feces, but this may vary within species and even between individuals in the same species (Maity *et al*., 2009). At a lower level of oxygen, this ratio was changed to $1 : 2.94 \times 10^4 : 2.16 \times 10^7$ and *E. coli* proliferation was higher (10^6) as it possessed elaborate genetic regulatory network for sensing oxygen (Holy *et al.,* 2012). It has been shown that immobilization for six hours initiates the increase of the concentration of *E. coli* in the proximal sections (the duodenum and the jejunum) of the digestive tract (Gritsenko *et al.,* 2000). This rapid expansion of *E. coli* population may encourage the growth of other strict anaerobes (*Bacteroidetes* sp. *Bifidobacterium* sp. and *Lactobacillus* sp.*)* and pathogen (*Salmonella* sp.*)* in anaerobic respiration (Gombosov *et al.,* 2011). But it is not clear, why *Bacteroidetes* sp. and lactic acid bacteria were lower than other anaerobes. The growth of Gram negative bacteria can cause a serious burden in the gut lumen due to poisoning with bacterial lipopolysaccharides.

The loss of body weight at hypobaric hypoxic conditions had been described in several studies (Benso *et al.,* 2007). In the present study, the final body weight of the army personnel (AP) was not changed significantly during the seven-day period of the experiment, but there was a tendency to weight loss (Table 2) which may be attributed to the higher metabolic rate, different energy output, and the loss of body water (Wall *et al.,* 2009). Initially the body temperature and SPO_2 were decreased, and at the seventh day of acclimatization they were increased due to the lower oxygen concentration in the air. In such conditions, the heart rate altered significantly due to the activity of autonomic nervous system (Bhaumik *et al.,* 2013).

The level of hemoglobin and HCT were increased during acclimatization to HA. Literature reveled that hypoxia causes the excessive secretion of erythropoietin (EPO) to increase blood RBC and Hb (Wickler *et al.,* 2000; Mizuno *et al.,* 2008) in order to compensate for the reduced blood oxygen content. In the current study, blood Hb and HCT were increased (Table 2) as describes by (Mairbaurl 2013). It was known several decades ago that erythrocytes are produced from the successive maturations of different erythroid progenitors which are responsive to erythropoietin (EPO). The rise in HCT and hemoglobin contents suggests that HA increases the EPO production (EPO is a glycoprotein hormone produced by the kidneys and secreted into the plasma), producing then haematological changes, (Gouttebargea *et al.,* 2012).

The alkaline phosphatase activity removed the phosphate from glutamine of the lipid moiety to reduce the LPS toxicity and create a less toxic situation (Bates *et al.,* 2006). The α-amylase activity digests the undigested polysaccharides to salvage energy and facilitated acid accumulation in the colon (Gloster *et al.,* 2008).

This study shows that the lower pressure of atmospheric oxygen at high altitude reduced the blood oxygen level that disturbed the physiological buffering system. As a result, gut microbiota and its associated enzymes are altered, perhaps causing the indigestion and the GI complications during the acclimatization at HA.

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