Hyperbaric environment up-regulates CD15s expression on leukocytes, down-regulates CD77 expression on renal cells and up-regulates CD34 expression on pulmonary and cardiac cells in rat

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ABSTRACT

Objective(s): The aim of this study was to estimate effects of hyperbaric (HB) treatment by determination of CD15s and CD11b leukocyte proinflammatory markers expression. In addition, this study describes changes in CD77 and CD34 expression on rat endothelial cells in renal, pulmonary and cardiac tissue following exposure to hyperbaric pressure.

Materials and Methods: Expression of CD11b and CD15s on leukocytes, as well as CD77 and CD34 expression on endothelial cells in cell suspensions of renal, pulmonary and cardiac tissue in rats after hyperbaric treatment and in control rats were determined by flow cytometry.

Results: Hyperbaric treatment significantly increased percentage of leukocytes expressing CD15s (from 1.71±1.11 to 23.42±2.85, P<0.05). Hyperbaric treatment significantly decreased sum percentage of CD77+CD34+ renal cells (from 16.35±5.5 to 4.48±1.28, P<0.05). Hyperbaric treatment significantly increased percentage of CD34+ pulmonary cells (from 3.27±2.01 to 11.92±6.22, P<0.05). Our study is the first reporting the hyperbaric environment influence on CD34+ heart cells in rats.

Conclusion: The current findings of increased percentage of leukocytes expressing endothelial selectin ligand CD15s after HB treatment, point its role in endothelial damage prevention. We found out a significantly increase in percentage of CD34+ cardiac cells as well as CD34+ pulmonary cells in rats after HB treatment which could be a part of repair mechanism of injured endothelium caused by hyperoxia.

Introduction

The key point of hyperbaric treatment is the elevated pO2 level both in the plasma and tissues. Normal alveolar pO2 level is reached at 1 ATA (ATA = 760 mmHg which is the normal atmosphere pressure at the sea level), while higher atmosphere pressures cause an increase of the level of oxygen dissolved in the plasma. SCUBA (SCUBA, self-contained underwater breathing apparatus) diving was shown to induce a significant increase of the total number of leukocytes, particularly neutrophils. Whereas the exact mechanisms that lead to endothelial dysfunction are still incompletely understood, hyperoxia-induced production of reactive oxygen species (ROS), reduction in the bioavailability of nitric oxide (NO) and direct mechanical damage to the endothelium during decompression, are considered to play an important role (1).

Recently, it has been demonstrated that a single air SCUBA dive induced a significant increase in the number of monocytes expressing CD15 as well as the increase in the small monocyte subpopulation highly expressing CD15s (2). Sialylated fucosylated glycans (sialyl Lewis x-type glycans or CD15s) are expressed on circulating granulocytes and monocytes and, upon recognition by endothelial selectins, mediate initial leukocyte endothelium interactions (3).

Zen et al showed directly that human leukocyte CD11b is a major membrane protein decorated
with CD15s and that CD15s-related moieties mediate the binding of CD11b with E-selectin (4). A later study demonstrated inhibition of leukocyte adhesion to endothelium by hyperbaric oxygenation. The mechanism of this inhibition is attributed to decreased granulocyte CD11b/CD18 expression (5). The expression of atherogenic adhesion molecule CD11b was found to be decreased after high frequency and long duration exercise (6). It has also been shown that a competitive marathon race can decrease neutrophil functions (oxidative burst activity and phagocytic activity) in athletes (7).

Caveolae comprise one subset of lipid rafts in cell surface. They are flask-shaped membrane invaginations formed from lipid rafts by polymerization of caveolins, which are integral membrane proteins that tightly bind cholesterol and sphingolipids. Caveolae have been found to be partaking in many physiological and pathological processes involving endothelial cells, such as atherosclerosis, hemostasis, and thrombosis. Caveolae of endothelial plasma membranes are rich in neutral glycosphingolipid, globotriaosylceramide, Gb3Cer or CD77. Excessive endothelial CD77 accumulation is associated with endothelial dysfunction (8). Hyperbaric oxygen treatment, a method based on 100% oxygen exposure, has a beneficial effect on renal dysfunction in sepsis caused by *Escherichia coli* (9). CD77 is a receptor for *Stx* (*Stx*, *Shigella* toxins) produced by *Shigella dysenteriae* type 1 and enterohemorrhagic *E. coli* that are most common cause of HUS (HUS, hemolytic-uremic syndrome).

Uschida *et al.* showed that specific antibodies for *Stx* positively stained pulmonary tissue from a patient who died of HUS associated with *Stx*-producing *E. coli* infection, indicating the deposition of *Stx* in the lung. Related experiments with normal pulmonary tissue revealed apparent *Stx* binding to both vascular endothelium and to portions of the pulmonary epithelium. In addition, CD77-positive lung carcinoma cell lines, which are derived from lung epithelium, showed reactivity to *Stx* and a high susceptibility to *Stx*, as determined by MTT assay (10).

Glomerular endothelial cells in humans are the primary target of the toxic effects of *Stx*, but why lesions in *Stx*-associated HUS preferentially localize to the renal microvasculature is still unclear (11). Kidney is a human organ that has a dramatic capacity to regenerate after injury. Whether stem cells are the source of the epithelial progenitors replacing injured and dying tubule, epithelium is currently an area of intensive investigation. The fundamental unanswered questions in this field include whether renal stem cells exist in adults, if they do, where are they located (interstitium, tubule, cortex, medulla) and what markers can be relied upon for the isolation and purification of these putative renal stem cell (12). Resident stem/progenitor cells of different human adult organs are known to express stem cell markers such as CD34, CD117 and CD133. As we know, CD34 is a sialomucin-type glycoprophosphoprotein, traditionally a marker of hematopoietic stem cells and was found on endothelial cells and fibroblasts as well (13). Despite its utility as a stem-cell marker, the function of CD34 has remained remarkably elusive. It is believed that CD34 promotes cell proliferation and / or blocks differentiation of progenitor cells, while other members of CD34 family stimulate the migration of hematopoietic cells, or play a role in cell morphogenesis. It is interesting to point out that members of the CD34 family can stimulate and block cell adhesion (14). Exercise and the improvement of cardiovascular health tend to promote higher levels of circulating CD34+ cells (15). Advanced age and chronic cardiovascular disease tend to decrease both the functionality and the total count of CD34+ cells (16, 17). In many current researches, the bone marrow-derived CD34+ cells have been evaluated as a tool to repair the endothelial damage caused by cardiovascular disease. New evidence supports both a role of transdifferentiation of CD34+ cells to cardiomyocytes (18) and their ability to fuse with existing cardiomyocytes (19). In recent review, Mackie and Losordo showed the preclinical evidence supporting the therapeutic potential of CD34+ cells in ischemic models, and the evidence for the clinical usefulness of CD34+ cells in the treatment of human ischemic disease (20).

Muller *et al.* demonstrated that CD34 is heterogeneously expressed by human pulmonary endothelial cells, and that expression is under influence of different physiological/pathophysiological factors, such as age or pulmonary hypertension (21).

Due to the described beneficial effects of hyperbaric treatment on the one hand, and its potential proinflammatory effect on the other hand, the aim of this study was to estimate effects of hyperbaric treatment by determination of CD15s and CD11b leukocyte proinflammatory markers as well as CD77 and CD34 expression on rat renal, pulmonary and cardiac cells.

### Materials and Methods

Experiments were performed with male Sprague-Dawley rats raised under controlled conditions (temperature of 22 ± 1°C and a light schedule of 14-hr light/10-h dark) at the Split University Animal Facility. Laboratory food and tap water were supplied *ad libitum*. Animals were bred and maintained according to the Guide for Care and Use of Laboratory Animals and the protocol was approved by the Ethics Committee of the Split University Medical School. Four weeks old rats were separated in 2 groups: the examination group (N=9) which underwent the hyperbaric treatment and untreated control group (N=5). Rats were exposed to hyperbaric pressure of air mixture (21% oxygen, 79% nitrogen) which equals the
immersion depth of 65 meters (7.5 ATA), in duration of 30 min. Decompression stops were 1 min at the depth of 15 m, 7 min at 12 m, 10 min at 9 m, 23 min at 6 m and 47 min at 3 m, according to US Navy decompression tables (http://www.usu.edu/scuba/navy_manual6.pdf). The same protocol was repeated next 2 days. In choosing the right protocol, we were guided by our previous results (22). The animals were exposed to hyperbaric treatment in a Comex hyperbaric chamber (Comex, Marseilles, France). The oxygen and carbon dioxide concentrations in the chamber were controlled by Servomex 570A oxygen analyzer (Servomex, Houston, TX, USA) and by Infrared carbon dioxide gas analyzer (Infrared Industries Inc, Santa Barbara, CA, USA).

In this study, the method for the preparation of samples for flow cytometry, as well as all antibodies used, were in accordance with our previous study (8).

**Flow cytometry of leukocytes**

Blood samples for flow cytometry were collected from jugular vein into glass vacuum tubes with EDTA anticoagulant, one hr after hyperbaric treatments and before sacrifice. One hundred µl of whole blood was pre-treated with FcR (Fc-receptor)-blocking reagent (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to prevent non-specific binding and it was incubated in the dark for 30 min on ice with 0.5 µg of primary anti-CD15s antibody produced in mouse (Pharmingen, San Diego, CA, USA). After two washes in 0.1 M PBS with 0.1% sodium azide, 0.5 µg of secondary FITC-conjugated, affinity chromatography-purified rabbit anti-mouse antibody (Pharmingen, San Diego, CA, USA) and 1 µg of phycoerythrin (PE)-conjugated antibody reactive to CD11b (IQ Test, Beckman Coulter, Marseille, France) were added to cells and incubated in the dark on ice for 30 min. After red blood cell lysis with Red Blood Cell Lysis Solution (Miltenyi Biotec, Bergisch Gladbach, Germany), 10^5 events were recorded on a Coulter Epics XL flow cytometer (Beckman Coulter Corporation, Miami, USA). Fluorochrome and isotype-matched controls as well as unstained cell samples were used as negative controls. Analysis of samples was done by WinMDI 2.9 analysis software. Results were expressed as percentage of cells showing expression of the assessed adhesion molecules.

**Flow cytometry of tissue cells**

After third hyperbaric treatment, rats were euthanized with prolonged exposure to diethylether and kidneys, lungs and heart were dissected from all animals.

Tissues were minced with scissors and incubated in a solution of 0.1 M PBS (phosphate buffer solution) with 0.1 % (for kidney) and 0.2 % (for heart and lung) collagenase type IA (Roche Diagnostics GmbH, Mannheim, Germany) in ratio: 100 mg tissue/5 ml collagenase in PBS. Cell suspensions were incubated for 30 min / 1 hr (kidney / heart, lung) at 37°C, with gentle stirring. After incubation, cell suspensions were filtrated through a 40-µm nylon mesh (Cell Strainer; BD Biosciences, San Jose, CA, USA) and suspended at 1.0 x 10^6 cells ml^{-1} in 100 µl 0.1 M PBS.

Monoclonal anti-CD34 antibody conjugated with Phycoerythrin Cyanin 5 (PC5, Beckman Coulter, Marseille, France) was used for detection of CD34 positive cells. Monoclonal anti-CD77 antibody conjugated with FITC (BD Pharmingen, Erembodegem, Belgium) was used for detection of CD77 positive cells.

Isolated tissue cells were incubated in dark at 4°C for 30 min with two antibodies for double cell labeling: 1 µg of anti-CD34-PC5 and 1 µg of anti-CD77-FITC. After two washes in 0.1 M PBS, cells were resuspended in 0.3 ml of 0.1 M PBS. 10^5 events were recorded on a Coulter Epics XL flow cytometer (Beckman Coulter Corporation, Miami, USA). Fluorochrome-minus-one controls as well as unstained cell samples were measured and processed as negative controls to set the appropriate regions. Analysis of samples was done by WinMDI 2.9 analysis software. Results were expressed as percentage of cells showing expression of the assessed adhesion molecules.

**Statistical analysis**

Data are reported as mean ± SD (SD, standard deviation). Total CD15s+ leukocytes were defined as a sum of % of CD11b-CD15s+ and % of CD11b+CD15s+ leukocytes and total CD11b+ leukocytes as a sum of % of CD11b+CD15s+ and % of CD11b+CD15s- cells. Total CD34+ cells were defined as a sum of % of CD34+CD77- and % of CD34+CD77+ and total CD77+ cells as a sum of % of CD34+CD77+ and % of CD34+CD77- cells. Due to relatively small sample, nonparametric Mann Whitney U test was used to test significance of differences between control and experimental group. Coefficient of correlation was calculated between variables: CD11b+ and CD15s+ leukocytes with CD34+ and CD77+ tissue cells.

All of the results were considered significant at 95% confidence level (P < 0.05) and were obtained by using software Statistica 12.0 (StatSoft, Tulsa USA).

**Results**

In this study an effect of 3 repeated hyperbaric treatments on percentage of CD11b+ and CD15s+ leukocytes was investigated.

The percentages of CD15s+CD11b- leukocytes were significantly increased (from 1.71 ±1.11 to 23.42 ±2.85, P<0.05) and total CD15s+ leukocytes were significantly increased (from 4.51 ±2.42 to 25.68 ±3.22, P<0.05) in group that went hyperbaric treatment after the first day. Hyperbaric treatment did not change percentage of total CD11b+ leukocytes (from 7.32 ±3.98 to 5.25 ±0.75) (Figure 1).
The percentage of CD15s+CD11b- leukocytes decreased from second to third day, although not statistically significant. After strong increase of percentage of total CD15s+ leukocytes, following first and second hyperbaric treatment, this percentage slightly decreased following third treatment.

Furthermore, the goal of this study was to determine expression of CD77 and CD34 on renal, cardiac and pulmonary rat cells after repeated hyperbaric treatment in comparison to non-treated animals.

Hyperbaric treatment significantly decreased sum percentage of CD77+CD34- and CD77+CD34+ renal cells (from 16.35±5.5 to 4.48 ±1.28, P < 0.05). The percentages of total CD34+ rat renal cells in the group exposed to hyperbaric treatment was also significantly lower compared to the control group, from 23.24± 8.38 to 10.76 ±6.32, P < 0.05 (Figure 2).

It is well known that lung epithelium is another target for Stxs, and Stx-mediated injury to lung epithelial cells is thought to play an important role in the pathogenesis of pulmonary involvement associated with *E. coli* infection (10).

Based on our results, hyperbaric treatment would not have beneficial effect on lung in conditions associated with *E. coli* infection as percentage of total CD77+ lung cells increased in rats that went hyperbaric treatment, from 3.41±2.11 to 23.53 ±13.09, P < 0.05 (Figure 3). Total CD34+ rat lung cells in the group exposed to hyperbaric treatment was significantly higher compared to the control group (from 3.27±2.01 to 11.92 ±6.22, P < 0.05).

The percentage of total CD34+ cells was significantly increased in cardiac tissue in group of rats that went hyperbaric treatment, from 4.98±3.17 to 33.79 ±14.69, P < 0.05. We found out that percentage of CD77+ cardiac tissue cells were significantly increased due to hyperbaric treatment, from 1.81±2.15 to 8.19 ±4.29, P < 0.05 (Figure 4).
Table 1. The correlations between the proportion of different leukocytes and cellular subpopulations from the heart, lung and kidney of rats that went hyperbaric treatment

| Tissue cellular subpopulations | Leukocyte populations | Total 11b+ | Total 15s+ |
|-------------------------------|-----------------------|------------|------------|
|                               | Total 11b+             | r          | P          |
| Cardiac                       | Total 34+              | 0.55       | 0.12       | -0.28      | 0.46       |
| Pulmonary                     | Total 77+              | 0.64       | 0.06       | -0.51      | 0.16       |
| Kidney                        | Total 34+              | 0.46       | 0.21       | -0.30      | 0.43       |
|                               | Total 77+              | 0.24       | 0.53       | -0.57      | 0.11       |
|                               | Total 34+              | 0.26       | 0.50       | 0.17       | 0.67       |
|                               | Total 77+              | -0.17      | 0.67       | 0.29       | 0.45       |

Correlation analysis results: r=coefficient of correlation, P=significance of correlation coefficient

The results of correlation analysis between leukocytes markers CD11b and CD15s with tissue antigens CD34 and CD77 in rats that went hyperbaric treatment are presented in Table 1. There is no statistically significant correlation between leukocytes markers and tissue antigens in rats that went hyperbaric treatment.

Discussion

The expression of atherogenic adhesion molecule CD11b was found to be decreased after high frequency and long duration exercise (23). Granulocyte-endothelial cell adhesion tests indicate that CD11b, the major membrane protein decorated with CD15s (4) is decreased after hyperbaric oxygen treatment. However, our results show unchanged CD11b leukocyte expression in rat following hyperbaric treatment. The opposite results obtained in our study could be explained by the different conditions in hyperbaric air treatment versus hyperbaric oxygen treatment. Several fold higher percentage of total CD15s+ than total CD11b+ leukocytes was detected in group that went hyperbaric treatment. That could be the consequence of higher affinity of anti-CD15s antibody to sialyl Lewis x-type glycoprotein of CD11b glycoprotein than the affinity of anti-CD11b antibody to peptide epitope Mac-1 of CD11b glycoprotein.

Result of significantly increased percentage of CD15s+ leukocytes after repeated hyperbaric treatment is in accordance with previously reported results and elucidated role of this protein in the acute inflammatory process (24).

Kidneys play a critical role in maintaining the homeostasis of blood. Edremitlioğlu et al showed previously that renal dysfunction in sepsis improved by the use of hyperbaric oxygen was accompanied by an increase of antioxidative defense mechanisms: the superoxide dismutase and catalase activities in the renal cortex, and an increase in the catalase activity in the renal medulla (9). The effects of hyperbaric treatment on healthy kidneys are still unknown. Daily secretion of urine increases for 500 ml during diving with air mixture (up to 3-49 ATA), although the intake of fluids and velocity of glomerular filtration remains unchanged (25). Furthermore, it has been demonstrated that Gb3 is over-expressed in proliferative endothelial cells of growing tumor relative to quiescent cells and it could be a viable alternative target for tumor immunotherapy and angiogenesis inhibition (26). Our study showed that percentage of CD77+CD34+ rat renal cells in the group exposed to hyperbaric treatment was significantly lower than in the control group, as well as the percentage of total CD77+ cells. Considering the fact that CD77 molecules are located next to Na+/K+-ATPase in caveolins of kidney epithelial cells (27), our results show the possible role of CD77 in mechanisms responsible for development of hyperbaric diuresis.

In adult kidneys, antibodies against CD34 label almost all endothelial cells (28). Podocyte luminal membrane domain contains other sialomucins of the CD34 family: podocalyxin and endoglin whose function is still poorly understood. Acevedo et al reported an increased expression of CD34 on renal glomerular cells of older diabetic animals which reflect involvement of CD34 in the pathogenesis of glomerular alterations related to age and diabetes (29). In addition, putative progenitor cell mobilization is higher with 2.5 versus 2.0 ATA of oxygen treatments, and all newly mobilized cells exhibit higher concentrations of an array of regulatory proteins (30). A single dive acutely induces vascular oxidative stress, causing transient endothelial dysfunction. Endothelial progenitor cells and circulating angiogenic cells contribute to endothelial repair, either by integrating in injured endothelium or by secreting angiogenic growth factors (31).

The present study, in contrary, showed a clear decrease in percentage of CD34+ renal cells after repetitive hyperbaric treatment. Hyperoxia-induced production of ROS, reduction in the bioavailability of nitric oxide (NO) and direct mechanical damage to the endothelium during decompression are considered to play an important role to endothelial dysfunction (1). It is speculative that ROS induced apoptosis of renal CD34+ is plausible mechanism for the observed decrease. In addition, it has been reported in rats that a decompression trauma acutely increased levels of interferulin-6 (32) and therefore we can speculate that the release of pro-inflammatory cytokines in response to hyperbaric treatment may account for apoptosis in endothelial renal cells.

The percentage of total CD34+ cells in lung tissue was also increased and we can assume that hyperbaric conditions induce pulmonary endothelial angiogenesis. Few recent studies showed how blood-derived CD34+ endothelial progenitor cells contribute to pulmonary angiogenesis. It has been concluded that circulating CD34+ endothelial progenitor cells, characterized by active cell division and an amplified transcriptional signature, transit into resident endothelial cells during compensatory lung growth. The authors discuss how therapeutic manipulation of these cells may be beneficial in a variety of lung diseases (33).

In this study, we used immersion depth of 65 meters, and assume that changing the hyperbaric conditions and changing depths would change results of measured antigens. Exposure to special environment
that excessive endothelial CD77 gycosphin- golipid accumulation leads to K (Ca) channel dysfunction (43). In study of Režić-Mužinić et al renal CD34+CD77+ cells showed the most sensitivity to elevated calcium (8). That results are significant in the view of recent finding of vascular endothelial-cadherin cleavage caused by Ca²⁺ influx that contribute to the dissolution of adherent junctions during endothelial cell activation and apoptosis (44). We speculate that CD77-CD34+ cells in kidney tissue and CD77-CD34+ cells in pulmonary tissue, due to their poorer lipid raft content, succumb at higher extent to the dissolution of adherent junctions during endothelial cell activation and apoptosis provoked by Ca²⁺ influx.

Conclusion

Results of this study recruit CD15s analyses for the majority investigations of leukocyte proinflammatory features and present CD15s+ leukocytes as intelligent cells critical for the regulation of the inflammatory process with ability to adjust to extreme conditions. Based on our findings, we also speculate that positive effects of hyperbaric oxygenation on renal dysfunction in sepsis caused by E. coli are mediated by the decreased percentage of CD77+ cells. Our result of increased percentage of CD34+ pulmonary cells after hyperbaric treatment support the hypothesis that endothelial progenitor cells play a very important role in lung growth in physiological and many pathophysiological conditions. For now, we can only speculate of beneficial effects of hyperbaric treatment in promoting heart angiogenesis as well as use of hyperbaric conditions as possible therapeutic method for ischemic cardiovascular diseases treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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