Modulation of small intestinal homeostasis along with its microflora during acclimatization at simulated hypobaric hypoxia

Atanu Adak, Kuntal Ghosh & Keshab Chandra Mondal *
Department of Microbiology, Vidyasagar University, Midnapore 721 102, India

Received 4 November 2013; revised 20 May 2014

At high altitude (HA) hypobaric hypoxic environment manifested several pathophysiological consequences of which gastrointestinal (GI) disorder are very common phenomena. To explore the most possible clue behind this disorder intestinal flora, the major player of the GI functions, were subjected following simulated hypobaric hypoxic treatment in model animal. For this, male albino rats were exposed to 55 kPa (~ 4872.9 m) air pressure consecutively for 30 days for 8 h/day and its small intestinal microflora, their secreted digestive enzymes and stress induced marker protein were investigated of the luminal epithelia. It was observed that population density of total aerobes significantly decreased, but the quantity of total anaerobes and *Escherichia coli* increased significantly after 30 days of hypoxic stress. The population density of strict anaerobes like *Bifidobacterium* sp., *Bacteroides* sp. and *Lactobacillus* sp. and obligate anaerobes like *Clostridium perfringens* and *Peptostreptococcus* sp. were expanded along with their positive growth direction index (GDI). In relation to the huge multiplication of anaerobes the amount of gas formation as well as content of IgA and IgG increased in duration dependent manner. The activity of some luminal enzymes from microbial origin like α-amylase, gluco-amylase, proteinase, alkaline phosphatase and β-glucuronidase were also elevated in hypoxic condition. Besides, hypoxia induced in formation of malondialdehyde along with significant attenuation of catalase, glutathione peroxidase, superoxide dismutase activity and lowered GSH/GSSG pool in the intestinal epithelia. Histological study revealed disruption of intestinal epithelial barrier with higher infiltration of lymphocytes in lamina propria and atrophic structure. It can be concluded that hypoxia at HA modified GI microbial imprint and subsequently causes epithelial barrier dysfunction which may relate to the small intestinal dysfunction at HA.

**Keywords:** Epithelial barrier, Growth direction index, Hypoxia, Microflora, Small intestine, Systemic inflammation

Mountains cover one-fifth of the earth’s surface; 38 million people live permanently at altitudes ≥2400 m, and 100 million people travel to high-altitude locations each year\(^1\). During ascent from sea level to high altitude (HA), barometric pressure of atmosphere falls exponentially that decreases the partial pressure of oxygen in the inspired gas of human. People like military personnel, veterans, athletes and travellers generally face such environmental hazards above 1,493 m and extreme altitudes above 5,486–6,096 m during acclimatization\(^2,3\). It causes physiological hypoxia in which an individual face an initial “struggle response” that induced several neurological complications collectively known as acute mountain sickness (AMS)\(^1,2,4\). Moreover, an individual exposed to other environment stressors like ultraviolet radiation, lower temperature and inability to maintain adequate personal hygiene and isolation from adequate medical care complicate the AMS. Apart from neurological and pulmonary syndrome many sojourners also experience several gastrointestinal (GI) disturbances like anorexia, epigastric discomfort, flatus expulsion, dyspepsia, nausea, severe acidity, vomiting, infectious diarrhoea and haematemesis etc\(^4,5\). The wide mucosal surface (200-300 m\(^2\)) of GI tract harbour and establish complex microbial ecosystem combining the gastrointestinal epithelium, immune cells and its resident microbiota. The microbiota is the most important and integral part of GI tract that participate in digestive, protective, structural and metabolic homeostasis\(^5\). A number of digestive enzymes secreted by microbial imprint hydrolyse all undigested or semi-digested food into absorbable form. The disturbance of GI microenvironment and its ecosystem is related to the weakening physio-chemical barrier through the induction tone of the local inflammatory responses\(^4,6\).
Still the relationship between intestinal dysfunction with microbial modification during hypobaric hypoxia is not elucidated. The present study has been undertaken to investigate the detrimental effects of hypobaric hypoxia at HA on GI microflora, in male albino rats model which were exposed to a simulated hypobaric hypoxic condition. Modulations of some indicator predominant microbial populations have been examined to assess pathophysiological status of small intestine.

**Materials and Methods**

Experimental animals and grouping—Healthy male albino rats (30; average body weight 145 ± 7 g) were used. The rats were divided randomly into following two groups of 15 each: hypobaric hypoxic (HH) and normobaric (NB) group. All animals were housed in polypropylene cages (34 × 28 × 19 cm) with normal 12:12 h L:D cycle. The animals were acclimatized for a week in laboratory environment. They were not under treatment of any medication and allowed to consume specific boiled homogenous diet (containing carbohydrates, 74.05%; proteins, 10.38%; fibre, 2.20%; iron, 56 ppm; calcium, 400 ppm and sodium, 500 ppm) throughout the experimental period and water ad libitum.

Exposure to hypobaric hypoxia and sample collection—The rats of hypobaric hypoxic (HH) group were exposed to 55 kPa (~4872.9 m altitude) for 8 h/day consecutively for 30 days at the same day time in a simulated hypobaric hypoxic chamber with adequate supplement of food and water. The NB group was maintained at normal atmospheric pressure (101.3 kPa) without interrupting their normal activity and circadian rhythm. At 10 days interval 5 rats in each group were sacrificed by deep anaesthesia with chloroform and part (2-3 cm) of the ileum was dissected aseptically. All procedures were performed in accordance with the animal care guidelines of Vidyasagar University, 3rd meeting held on 25.09.12 (item no. 3.ii).

Enumeration of microbial population—The contents of ileum segments were suspended in sterilized phosphate-buffer saline (PBS; pH 7.0 and 9 g/L NaCl) and it was vortex thoroughly. The suspension was used and some indicator cultivable bacterial populations in the ileum were enumerated by standard spread plate technique in the selective media following the instructions of the HiMedia manual (www.himedialabs.com). The total aerobes and anaerobes were cultured on single-strength trypticase soya agar and reduced wilkins chalgren agar (supplemented with sodium succinate, hemin, vancomycin, menadione, oleandomycin phosphate polymyxin B and nalidixic acid). The population density of *Escherichia coli*, *Bacteroides* sp. and *Clostridium perfringens* were enumerated by culturing on Mac-Conkey, bacteroides bile esculin agar (supplemented with gentamicin 100 mg/L) and perfringens agar base (supplemented with supplement I = sodium sulpha party 100 mg/L and supplement II = oleandomycin phosphate 0.5 mg/L and polymyxin B 10 000.0 IU/L). *Bifidobacterium* sp., *Lactobacillus* sp. and *Peptostreptococcus* sp. were cultivated respectively on bifidobacterium, rogosa SL and KF streptococcal agar. Anaerobic conditions were maintained in a CO2 incubator [Heal force Air jacket (HF 151 UV, 1 unit)], filled with 10% CO2 and H2 and the population density was expressed as logarithm (10 base) value of colony forming unit (CFU)/g of wet ileum content.

Growth direction index (GDI)—The CFU/g of bacterial populations in logarithmic values of NB group tallied with the HH group. When control log10CFU/g was higher in comparison to test log10CFU/g, the GDI was denoted as negative and reverse event was followed as positive. This will show a straight forward portrait about expansion and contraction of a microbial population in a particular ecosystem.

Measurement of gas volume—The ileum content (100 µL) was added in 30 mL of Mac-Conkey broth and volume of the gas was measured by an inverted volumetric Durham’s tube after 24 h of incubation at 37 °C. Gas formation ability was expressed in mL/g of ileum content.

Faecal enzymes activity—The faecal aliquot was centrifuged at 10,000 rpm for 5 min, the supernatant was collected and α-amylase and gluco-amylase activity was determined by glucose oxidase-peroxidase and dinitro salicylic acid method. Proteinase, alkaline phosphatase and β-glucuronidase activity were assayed with some modifications. Proteinase activity was assayed by using 0.8 mL of 1% (w/v) azocasein as the substrate and 0.2 mL of faecal aliquot and incubated for 45 min. The reactions were stopped by equal volume of 5% (w/v) trichloroacetic acid and 1 mL of 1 (N) NaOH was added, the absorbance was taken at 450 nm. Alkaline phosphatase activity was assayed by using 0.1 mL of 50 mM para-nitrophenylphosphate and 50 µL faecal
aliquot and 0.7 mL of 50 mM tris buffer (pH 8.5) and incubated for 1 h at 37 °C. The reaction was stopped by 0.1 mL of 1(N) perchloric acid and 2.5 mL of 0.50 mM NaOH. The absorbance was taken at 420 nm. The activity of β-glucuronidase was determined by using 0.7 mL of 1.2 mM phenolphthalein glucuronide and 0.7 mL of 100 mM sodium acetate buffer (pH 3.8) and 0.1 mL faecal aliquot. It was mixed and incubated at 37 °C for 30 min. Thereafter 5 mL of 200 mM glycine buffer (pH 10.4) was added and OD was taken at 540 nm. Total protein in the faeces was estimated and the enzyme activities were expressed in specific activity (U/mg of protein).13

Analysis of antioxidant parameters—The dissected segment of ileum was transferred in a chilled phosphate-buffer saline (PBS; pH 7.0). The inner content was gently washed thereafter it was opened vertically and epithelial cells were scraped out with a scalpel. The scraped cell (~10 mg) was suspended in chilled PBS (pH 7.0) and cell membrane was disrupted by sonicator [SONAPROS-PR 250 MP]. The sonication was performed with a titanium horn (3 mmø) at 20 kHz for 15 min at pulse rate of 2 sec at 15% amplitude. The temperature was maintained at 30 °C throughout the operation process by surrounding the suspension in ice bath. The suspension was centrifuged at 10,000 g and the supernatant was collected. Activities of antioxidant parameters like superoxide dismutase (SOD), catalase (CAT) were measured by studying the inhibition of pyrogallol auto-oxidation and catalysis of H2O2 at 420 and 240 nm13,14. The reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined using enzymatic recycling method15,16. Lipid peroxidation level was evaluated by the thiobarbituric acid reaction for malondialdehyde (MDA) formation at 532 nm.17

Histological study by light microscopy—About 2-3 cm of isolated ileum part was fixed in the Carnoy’s solution (60% ethanol, 30% chloroform and 10% glacial acetic acid). After 2 h fixation, tissues were dehydrated in graded alcohol and embedded in paraffin and cut into 3-5 μm thin sections. After periodic acid Schiff (PAS) staining, intestinal morphology like villus height, goblet cells number, tissue debris in lumen and modification of mucosal layer was observed using a Nikon Eclipse LV100POL polarized light microscope.

Scanning electron microscopic analysis—Fresh small intestinal tissue was rinsed with cold saline (0.9% NaCl) and cut into 5 mm × 5 mm sections, fixed in 2.5% glutaraldehyde, 10% osmium and dehydrated in sucrose solution containing PBS. Then it was gold coated and observed under scanning electron microscope (FEI Quanta -200 MK2). The arrangement of microvilli, deformed and exfoliated villi and the intercellular space between epithelia were examined.

Immunoglobulins assay—The luminal content (20 μL and 10 μL) was mixed with the 500 μL of activation buffer and 50 μL anti- rat IgA and IgG antibody was added respectively. After 5 min of agglutination reaction turbidity was measured at 340 nm and the concentration of IgA and IgG was expressed as mg/g of the intestinal content.

Statistical analysis—The data were presented as the arithmetic mean of five replicas (mean ± SD). Variations in microbial count were examined by one-way ANOVA (Kruskal-Wallis). Significant variation was measured by using Sigmaplot 11.0 (USA) software and accepted at the level of 5 and 1% (P<0.05 and P<0.01).

Results
During experimental period no morbidity of rat was recorded. The total aerobes in the ileum content were 4.75 that decreased to 2.61 with negative GDI of 1.81 after 30 days of acclimatization at 55 kPa. This reduction was 138 folds in respect to the population size of NB group (Table 1). In contrast, the quantity of total anaerobes significantly (P<0.05) increased from 6.02 to 8.04 (log10 CFU/g) with positive GDI (1.33), which seemed to be 105 folds higher than the control population size. After 30 days of hypobaric hypoxic stress the population ratio of total aerobes and anaerobes changed from 1:1.26 (ratio of log10 CFU/g of the population) to 1:3.08 in HH group. Among these groups these were modified to 2.19, 4.59 and 5.21 respectively in HH group. Among these groups
The population density of different bacteria was expressed (mean of 5 replicates in log_{10} CFU/g of NB group) that adjusted to 1:1.97:2.24 (ratio of log_{10} CFU/g of HH group) due to enlargement of population size by 51 and 31 fold during 30 days of hypoxic adaptation. The levels of IgA and IgG in the luminal content were 1.9 and 3.06 mg/g (in NB group) that progressively increased to 6.89 and 8.27 mg/g after 30 days of hypobaric hypoxic adaptation.

The activity of SOD was 106.35 U/mg of protein (in NB group) of small intestinal epithelia that significantly (P<0.05) decreased to 32.26 U/mg of protein during adaptation 30 days in hypoxia (Table 3). The CAT activity was 238.27 U/mg of protein in NB group. It also significantly (P<0.05) reduced respectively to 165.54, 103.62 and 79.83 U/mg of protein after 10th, 20th and 30th day in HH group. Consequently, the activities of SOD and CAT were 53.77 and 60.95 respectively. The population density of different bacteria was expressed (mean of 5 replicates in log_{10} CFU/g±SD). Superscripts (a, b, c, d) in a row are significantly different at P<0.05. 

The capability of gas-formation by composite microflora in NB group was 6.5 mL/g that polynomially (R² = 0.97) increased to 22.6 mL/g after 30 days of adaptation at hypoxia.

Apart from higher gas formation in ileum, basal activity of some microbes associated enzymes like α-amylase, gluco-amylase, proteinase and β-glucuronidase enhanced in HH group after 30th day of familiarization to the simulated hypoxia (Table 2).

### Table 1—Alteration of some indicator bacterial populations of small intestine and its change with GDI after 30 days of hypobaric hypoxia

<table>
<thead>
<tr>
<th>Microbial parameters</th>
<th>NB group</th>
<th>Hypobaric hypoxic exposure duration (in days)</th>
<th>GDI (log_{10}NB/log_{10}HH) and folds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Total aerobes</td>
<td>4.75±0.14a</td>
<td>3.54±0.11b</td>
<td>2.56±0.09c</td>
</tr>
<tr>
<td>Total anaerobes</td>
<td>6.02±0.17c</td>
<td>6.19±0.15c</td>
<td>7.41±0.13b</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>2.76±0.03d</td>
<td>3.55±0.08c</td>
<td>4.01±0.06b</td>
</tr>
<tr>
<td>Bifidobacterium sp.</td>
<td>2.19±0.04e</td>
<td>2.15±0.06c</td>
<td>2.17±0.04bc</td>
</tr>
<tr>
<td>Bacteroides sp.</td>
<td>3.12±0.09f</td>
<td>3.98±0.11c</td>
<td>4.32±0.08b</td>
</tr>
<tr>
<td>Lactobacillus sp.</td>
<td>4.93±0.07g</td>
<td>4.95±0.10c</td>
<td>5.01±0.11bc</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>4.30±0.09f</td>
<td>5.22±0.08c</td>
<td>5.75±0.11b</td>
</tr>
<tr>
<td>Peptostreptococcus sp.</td>
<td>5.36±0.12d</td>
<td>5.63±0.11c</td>
<td>6.31±0.14b</td>
</tr>
</tbody>
</table>

The population density of different bacteria was expressed (mean of 5 replicates in log_{10} CFU/g±SD). Superscripts (a, b, c, d) in a row are significantly different at P<0.05.

### Table 2—Modification of some luminal enzymes activities in NB group and HH group during 30 days hypobaric hypoxic acclimatization

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>NB</th>
<th>Hypobaric hypoxic exposure duration (in days)</th>
<th>Increase after 30 days over NB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>α-amylase</td>
<td>305.22±12.11c</td>
<td>313.12±13.45c</td>
<td>387.29±15.11b</td>
</tr>
<tr>
<td>Gluco-amylase</td>
<td>127.66±6.12d</td>
<td>143.32±6.75c</td>
<td>188.16±7.33b</td>
</tr>
<tr>
<td>Proteinase</td>
<td>7.76±0.87b</td>
<td>5.26±0.35c</td>
<td>8.19±0.91b</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>3.42±0.34b</td>
<td>3.95±0.38bc</td>
<td>4.14±0.44bc</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>6.39±0.25d</td>
<td>7.43±0.34c</td>
<td>8.56±0.51b</td>
</tr>
</tbody>
</table>

Specific activities of enzymes were expressed as U/mg of protein. Superscripts (a, b, c, d) in a row are significantly different at P<0.05.
activity, GSH and GSSG pool in the small intestinal epithelia were declined gradually after 10th, 20th and 30th day in HH group. In relation to lower level of antioxidants the formation of MDA in small intestinal epithelia in HH group was progressively elevated \((P<0.05)\) from 3.17 mM/mg of protein (in NB group) to 8.82 mM/mg of protein in HH group after 30 days acclimatization in hypoxia.

Histological study of small intestine by PAS staining showed that thinner layer of mucus along the surface of villi with reduction of goblet cell in HH group (Fig. 1). Besides, the villus length of small intestine shortened accompanying disordered arrangement and edema with higher infiltration of inflammatory cells in lamina propia. Tissue debris in the lumen of small intestine was noted in the HH group but there were no such noxious and debris matter present in NB group. Scanning electron microscopy also revealed severely injured intestinal mucosa as well as evident atrophy and disordered villi in HH group in respect to orderly intestinal villi of NB group (Fig. 2).

**Discussion**

Acute exposure to the hypoxic atmosphere at high altitude (above 8,000 metres) stimulated the sympatho-adrenal and central nervous system that cause in pathophysiological and metabolic malfunctions\(^1,18\). The role of enteric microbiota in bidirectional gut-brain interaction in health and disease has been reported. The intricate network of commensal bacterial flora and GI tract is co-evolved toward a mutually beneficial state\(^19\).

Normally, the proximal part of small intestinal mucosa was colonized with \(10^7-10^9\) number different (300-500) microbial species that formed a stable alliance with the host by a predominance of gram-
negative organisms and anaerobes. The environmental stress at HA caused hypoxia, that in turn reduced the oxygen delivery to peripheral tissue and cause cellular dysoxia. This higher anaerobic state of epithelia decreased the population of total aerobes. But it favoured the growth of total anaerobes in luminal microenvironment. The metabolic and respiratory networks of prokaryotes are drastically modulated by ambient oxygen tensions however micromolar concentrations of oxygen are sensed and transduced into changes in gene expression.

In *E. coli* there is a graded series of responses which facilitated its growth from microaerobic to aerobic range. This remarkable adaptability and aerotaxis ability of *E. coli* induced to increase in population size at the altered hypoxic microenvironment. This proliferation regulated the growth of other anaerobes like *Bacteroides* sp., *Lactobacillus* sp and *Bifidobacterium* sp. But it could not be confirmed from the experiment that why the enlargement of *Bifidobacterium* sp. was lower than other microbial population. Higher colonization of obligate anaerobes in the lower oxidation reduction potential of small intestine encouraged the establishment of other strict anaerobes like *C. perfringens*, *Peptostreptococcus* sp. This modified microbial imprint in the small intestine may influence the adhesive and invasive capacity with epithelial cell and caused opportunistic infection during acclimatization in hypoxemia.

Asides, hypoxia causes an immediate increase in gastric vagal nerve activity exerted a tonic inhibitory effect which in turn decrease the gastric tone and amplitude of contraction. The lower gastrointestinal motility and reduced delivery of gastric content resulted in decrease of absorption and self cleaning ability that extended the stay time in a nutrient rich environment. It may encourage proliferation of different opportunistic pathogenic bacteria as well as immuno modulation of epithelia. In a rat model, the migrating motor complex, often referred to as the *housekeeper of the gut*, was critical to the prevention of bacterial overgrowth in the upper small bowel.

Depletion of oxygen helped over population of different microbes that involved in higher anaerobic digestion and produced excess flatus and acid in the small intestine.

The microbial population secrete an array of enzymes to digest the unabsorbed dietary substance to salvage energy and provide it to the epithelia. Malabsorption of nutrients in hypoxia induced the activity of α-amylase, gluco-amylase by which overpopulation of microbes struggled to harvest energy and survive in the diverged ecological niche. Besides, protein was also utilized as a nitrogen source from which less energy and more toxic metabolites like urea and ammonia were produced. It may diffuse through the epithelial barrier and enter into the systemic circulation and complicated the acute mountain sickness (AMS). Moreover higher activity
of bacterial \(\beta\)-glucuronidase may cleave acyl glucuronides to their aglycone that reabsorbed through the hepatobiliary system and increase the load of carcinogen in the lumen of small intestine\(^3\). Therefore, consumption of any nonsteroidal drugs for the management of hypobaric hypoxia may initiate a cascade of events leading to epithelial damage and inflammatory responses which is triggered by higher permeability of the gut mucosa. These were also observed in the human gastrointestinal tract during high altitude acclimatization for 15 days at Leh (~3,505 m)\(^5\). But it was not clear from the experiment whether the sole source of these enzymes was either microbial or rat intestinal epithelia. Consequently, burden of lipopolysaccharide (LPS) due to enlargement of gram negative bacterial population heightened the level of alkaline phosphatase that removes the phosphate from glutamine of the lipid moiety to neutralize this LPS toxicity\(^{24}\). The activation of local innate immune activity stimulated the local gut-associated immune system which secretes immunoglobulin like IgG and IgA in the gut lumen that was also observed in human and rat model during acclimatization in hypoxia\(^2,4,5\).

Further, hypoxemia induced microvascular dysfunction during HA acclimatization altered cellular metabolic pathways which likely results in ATP depletion, acidosis and altered ion pump activity. It reduced cellular viability and increased paracellular permeability and produced excessive reactive oxygen species (ROS)\(^4,6,25\). This overwhelmed of ROS declined the SOD and CAT level of the intestinal epithelia (Table 3) with lower redox pool of GSH/GSSH. Therefore, unpaired electrons of the ROS damaged the lipid bilayer membrane of intestinal epithelia and resulted in higher formation of MDA in the small intestinal epithelia.

The discontinuous layers of mucus on small intestinal epithelia confer various ecological functions to the indigenous GI microflora. Normally, it lubricates the lumen and acts as innate immuno barrier to protect against the mechanical, chemical injury and bacterial invasion\(^23\). The composition and expression was changed in broad range of luminal insults, including changes in the normal microbiota. The reduction in number of goblet cells in HH group indicated that hypobaric hypoxia caused cytoclasia of goblet cells and dysfunction of the immuno barrier. Besides, higher infiltration of inflammatory cells in lamina propria, shortening of villus length and tissue debris in lumen of HH group indicated the induction of severe inflammation, damage and necrosis of the mucosal layer of intestine. This injury conferred the opportunity to pathogenic microbiota to adhere and invade in the systemic circulation and may complicate the AMS during hypoxic acclimatization. The SEM also revealed intestinal mucosal atrophy, mucosal barrier dysfunction that also strongly correlated with oxidative damage of epithelial layer.

**Conclusion**

It can be concluded from the results that hypoxic stress modulated indigenous GI microflora and the magnitude of association with host. The expansions of anaerobic bacterial populations coupled with the higher acid and gas formation in ileum. Distortions of microbial ecology and its functional activities in ecological niche, change luminal microenvironment that directly or indirectly induced inflammatory response. The damage of gut epithelial barrier may facilitate the influx of endotoxin and other noxious luminal content into the systemic. It may activate the systemic inflammation and accelerate the complication of AMS.

**Acknowledgement**

One of the authors A. A. is grateful to Department of Science and Technology, India for the INSPIRE Fellowship. Thanks are also to the Director, Defence Institute of Physiology & Allied Sciences, Delhi - 54 for financial support.

**References**