Serum levels of nitric oxide and endothelin-1 in vasculopathy managed with hyperbaric oxygen therapy

Sasa Dragic[1,4]*, Danica Momcicevic[1], Biljana Zlojutro[1], Milka Jandrić[1], Tijana Kovacevic[2,5], Vlado Djajić[1,5], Aleksandar Gajić[3], Goran Talić[3,5] and Pedja Kovacevic[1]

[1]Medical Intensive Care Unit, University Clinical Centre of Republic of Srpska, Banja Luka, Republic of Srpska, Bosnia and Herzegovina

[2]Pharmacy department, University Clinical Centre of Republic of Srpska, Banja Luka, Bosnia and Herzegovina

[3]Institute for the Physical Medicine and Rehabilitation "dr Miroslav Zotovic" Banja Luka, Republic of Srpska, Bosnia and Herzegovina

[4]Pan- European University "Apeiron", College of Health Sciences, Banja Luka, Republic of Srpska, Bosnia and Herzegovina

[5]Faculty of medicine, University of Banja Luka, Banja Luka, Republic of Srpska, Bosnia and Herzegovina

Abstract

Background: Roles of nitric oxide (NO) and endothelin-1 (ET-1) in the local regulation of blood flow under physiological conditions are important and well known, while data on their effects and interactions in conditions of hyperbaric hyperoxia is still insufficient.

Methods: This was a prospective observational study which included patients who underwent HBOT in accordance with existing therapeutic protocol for PAD during time period of six months, between January and July of 2016. Clinical stage of PAD according to Fontain was taken into account, as well as risk factors, demographic, anthropometric and clinical characteristics of studied patients.

Results: The study included 64 patients with a mean age (± Sd) 60.2 ± 12.7 years, of whom 28 were female. Patients' NO serum levels generally increased after HBOT ($NO_{before\ HBOT}$ 21.6 ± 9.2 vs. $NO_{after\ HBOT}$ 23.5 ± 10.6 (p=0.2)), except in patients with stadium IV of PAD who had lower serum NO levels after HBOT ($NO_{before\ HBOT}$ 23.6 ± 11.5 vs $NO_{after\ HBOT}$ 20.4 ± 7.0 (p=0.4)), although these differences were not statistically significant. On the contrary, in all studied patients ET-1 level increased significantly after HBOT ($ET-1_{before\ HBOT}$ 4.2 ± 11.6 vs. $ET-1_{after}$ 18.3 ± 21.0 (p<0.001)) including patients with stadium IV of PAD.

Conclusion: Treatment of PAD using HBOT leads to the predominance of vasoconstrictor effects probably caused by elevation of serum ET-1 concentrations, while other factors such as exposure time to hyperbaric conditions, activation of antioxidant molecules, and the influx of other interfering substances must be considered in interpreting the effects of NO molecules.

Introduction

Tissues regulate blood flow in regards to its own metabolic needs, and this regulation is in short term done by changing the diammeter of small blood levels while in long term it depends on local conditions and adaptation mechanisms [1,2]. Endothelial cells of small arteria and arterioles wall produce vasoactive substances, some of which are potent vasodilatators – like nitric oxide (NO) and others are potent vasoconstrictors – such as endothelin - (ET-1) [3].

NO is also considered responsible for platelet aggregation, non-adrenergic-non-cholinergic neurotransmission and cytotoxic reactions [4], while ET-1 has a 100-fold stronger vasoconstrictor effect than adrenaline and may exert effects on muscle contractility, secretory activity, cellular transport management, gene expression, cell growth and proliferation as well as modulation of the immune response [5,6].

Homeostasis of vasoactive substances is impaired in many diseases; one of them is peripheral arterial disease (PAD) where HBOT is used as one of possible treatment modalities. The effects of HBOT on suppression of gas gangrene toxin production, on NK cell activity, leukocyte adhesion, vasoconstrictor effects in normal blood vessels under hyperbaric conditions, on fibroblast production, osteoclast activity, modeling of immunosuppressive properties and diminished interleukin 1 are well known [7]. Oxygen availability is known to affect vascular tone control as it leads to changes in the production and sensitivity of blood vessel walls with various vasoactive substances such as arachidonic acid metabolites and NO [8-14].

The mechanisms by which HBOT exerts positive effects cannot be explained solely by the compensation of oxygen deficiency [15,16]. Studies have shown that inhalation of oxygen at 2 bars does not act vasoconstrictively until the lack of oxygen in the tissues is corrected. HBOT increases oxygen supply to hypoxia-compromised tissues and cells, reduces the build-up of lactic acid and other metabolites in the muscle and helps developing of collateral circulation. If this type of therapy is combined with vasodilators, prostaglandin infusions, sympathetic denervation, surgical revascularization procedures and rehabilitation, excellent results can be achieved [17].

Most PAD patients have impaired vascular function including vascular regulation. So far, there have been various controversies in

the knowledge and findings related to effects of NO. NO is known to have vasodilatory effects, it increases angiogenesis and protect endothelial cells from apoptosis. Besides, NO inhibits the migration and proliferation of smooth muscle vascular cells and reduces platelet activation, while NO donors increase the amount of collagen in fibroblasts by promoting normal wound healing [18-21].

Experimental [22] and clinical data suggest that intermittent HBOT [23] decreases tissue edema, increases NO synthesis, changes vascular reactivity to stimuli [24] and inhibits neuroinflamatory factors expression and apoptotic pathways [25]. Although NO is considered responsible for positive effects of HBOT, other vasoactive substances such as ET-1 should not be overlooked, since some experimental data show changes in ET-1 levels under hyperbaric conditions [26].

Materials and methods

Study design and setting

This was a prospective observational study which included patients who underwent HBOT in accordance with current therapeutic protocol for PAD during time period of six months, between january and july of 2016 at the Institute of Physical Medicine and Rehabilitation "Dr. Miroslav Zotovic" Banja Luka - Centre for hyperbaric medicine and chronic wound treatment. The study was approved by the Ethics Committee of the Institute for the Physical Medicine and Rehabilitation "dr Miroslav Zotovic" Banja Luka. The informed consent was signed by the patient or authorised representative of the patient. Analysis of NO and ET-1 serum levels were performed in two different certified laboratories. SH animals were divided in subgroups:

Patients

The Centre for Hyperbaric Medicine and Chronic Wound Treatment is the only centre in the Republic of Srpska that uses the method of HBOT in the treatment of PAD and covers the territory of about 1.3 million inhabitants. Inclusion criteria for this study were age above 20 years, signed consent to participate in the study (informed consent), documented diagnosis of PAD including the stage of the disease according to Fontain [27], physician recommendation for HBOT and cardiologist approval for HBOT. Exclusion criteria were existence of upper respiratory tract infection, emphysema with hypercapnia, febrile condition, spontaneous pneumothorax in medical history, previous reconstructive operations of the middle ear, previous operations on the chest, confirmed changes in radiography or CT of the chest, viral infections and claustrophobia. According to Fontain's classification of PAD, the study included patients with stage II (claudicatio intermitens as the dominant symptom), III (resting pain as the dominant symptom), and IV (the predominant finding is irreversible ischemia with necrosis and gangrene). Prior to inclusion in the study, each patient was interviewed and demographic data (gender and age) and anthropometric data (height and weight from which BMI was calculated) were recorded for patients who provided written consent, documented medical history and current comorbidities (mandatory for diabetes mellitus), current therapy (mandatory for acetylsalicylic acid and insulin), and smoking status.

HBOT treatment protocol

HBOT of studied patients was provided using modern multiseat hyperbaric chamber type Haux-Starmed 2500. The conditions to which all subjects were subjected in accordance to therapeutic protocol were to achieve a maximum of 2.2 atm, ie 1.2 bar (corresponds to a dive at 10 meters depth) at FiO2 1.0 (100% O2). Patients inhaled oxygen via individual masks and one treatment lasted 60 minutes (10 minutes to achieve compression, treatment under achieved conditions for 40 minutes and 10 minutes to decompress). Each of these patients were submitted to 10 therapeutic procedures.

Blood sampling

Blood for analysis was obtained by venipuncture of the cubital vein. All principles of asepsis were followed during blood sampling. A total sample of 4 milliliters of blood was taken from each patient; the first sample of 2 milliliters was taken 15 minutes before the first treatment in the hyperbaric chamber, and the second sample of 2 milliliters was taken 5 minutes after the completion of the tenth treatment.

NO sereum levels measurement

NO levels were determined by spectrophotometry using the Griess reagens (Griess method). Immediately after sampling, the samples were treated with 30% $ZnSO_4$ to deproteinize the blood and release hemoglobin bounded NO_3^{2-}. After adding 0.05 milliliters of 30% $ZnSO_4$ to one milliliter of heparinized blood diluted with 0.9% NaCl in ratio 1:1, it was centrifuged for 10 minutes, and separated supernatants were stored in the freezer at -80 °C. NO concentration was measured using the classical colorimetric Griess reaction, the conversion of NO_3^{2-} to NO_2^{2-} by elemental zinc followed by measuring NO_2^{2-} concentration. 8 milligrams of elemental zinc powder suspended in 0.4 milliliters of purified water was added to 1 milliliter of deproteinized blood. 0.032 ml of 5% acetic acid and purified water of up to 2 milliliters were then added to the sample and stirred using electromagnetic vibrator for 5 minutes at room temperature. After that, sample was centrifuged for 2.5 minutes at 700 g. 1 ml of supernatant was than aded to 1 ml of freshly prepared Griess reagens (mixture of equal parts of 0.1% solution of naphthylethylenediamine dichlond in purified water and 1% sulfanilamide in 5% H_3PO_4 solution which are stirred and left to cool for 12 hours before use). After stirring for 10 minutes on a vibrator at room temperature, light absorption (optical density) was measured at 546 nm using a spectrophotometer. The concentration of NO (in mmol/L) was determined from a standard curve with known concentrations of $NaNO_2$ (from 1.56-100 mmol/L). Purified water with Griess reagens was used as a blank determination. The mean value of three consecutive measurement performed on the same sample was taken as the definitive level of NO.

ET-1 serum level measurement

Serum was separated from the whole blood using water bath at 37 °C, after which it frozen at -80 °C until analysis. Determination of serum ET-1 levels was performed with EIA methodology based on an immunometric assay, the so-called. "Sandwich technique" using the Endothelin-1 ELISA kit - IBL Hamburg, Germany. A 96-well tile is required to perform this technique. Within each recess (pond) are binding antibodies fixed to the pond wall. These antibodies have a specific affinity for ET-1 molecules. After the addition of the sample to each individual pond, a binding reaction of all ET-1 molecules present in the test sample occurs with binding antibodies fixed to the pond wall. After the sample had been added to the ponds, a solution of acetylcholinesterase: Fab conjugate (antibodies) was added. The role of Fab conjugates is to bind ET-1 molecules, but for the second epitope (on the opposite side) relative to the "binding" antibodies. This creates complexes made of binding antibodies, ET-1 and acetylcholinesterase: Fab conjugates or sandwiches. As sandwiches are now firmly attached to the base of each pond, the rest of the contents are washed out with buffer fluid. After washing, Ellmans reagens was added. The role of

this reagens is to change the color (to yellow) in reaction with the Fab conjugate. The concentration of ET-1 is determined messuring change in color intensity. This measurement was performed electronically using an ELISA reader (Elx 800 Universal Microplate Reader Biotek Instruments, INC) at a wavelength of 405 nm. An automatic ELISA washer from the same manufacturer was used to rinse the plate. The standard curve was obtained from known ET-1 activities within the kit. Blinds were tested using 2 ponds to which no Fab conjugate was added, while the rest of the procedure was the same. The values obtained are expressed in pg/ml. Since 8 standard probes were used to obtain the standard curve, with decreasing concentrations in the S1 - S8 standards, the S1se chamber contained a concentration of about 250 pg / ml, in the next 125 pg / ml, and further 62.5 pg / ml, 26.3 pg / ml and in each of the following two times less than in the previous one, while the S8 sample contained only diluted human plasma from the kit itself, with the manufacturer's guarantee that the concentration of ET-1 was less than 1.5 pg / ml. Thus, for the adsorbents obtained below the adsorbance values for standard S8, we can interpret the serum ET-1 levels of subjects below 1.5 pg/ml corresponding to the expected (normal) values in potentially healthy subjects. For these adsorption findings, a result of 1.5 pg/ml was added in the table. The mean value of three consecutive measurement performed on the same sample was taken as the definitive level of ET-1.

Statistical analysis

The obtained results were stored in table (MS Excel 2013), and the SPSS software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) was used for all statistical analysis. Data were processed by standard statistical methods, both from the domain of descriptive statistics (basic descriptive measures: mean, mode, median, standard deviation) and from the area of statistical inference (Student's t test for small independent samples, Student's t test for small dependent samples, χ^2 test, ANOVA test). Values of $p < 0.05$ were considered statistically significant.

Results

During the study period, total of 64 patients (36 men and 28 women) treated at the Center for Hyperbaric Oxygen Therapy and

Treatment for Chronic Wounds were included using previously defined criteria. Other descriptives are shown in Table 1.

Mean concentrations of NO and ET-1 (± SD) in selected groups of patients before and after HBOT and statistical analysis results are shown in Table 2.

Analyzing baseline NO concentrations (before HBOT), we found significantly higher NO serum concentrations in the age group of 20-29 years compared to other age groups, but we did not comment on these results because the sample was too small. Besides this, lower baseline concentrations NO were found in diabetic compared to non-diabetic patients and non-smokers but difference was not statistically significant.

Analyzing baseline ET-1 concentrations, we found lower serum concentrations in males compared to females as in non-smokers versus smokers, in those receiving insulin versus in non-insulin recipients - but without statistical significance. We found significantly higher serum levels of ET-1 in diabetic patients compared to non/diabetic patients.

Discussion and Conclusion

In our study, we showed the prevalence of vasoconstrictor substances (ET-1) in prolonged exposure to hyperbaric hyperoxia during PAD treatment, while the level of vasodilator substances (NO)

Table 1. Descriptive parameters of studied patients

	N (%)
Sex ♂	36(56.3)
Age 20-39	6(9.4)
Age 40-59	22(34.4)
Age 60-69	25(39)
Age >70	11(17.2)
Stage II PAD	30(46.9)
Stage III PAD	21 (32.8)
Stage IV PAD	13(28.3)
Smokers	15(23)
Diabetes	52(81)
Acetilsalicil acid	17(27)
Insulin	35(55)
Body mass index >24.9	44(68.7)

Table 2. Mean concetrations of NO and ET-1 (± SD) before and after HBOT

	NO$_{before}$ (mean ± Sd) (mmol/l)	NO$_{after}$ (mean ± Sd) (mmol/l)	p*	ET-1$_{before}$ (mean ± Sd) (pg/ml)	ET-1$_{after}$ (mean ± Sd) (pg/ml)	P*
All patients	21.6 ± 9.2	23.5 ± 10.6	0.2	4.2 ± 11.6	18.3 ± 21.0	<0.001
Sex ♂	22.3 ± 8.9	24.5 ± 9.0	0.2	1.5 ± 0.0	14.0 ± 3.1	<0.001
Sex ♀	21.1 ± 9.5	22.7 ± 11.7	0.5	6.5 ± 2.6	21.9 ± 27.9	<0.001
Stage II (Fontain)	21.9 ± 9.6	26.2 ± 12.1	0.04	6.3 ±15.9	20.8 ± 29.5	<0.001
Stage III (Fontain)	20.0 ± 6.9	21.6 ± 9.4	0.5	1.5 ± 0.0	14.0 ± 3.4	<0.001
Stage IV (Fontain)	23.5 ± 11.5	20.4 ± 7.0	0.4	3.8 ± 8.2	19.6 ± 11.9	<0.001
Age ≤ 65	21.0 ± 9.0	23.9 ± 10.7	0.085	4.5 ± 13.6	19.2 ± 26.0	<0.001
Age > 65	22.6 ± 9.6	22.9 ± 10.6	0.904	3.7 ± 7.7	17.0 ± 8.9	<0.001
Smoker/Yes	19.6 ± 7.1	21.5 ± 7.7	0.424	8.9 ± 21.7	29.2 ± 41.0	0.002
Smoker/No	22.2 ± 9.7	24.1 ± 11.3	0.293	2.8 ± 5.4	15.1 ± 6.4	<0.001
Insulin/Yes	29.9 ± 8.6	23.5 ± 9.6	0.195	2.6 ± 4.8	14.7 ± 4.7	<0.001
Insulin/No	22.5 ± 10.0	23.6 ± 11.8	0.637	6.2 ± 16.3	22.6 ± 30.5	<0.001
Acetilsalicilacid/Yes	20.4 ± 6.8	23.90 ± 11.65	0.207	3.0 ± 6.1	16.6 ± 8.6	<0.001
Acetilsalicilacid/No	22.1 ± 10.0	23.4 ± 10.3	0.456	4.7 ± 13.0	18.9 ± 24.0	<0.001
Diabetes/Yes	20.9 ± 8.0	23.1 ± 9.9	0.17	2.8 ± 5.6	15.5 ± 7.5	<0.001
Diabetes/No	24.8 ± 13.2	25.5 ± 13.4	0.863	10.3 ± 23.8	30.4 ± 45.5	0.009
BMI ≥ 24.9	20.6 ± 7.3	23.1 ± 10.2	0.105	4.2 ± 12.8	18.9 ± 24.3	<0.001
BMI < 24.9	23.92 ± 12.37	24.7 ± 11.5	0.814	4.2 ± 8.4	17.1 ± 11.1	<0.001

Student's t-test

had variable trends depending on disease stage, condition, demographic parameters and the presence/absence of observed risk factors.

By analyzing NO serum levels before and after treatment of PAD with HBOT, we noted an increase in serum NO concentrations without statistical significance in most groups (except for stage II disease where increase was statistically significant), while a marked decrease in serum NO concentrations after treatment was only detected in subjects with stage IV of PAD and in those on insulin, but with no statistically significant difference.

The absence of a statistically significant increase in serum NO concentrations can be interpreted by prolonged exposure to hyperbaric conditions, oxidative stress, and enhanced generation of superoxide anions responsible for neutralizing NO molecules [28-31]. This claim is supported by the observed decrease in serum NO concentrations after HBOT in patients with the highest disease stage (Fontain stage IV), where local inflammatory status is exacerbated by oxidative stress responsible for neutralizing NO molecules [30,32]. Besides this, literature data indicate a strong association of C reactive protein as an indicator of inflammation (and PAD is basically an inflammatory process) with stimulation of endothelial procoagulant tissue factors, leukocyte adhesion molecules and chemotactic substances, as well as inhibitors of endothelial NO synthetase, reduction in NO production [33]. Findings of other authors show that NO is one of the key molecules in realizing the beneficial effects of oxygen administration under conditions of hyperbaric hyperoxia, which depends on its concentration, which is further dependent on the time of exposure and activation of antioxidant mechanisms [34]. There are studies reporting the modulation of NOS activity under hyperbaric conditions in in vivo and in vitro conditions. An increase in NO synthesis was observed after exposure to hyperbaric conditions, which was inhibited by the inhibitor of NO synthetase - L NMME (L nitro arginine methyl ester) [35].

HBOT is known to induce systemic vasoconstriction (in the brain as well) and this is due to the inactivation of NO by superoxide anions [36]. Some earlier studies showed an oxidative stress augmentation after prolonged exposure to hyperbaric hyperoxia, which supports the claims made above [37]. Since exposure time which leads to optimal results hasn't been specified yet, it is very difficult to quantify the antioxidant mechanisms, [38] and it requires further research in this field.

Looking at the insulin induced fall in serum NO concentrations after HBOT, the explanation for this could be found in the fact that insulin resistance leads to the release of free fatty acids, which further leads to the activation of protein kinase C, inhibition of phosphatidyl inositol 3 kinase (endothelial NO synthetase agonist) and to increased production of superoxide radicals that impair NO homeostasis [39].

Serum ET - 1 concentrations were significantly higher after treatment of PAD with HBOT in all observed patients' groups. This finding is expected, and in the interpretation of these results, we may refer to other studies that have demonstrated that prolonged exposure to hyperbaric conditions leads to the predominance of vasoconstrictor mechanisms involving ET-1 [40,41]. These effects are predominantly exerted by ET-1 via the ET-A receptor, whereas the initial short-term vasodilation effect is explained by the autocrine feedback of ET-1 through ET-B receptors on endothelial cell membranes, which promotes the transient formation of prostacyclin and NO that induce vasodilation [42,43].

We found lower baseline serum ET -1 levels in men compared to women, in diabetic versus non-diabetic patients, and in those on insulin therapy compared to those who do not take insulin - but without a statistically significant difference. On the other hand, we found significantly higher serum concentrations of ET -1 in smokers than non-smokers. To date, data on gender dimorphism in baseline ET-1 levels were not present, while smoking is known to have a negative effect on the ability of blood vessel endothelium to respond by adequate secretion of vasoactive substances [44]. Considering some earlier findings, we expected significantly higher ET-1 levels in diabetic patients [45], which was not the case in our study. The effect of hyperglycemia on blocking the function of endothelial NO synthetase and enhancing the production of free radicals that interfere with vasodilatory homeostasis is also well known [46].

The limitations of this study were firstly the inability to accurately determine the serum concentration of ET-1 lower than 1.5 pg/ml, as well as the design of the study, which predicted that blood would be sampled only before the start of treatment and 10 days after the treatment, a fundamental obstacle in quantifying the effects of prolonged exposure to hyperbaric conditions.

In conclusion, the key finding of this study is that treatment of peripheral arterial disease with hyperbaric oxygen therapy leads to an overcoming of vasoconstrictor effects probably caused by elevation of serum ET-1 concentrations, while other factors such as exposure time to hyperbaric conditions and inflammation rates which are in accordance to stage of disease, activation of antioxidant molecules as well as the effects of other substances that interfere with the effects of NO must be considered in interpreting the effects of NO molecules.

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