Biochemical Study of the Endothelial function in persons living at High Altitude in Saudi Arabia

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ABSTRACT

The aim of this study was to investigate the biochemical effect of high altitude on modulation of the adaptive response of endothelial functions in persons’ living at high altitude in Saudi Arabia. Materials and Methods: this study included 90 apparently healthy individuals, composed of 2 groups: High altitude group; 45 apparently healthy male volunteer subjects from Abha city, (2,200 meters above sea level). Sea level group; 45 apparently healthy control male volunteer subjects from Makkah. Laboratory measurements for all subjects were performed including: Fasting blood sugar, kidney and liver function tests, and plasma lipid profile. In addition, parameters of vascular endothelial functions including: Nitric Oxide (NO) in the form of serum nitrite and nitrate (NO2-/NO3-), serum endothelin-1 (ET-1) and vascular cell adhesion molecule (VCAM-1) were also measured. Results: Measurements confirmed higher levels of ET-1 in high altitude subjects compared with those at sea level (P < 0.001). Serum VCAM-1 in high altitude subjects are higher compared with those at sea level (P = 0.015). Our results also, confirmed higher levels of NO in high altitude subjects compared with those at sea level (P = 0.003). conclusion: our data suggest that chronic hypoxia at high altitude may lead to higher circulating concentrations of these important vasoactive compounds (VCAM-1, ET-1 and NO), either as a direct result of hypoxia or as adaptive response to chronic high-altitude exposure. This process could imply a potential role for the measurement of these compounds in the prognostic evaluation of patients with cardiovascular diseases.

KEYWORDS: Endothelin-1, VCAM-1, Nitric Oxide, High altitude, Endothelial function

INTRODUCTION

High altitude residents have adaptive mechanisms to survive in such hypoxic environment [1]. These adaptive mechanisms, although generally tolerated by most healthy subjects, may induce major problems in patients with preexisting cardiovascular diseases (CVD) [2]. High altitude acclimatization and adaptation mechanisms have been well clarified [3]. The knowledge about the risk factors, molecular mechanisms and related intracellular signaling pathways, and non pharmacological and pharmacological treatment options regarding endothelial dysfunction is still accumulating [4]. A number of physiological changes occur with increasing altitude, with hypoxia probably the most important effect [5]. Subjects apparently well adapted to these altitudes for many months or years develop chronic mountain sickness (CMS), also called Monge's disease [6]. Responses to continued hypoxaemia and tissue hypoxia may prove detrimental in the long term. For example, CMS occurs in natives or long-life residents living above 2,500 meters [7,8]. Defense mechanisms include erythropoiesis and angiogenesis to augment red blood cell mass and oxygen delivery, and metabolic re-modeling that increases utilization of oxygen-efficient fuel substrates such as carbohydrates [9]. These
adaptive mechanisms, although generally tolerated by most healthy subjects, may induce major problems in patients with preexisting cardiovascular diseases [10]. Exposure to high altitude may unmask coronary artery disease, left ventricular dysfunction, or pulmonary hypertension that was asymptomatic at sea level [11,12].

The healthy endothelium is a dynamic organ that regulates vascular tone by balancing production of vasodilators and vasoconstrictors in response to a variety of stimuli [13,14]. The endothelium, senses mechanical stimuli, such as pressure stress, and hormonal stimuli, such as vasoactive substances. In response, it releases agents that regulate vasomotor function, trigger inflammatory processes, and affect hemostasis [15]. Endothelial dysfunction has varied clinical implications in many diseases [16, 17]. Endothelial cell dysfunction (ECD) is a broad term which implies dysregulation of endothelial cell functions, including impairment of the barrier functions of endothelial cells and vasodilatation. Also, endothelial dysfunction can be defined as an imbalance between vasodilating and vasoconstricting substances produced by (or acting on) endothelial cells [18]. Several factors contribute to ECD including smoking, high blood pressure, diabetes, high cholesterol levels, obesity, hyperglycemia, advance glycation end products (AGEs), and genetic factors [19].

Endothelin-1 (ET-1) and endothelial nitric oxide (NO) synthase (eNOS) are a pair of antagonistic peptides present in endothelial cells [20]. ET-1 is a potent endogenous vasoconstrictor whereas NO is a vasodilator [21]. Both NO and ET-1 are produced locally by the vascular endothelium and modulate both vascular reactivity and vascular smooth muscle cell proliferation [22, 23]. ET-1 is the most potent internal vascular contracting factor currently known produced by the vascular endothelium and is implicated in various CVD states [3, 24].

Cell adhesion molecules are critical to many normal physiological processes. Given their widespread importance it is not surprising that cell adhesion molecules have also been implicated in many diverse pathological processes such as inflammation and wound healing, septic shock, transplant rejection, cancer, and atherosclerosis. Understanding of the role of cell adhesion molecules in these processes has suggested their use as either diagnostic or prognostic markers, or as potential targets for therapeutic intervention [25]. Vascular cell adhesion molecules -1 (VCAM-1) is an adhesion molecule, expressed on activated endothelium, involved in leukocyte attachment and transmigration and is a therapeutic target in inflammatory disease [26].

NO is synthesized from L-arginine by a family of nitric oxide synthases (NOS). Three NOS isoforms have been characterized: neuronal NOS (nNOS) primarily found in neuronal tissue and skeletal muscle; inducible NOS (iNOS) isolated from macrophages and many other cells; and endothelial NOS (eNOS) present in vascular endothelial cells, cardiac myocytes, and in blood platelets [27]. NO is a signaling for the control of blood pressure, blood flow, and other body functions [28] and also, it is an important antioxidant [29], and cellular energy production by mitochondria [30]. NO, the predominant mediator of normal vascular function, causing smooth muscle dilation and myofibrillar relaxation in response to stimulation by endogenous factors such as bradykinin, acetylcholine, and catecholamines, as well as ischemia [31]. The assessment of an altered NO availability is of potentially important diagnostic and prognostic significance. The plasma level of NO₂− i.e., the sum of NO₃− and NO₂−, is frequently used to assess NO bioavailability in vivo [32].

**The aim of the study**, therefore, was to investigate the biochemical effect of high altitude on modulation of the adaptive response of endothelial functions in persons’ living at high altitude in Saudi Arabia.

**MATERIALS AND METHODS**

**Subjects:**

To avoid the effect of gender difference, this study included 90 apparently healthy men with age range 35 – 55 (mean 45) years. All individuals selected for this study were living in the same area for the last five years and they were divided into 2 groups:

1- High altitude group: This group consisted of 45 apparently healthy volunteer men from Abha city, which is the capital of Aseer province in Kingdom of Saudi Arabia (2,200 meters, 7,200 ft, above sea level) representing High altitude group.

2- Sea level group: This group consisted of 45 apparently healthy volunteer men from Makkah (the holy city) representing sea level group.

**To be eligible for the study, all subjects had to fulfill the following criteria:**

No smoking, no history and clinical signs of heart failure, autoimmune disease, or any other chronic illness, such as diabetes, cancer, chronic liver disease, and chronic renal disease. Also, subjects with any infections, acute or chronic inflammatory disease, and patients with neural complications e.g., peripheral neuropathy were excluded from this study. All subjects were subjected to a thorough history taking and general medical examination with special stress on cardiovascular examinations. Blood pressure measurements as well as ECG were performed. Written informed consent was obtained from all volunteers according to the declaration of Helsinki and the study was approved by the Ethics Committee of College of Medicine, King Khalid University.

**Blood sampling:**

For all subjects, the following were done: Fasting venous peripheral blood samples (10 ml each from the antecubital vein) were obtained from all subjects. Blood samples were drawn using a 25-gauge needle, avoiding hemolysis, into plain tubes. The blood was centrifuged and the serum was separated into 0.5 ml aliquots and frozen at - 80°C for subsequent analysis. Several serum biochemical measurements for healthy subjects were made which include routine chemical analysis including:

1- Fasting blood sugar, kidney and liver function tests.

2- Lipid profile including: total cholesterol (TC), High density lipoprotein cholesterol (HDLC), Low density lipoprotein (LDLC) and triglycerides (TG).

3- Measurement of parameters of vascular endothelial functions including:

a- Endothelin-1 (ET-1)

b- NO oxide synthase (eNOS)

c- Endothelial nitric oxide (NO) synthase (eNOS)
b- Vascular Cells adhesion molecule-1 (VCAM-1)
c- Nitric oxide in the form of serum nitrite and nitrate concentrations.

Methods:
The biochemical parameters including kidney function tests, liver function tests and lipid profile were determined using commercial reagents with an automated chemical analyzer (Abbott analyzer, Abbott Laboratories, Abbott Park, Chicago, IL).

Determination of serum Endothelin-1: Measurement of the serum endothelin-1 was performed by human endothelin-1 immunoassay kit (Cayman chemical, Ann Arbor, MI, USA) using monoclonal antibody specific for ET-1. The concentration is determined by measuring the enzymatic activity of acetylcholinesterase at 412 nm against the standard curve using the plate reader (Human, Wiesbaden, Germany). The intra-assay coefficient of variation was 4.4% and the inter-assay coefficient of variation was 5.2% [33].

Determination of serum vascular cell adhesion molecule-1 (VCAM-1): Serum levels of VCAM-1 was assessed by commercial ELISA: Human serum VCAM-1 ELISA kit (Abnova, Jhongli, Taiwan), following the manufacturers’ instructions. ELISA experiments were run simultaneously on all samples previously frozen at ~80°C. The test is using Biotin / Avidin conjugate and the absorbance is measured at 450 nm using the plate reader (Human, Wiesbaden, Germany). The sensitivity of the test was determined to be 0.6 ng/ml. The intra-assay coefficient of variation was 3.1% and the inter-assay coefficient of variation was 5.2%.

Determination of serum Nitrate / Nitrite: We used colorimetric assay kit (Cayman chemical, Ann Arbor, MI, USA) in two-step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of the Griess reagents which convert nitrite into a deep purple azo compound [34]. Photometric measurement of the absorbance at 540 nm accurately determines the concentration. The serum samples were first thawed, then deproteinized by adding zinc sulfate. Deproteinization is a necessary step in the measurement of serum nitrite concentrations [35]. Ten microlitres of 1.5 g/mL zinc sulphate solution was added to 1mL of serum, vortexed for 1 minute, and centrifuged at 10,000 g for 10 minutes at room temperature (RT=25°C). The supernatant was pipetted out and centrifuged again at 10,000 g for 10 minutes. The clear serum (100 μL) was applied in duplicate to a 96-well ELISA plate, 100 μL of vanadium (III) chloride (8 mg/mL) was added to each well (for reduction of nitrate to nitrite) followed by the addition of 100 μL of Griess reagent (equal mixture of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine hydrochloride in distilled water). The plates were incubated for 30 minutes at RT and the optical density was measured at 540 nm using the plate reader (Human, Wiesbaden, Germany). A two-fold dilution series (0.193 - 100 μM) of NaNO₂ was prepared from 100 μM NaNO₂ solution using distilled water. Each dilution (100 μL) was mixed with an equal volume of Griess reagent, and the optical density (OD) was measured at 540 nm. A standard curve was plotted. The intra-assay coefficient of variation was 3.4% and the inter-assay coefficient of variation was 2.7%.

Statistical analysis of final results
The data were statistically analyzed using the Statistical Package for the Social Sciences software (SPSS version 13.0). The data were categorized as parametric and showed preserved normality, using kolmogrov-Smirnov test, and were presented as mean ± standard error of mean (SEM). The independent sample t-test was applied for analysis of difference between each two groups. A probability (P value) of 0.05 was considered to be statistically significant.

RESULTS
The data presented in table (1) showed that the high altitude subjects had average total cholesterol of 4.11 mmol/l, HDLc 1.00 mmol/l, LDLc 2.85 mmol/l and TAG 1.09 mmol/l. Sea level subjects had average total cholesterol 4.61 mmol/l, HDLc 1.18 mmol/l, LDLc 2.91 mmol/l and TAG 1.26 mmol/l. Our results also showed non-significant differences of lipid profile levels between subjects of high altitude and sea level, except the higher serum level of total cholesterol in healthy individuals of sea level compared to those of high altitude (P = 0.015).

ET-1 level was measured in serum samples of high altitude and sea level subjects. The high altitude subjects had average ET-1 serum levels of 2.30 ± 0.02 pg/ml and, whereas sea level subjects had average serum ET-1 level of 1.99 ± 0.05 pg/ml (Table 2). Measurements confirmed the significant higher levels of ET-1 in high altitude healthy subjects compared with those at sea level (P < 0.001).

Table 1: Comparison of Serum levels of lipid profile parameters in healthy male subjects of high altitude vs. of sea level.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TC (mmol/l)</th>
<th>HDLc (mmol/l)</th>
<th>LDLc (mmol/l)</th>
<th>TAG (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects of high altitude (N = 45)</td>
<td>4.11 ± 0.08</td>
<td>1.00 ± 0.023</td>
<td>2.85 ± 0.10</td>
<td>1.09 ± 0.05</td>
</tr>
<tr>
<td>Subjects of sea level (N = 45)</td>
<td>4.61 ± 0.10</td>
<td>1.18 ± 0.022</td>
<td>2.91 ± 0.10</td>
<td>1.26 ± 0.05</td>
</tr>
<tr>
<td>P</td>
<td>0.015</td>
<td>N.S</td>
<td>N.S</td>
<td>N.S</td>
</tr>
</tbody>
</table>

NS = Non Significant.
Table: 2 Comparison of Serum levels of Endothelin-1 (ET-1), Vascular cell adhesion molecule-1 (VCAM-1) and Nitrite/Nitrate (NO$_3^-$/NO$_2^-$) in healthy male subjects of high altitude vs. of sea level. Data expressed as Mean ± Standard Error of Mean.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ET-1 (pg/ml)</th>
<th>VCAM-1 (ng/ml)</th>
<th>NO$_3^-$/NO$_2^-$ (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects of high altitude</td>
<td>2.30 ± 0.02</td>
<td>1260.9 ± 47.6</td>
<td>120.1 ± 2.5</td>
</tr>
<tr>
<td>(N = 45)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subjects of sea level</td>
<td>1.99 ± 0.05</td>
<td>1057.5 ± 25.8</td>
<td>106.5 ± 1.5</td>
</tr>
<tr>
<td>(N = 45)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.001</td>
<td>0.013</td>
<td>0.003</td>
</tr>
</tbody>
</table>

VCAM-1 level was measured in serum samples of high altitude and sea level subjects. The high altitude healthy subjects had average VCAM-1 serum levels of 1260.9 ± 47.6 ng/ml, whereas sea level subjects’ had average VCAM-1 serum levels 1057.5 ± 25.8 ng/ml (Table 2). Measurements confirmed the significant higher levels of VCAM-1 in high altitude healthy subjects compared with those at sea level (P = 0.013).

The high altitude subjects had average serum level of nitrate/nitrite of 120.1 ± 2.5 nmol/ml, whereas the sea level subjects had average serum nitrite/nitrate of 106.5 ± 1.5 nmol/ml (Table 2). Measurements confirmed higher levels of nitrate/nitrite in high altitude healthy subjects compared with those at sea level (P = 0.003).

DISCUSSION

To the best of our knowledge, this is the first biochemical study to directly investigate the biochemical impact of high altitude on endothelial functions in human in Saudi Arabia. The interactions among various biomarkers remained unexplored under the stressful environment of high-altitude [7]. At high-altitude, differences in barometric pressure result in hypoxia which is subject to adaptation [36,37]. High-altitude exposure triggers a series of physiologic responses intended to maintain an adequate tissue oxygenation [10]. The healthy endothelium is a dynamic organ that regulates vascular tone by balancing production of vasodilators and vasoconstrictors in response to a variety of stimuli [31]. Circulating endothelial-derived/associated markers have been proposed as potential alternatives for evaluation of the endothelium in condition of vascular disorders [32,38].

ET-1 is the most potent internal vasoconstrictor factor currently known produced by the vascular endothelium and is implicated in various CVD states [3,24]. Because ET-1 plays a pathophysiologic role in various forms of cardiovascular disease, it has been suggested as a potential marker of endothelial dysfunction [13]. Our study showed significantly higher levels of ET-1 in high altitude healthy subjects (Mean = 2.3 pg/ml) compared with those at sea level (mean =1.99 pg/ml) (P < 0.001). These findings support the previous results that hypoxia leads to higher ET-1 levels and contributes to the increased hypoxic vasoconstriction [39-41]. In the Mount Everest study it has been observed that at high altitude an increase of plasma ET-1 compared to values found at sea level. These changes tended to decrease rapidly with the return to lower altitudes [42]. Also, Morganti et al [43] concluded that hypoxia appears to be a potent driving stimulus for ET-1 secretion in humans and that the circulating levels of ET-1 may have a relevant function in the adaptation of the pulmonary circulation to high altitude.

Hypoxia stimulates the induction of genes encoding growth factors for blood vessels and remodeling enzymes [44]. The elevated level of ET-1 in high altitude subjects could be explained by the up-regulation of ET-1 gene expression by hypoxia which is a potent modulator of gene expression influencing the expression of approximately 1.0% of the genes in the genome [45]. It is usually thought that ET-1 acts a paracrine/autocrine factor rather than as a classical circulating hormone [43]. Because ET-1 plays a pathophysiologic role in various forms of cardiovascular disease, it has been suggested as a potential marker of endothelial dysfunction [13]. Also, due to the potent vasoconstricting effects of ET-1 and its involvement in various CVD, blockade of the ET-1 receptor has received considerable attention [21,33]. ET-1 antagonists are promising new agents in the treatment of cardiovascular diseases [28,46].

Endothelial dysfunction is also characterized by increased production of endothelium-derived contracting factors, including angiotensin II and prostanoids [47] which will lead to increase in systemic blood pressure especially with significant changes at 1200 to 3000 m above sea level [48]. This leads to cell growth and inflammation by the activation of nuclear factor-κB (NF-κB), vascular cell adhesion molecule (VCAM) and interleukin-6 (IL-6). Because endothelial dysfunction is paralleled by arterial inflammation, markers of endothelial dysfunction include soluble forms of intercellular adhesion molecule-1 (ICAM-1), VCAM-1, and E-selectin, which can be assessed in plasma [13]. VCAM and cytokine action increases the adhesiveness of the endothelium and subsequently the binding of inflammatory cells to the endothelial surface.
leading to vascular inflammation and thrombosis [49]. Our study showed significantly higher levels of serum VCAM-1 in high altitude subjects compared to those at sea level (P ≤ 0.015). The elevated serum VCAM-1 levels in high altitude residents could be explained by the up regulation of VCAM-1 gene. It is known that the hypoxic inducible factor-1 (HIF-1) is involved in the up regulation of several genes including VCAM-1 [39].

In our study, NO blood levels were estimated by measuring its stable metabolites, nitrite (NO$_2^-$) and nitrate (NO$_3^-$). The serum nitrate/nitrite levels were significantly higher in high altitude healthy subjects compared with those at sea level (P = 0.003). Cultured endothelial cells have shown an increased production of NO, expression of endothelial NO synthase under hypoxia at the transcriptional level [50,51]. Our findings are consistent with other observations of elevated NO metabolite concentrations in the blood of highlanders [11,46,52]. Activation of NO system in high altitude is a probable adaptive mechanism of cardio-vascular system to hypoxia and micro-vascular blood flow. Such increased NO stable metabolites blood level in high altitude residents may explain the vaso-dilative reserve and strengthening of cardiac pump and contractile functions for adequate tissue perfusion and optimal oxygen supply [4,23]. Also, Rich & McLaughlin [53] and Reeves & Leon-Velarde [54] supported the notion that high nitrite/nitrate levels in high altitude residents might be an integral part of the human physiological response to hypoxia. Increased longer-lived NO metabolites such as nitrite and nitrate levels were associated with changes in microcirculatory blood flow which may affect local tissue oxygen delivery through cGMP-independent mechanism [52].

Our study is limited regarding the changes in NO metabolite levels in vascular tissue, skeletal muscle, or red blood cells, which also expected to contribute to the physiological adaptations. Moreover, it remains unclear whether NO itself or a change in NO-related post-translational modification of proteins or transcription factors, may contribute to beneficial adaptation to hypoxia. Although the main objective of our study was to assess the quantitative changes of the NO metabolites in high altitude, several biochemical parameters variables are worthy of further investigation. Our results suggest that NO is an integral part of the human physiological response to hypoxia. These findings may be of relevance not only to healthy subjects exposed to high altitude but also to patients in whom oxygen availability is limited through disease affecting the heart, lung or vasculature [23]. Overproduction of NO by endothelial cells in response to stimulation was intended to protect the cells, rather than damage cells [55].

**CONCLUSION**

In conclusion, our data suggest that chronic hypoxia at high altitude may lead to higher circulating concentrations of these important vasoactive compounds (VCAM-1, ET-1 and NO), either as a direct result of hypoxia or as adaptive response to chronic high-altitude exposure. This process could imply a potential role for the measurement of these compounds in the prognostic evaluation of patients with CVD.

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**Conflict of Interest**

Authors received no financial support from any industry to conduct this study, and no conflicts of interest in performing this study that might bias the interpretation of results.

**REFERENCES**


42. Lüscher TF, Barton M. Endothelins and Endothelin Receptor Antagonists. Therapeutic Considerations for a Novel Class of Cardiovascular Drugs. Circulation 2000; 102: 2434-2440.


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