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## Early and Late Effects of Hyperbaric Oxygen Treatment on Oxidative Stress Parameters in Diabetic Patients

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### Summary

Exposure to hyperbaric oxygen leads to increased amount of reactive oxygen species (ROS) that are derived from various sources. After the discovery that ROS can function as signaling molecules, the idea of ROS being hazardous to biological tissues has been challenged. The aim of this study was to examine the changes in oxidative stress parameters in diabetics undergoing hyperbaric oxygen therapy (HBOT) due to foot ulcers. Twenty patients, who received HBOT for diabetic foot ulcers, were included in the study. Blood samples were taken before HBOT and 30 min after exit from the chamber, on the day of the first and the 15th HBOT sessions. They were used for the determinations of malondialdehyde (MDA), 8-isoprostane and advanced oxidation protein products (AOPPs). 8-Isoprostane and AOPP levels were not altered significantly after the first HBOT session, while both were increased on the fifteenth day ( $p < 0.05$ ). MDA was significantly increased only after the first HBOT session, and remained unchanged on the fifteenth day (within-day variations). Plasma AOPP levels were lowered significantly after fifteen consecutive HBOT sessions (between-day variations). Decreased AOPP levels suggest that increased oxygenation of tissues due to HBO therapy may activate some endogenous factors that prevent hazardous effects of the disease itself.

### Key words

Isoprostanes • Hyperbaric oxygen • Malondialdehyde • Advanced oxidation protein products • Diabetic foot

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### Introduction

Hyperbaric oxygen (HBO) therapy has been successfully used for the treatment of a variety of clinical conditions related to hypoxia, including acute carbon monoxide intoxication, air embolism, soft tissue infections, radiation necrosis and impaired wound healing (Wattel 1998, Feldmeier 2003). Exposure to HBO leads to an increase in the amount of dissolved oxygen and reactive oxygen species (ROS) in the blood. An excessive generation of highly reactive oxidants results in tissue damage, called as oxidative stress. ROS are derived from a variety of sources, such as the xanthine oxidase system, activated neutrophils, the electron transport chain of mitochondria, and the arachidonic acid pathway. Since free radicals have very short half-lives, the clinical assessment of oxidative stress *in vivo* is based on the measurement of different stable oxidized products of modified lipids, proteins, carbohydrates and nucleic acids. Malonyldialdehyde (MDA), one of the most-widely used biomarkers of oxidative stress, is produced enzymatically by the breakdown of unstable hydroperoxides during peroxidation of unsaturated fatty acyl moieties. 8-Isoprostane (8-isoPGF<sub>2α</sub>), an isomer of F<sub>2</sub>-isoprostanes, is produced nonenzymatically from arachidonic acid, and used as a stable index of free-radical attack on membrane phospholipids (Roberts and Morrow 2000). Proteins are also susceptible to oxidant-mediated injury, forming cross-linkage and aggregation products that are resistant to proteolysis. Markers of protein oxidation were characterized by Witko-Sarsat *et*

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*al.* (1996), and named as advanced oxidation protein products (AOPPs), since they share several homologies with advanced glycosylation end-products (AGEs), which also indicate oxidant-mediated damage.

The oxidative effects of HBO have been investigated in animals and humans (Jamieson 1991, Oter *et al.* 2005, Dennog *et al.* 1999, Eken *et al.* 2005). Dennog *et al.* (1999) have determined the antioxidant response to a single HBO exposure in healthy humans, finding no significant differences in antioxidant levels and antioxidant enzymes involved in the primary defense against oxidative damage before and 24 h after the HBO session. However, the conditions of HBO therapy are not applicable to above-mentioned study, because the patient is repeatedly exposed to hyperbaric oxygen depending on the underlying disease. Recently, Eken *et al.* (2005) studied the effects of HBO therapy on oxidative stress in fifteen patients with various pathologies and reported that HBO did not cause any significant changes on erythrocyte antioxidant capacity and lipid peroxidation.

There is increasing evidence about the ability of HBO to induce cellular protection in a similar manner with other protective oxidative stress mechanisms, as reviewed recently (Yogarathnam *et al.* 2006). Repeated HBO exposure significantly attenuated the inflammatory mediators, free radicals, and mortality in endotoxic rats (Lin *et al.* 2005). Preconditioning by HBO treatment protected liver against subsequent ischemia-reperfusion injury in rats (Yu *et al.* 2005), and induced tolerance against spinal cord ischemia in rabbits (Nie *et al.* 2006). These protective effects of HBO may be related to the fact that reactive oxygen species can trigger a wide variety of cellular mechanisms by functioning as signal molecules (Herrlich and Bohmer 2000, Rosette and Karin 1996, Dalton *et al.* 1999)

Diabetic patients have significant defects of antioxidant defense elements, and the generation of reactive oxygen species is one of the major determinants of diabetic complications (Opara 2002, Martin-Gallan *et al.* 2003, Abou-Seif and Youssef 2004, Gil-del Valle *et al.* 2005, Davi *et al.* 2005). Diabetic patients with unhealing foot ulcers usually undergo HBO treatment. However, to our knowledge, there is no study with regard to the effects of HBO therapy on oxidative stress parameters in diabetics who are already characterized by increased production of reactive oxygen species.

The aim of this study was to examine the early and prolonged effects of hyperbaric oxygen on the levels of MDA, 8-isoPGF<sub>2α</sub> (8-isoprostane) and AOPPs in non-

insulin dependent diabetic patients who were exposed to hyperbaric oxygen for the treatment of foot ulcers.

## Methods

### Subjects

Twenty type 2 diabetic (T2DM) patients with foot ulcers who received hyperbaric oxygen therapy were included in the study. Local ethics committee approved the study protocol and all study subjects gave their informed consents. Patients were followed by the same physician responsible for diabetes control, wound care and antibiotic therapy according to the clinical and laboratory findings, and were given a diet depending on their metabolic needs without vitamin supplementation. Six patients were on statins owing to high levels of plasma lipids. Glucose levels were measured on the days when the parameters of oxidative stress were measured. No significant difference occurred in the glucose levels of patients during the experimental period. The characteristics of patients are shown in Table 1.

**Table 1.** Characteristics of the patients – fasting blood glucose and HbA<sub>1c</sub> levels on the day of admission.

Number of patients	20
<i>Age (years) *</i>	55 (41-70)
<i>Men/Women</i>	14/6
<i>Intake of alcohol (yes/no)</i>	6/14
<i>Smokers/non-smokers</i>	13/7
<i>BMI (kg/m<sup>2</sup>) *</i>	25.3 (19.4-29.7)
<i>Duration of diagnosed DM (years)</i>	8.5 (1-25)
<i>Fasting blood glucose (mg/dl) **</i>	223 ± 77
<i>HbA<sub>1c</sub> (%) **</i>	8.8 ± 1.3

\* median (range); \*\* mean ± S.D. HBO treatment

All subjects underwent HBO therapy according to a routine therapy protocol for diabetic foot ulcers with healing problems, determined by the European Committee on Hyperbaric Medicine and also by the Hyperbaric Oxygen Committee of Undersea and Hyperbaric Medicine (Wattel 1998, Feldmeier 2003). HBOT was carried out in a multiplace hyperbaric chamber once a day, and six days in a week. The treatment protocol was inhalation of 3x25 min periods of 100 % oxygen at a pressure of 2.4 ATA, interspersed with 5 min periods of air breathing.

### Sample collection

Venous blood samples (10 ml) were collected from each patient in standard sterile vacuum tubes containing EDTA. Samples were taken before HBO therapy and 30 min after the exit from the chamber, on the day of the first and 15th HBO sessions. Blood samples were immediately centrifuged at 600 g for 15 min. Aliquots of plasma were stored at  $-80^{\circ}\text{C}$  for 15 min. Aliquots of plasma were stored at  $-80^{\circ}\text{C}$  for MDA and AOPP determinations. For the 8-isoprostane measurement, samples were kept at  $-80^{\circ}\text{C}$  in the presence of 0.005 % BHT (butylated hydroxytoluene) to prevent oxidative formation of 8-isoprostanes.

### Analytical procedures

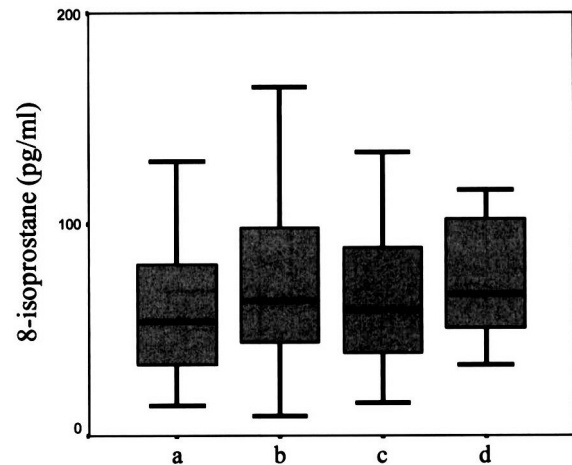
**8-Isoprostane:** The concentration of total (free and phospholipid-bound)  $\text{F}_2$ -isoprostane (8-isoPGF $_{2\alpha}$ ) was analyzed using commercially available enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI). Prior to analysis, plasma samples were hydrolyzed for separating the esterified 8-isoprostane in lipoproteins and purified for elimination of the contaminants. Purification of samples was made using affinity column (Cayman Chemicals, cat No: 416358). Each column was not used more than one regeneration cycle. The assay depends upon the competitive binding between the 8-isoprostane and an 8-isoprostane-acetylcholinesterase (AChE) conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. The concentration of 8-isoprostane was determined by interpolation from a standard curve. The detection limit was 5 pg/ml. Plasma samples were not diluted and all values were within the detection limits (5-500 pg/ml). Microtiter assay plates were scanned with a computer-controlled adjustable wavelength microtiter plate reader (ELx800 Instruments). Results were expressed as pg/ml.

**Malondialdehyde (MDA):** This was determined in plasma by the 2-thiobarbituric acid reactive substances (TBARS) method (Buege and Aust 1978). 1,1,3,3-Tetraethoxypropane (Sigma, UK) was used as a standard. Results were expressed as  $\mu\text{mol/l}$ .

**Advanced oxidation protein products (AOPPs):** AOPPs were determined in plasma using the method devised by Witko-Sarsat *et al* (1996). AOPP concentrations were expressed as  $\mu\text{mol/l}$  of chloramine-T equivalents.

### Statistics

Results were expressed as means  $\pm$  S.D., and analyzed using repeated measures for two-way ANOVA



**Fig. 1.** 8-Isoprostane levels in 20 diabetic patients. Horizontal lines in boxes represent the median values, the boxes express 50 % distribution, the upper lines express 75 %, and the lower 25 % distributions. (a) 1<sup>st</sup> day before HBOT, (b) 1<sup>st</sup> day after HBOT, (c) 15<sup>th</sup> day before HBOT, (d) 15<sup>th</sup> day after HBOT. *c* versus *d*:  $p < 0.05$ .

after the logarithmic transformation of data for AOPP and isoprostanes.  $P < 0.05$  values were regarded as significant. Pearson correlation analysis was performed to see any relation between oxidative markers. All analyses were performed with the SPSS statistical program (10.0 software version, SPSS Inc, Chicago, IL, USA).

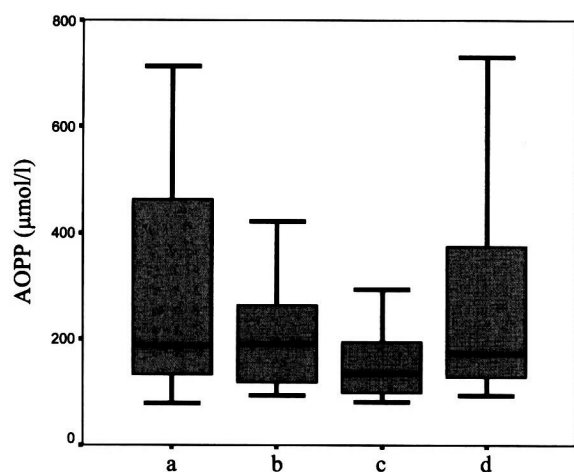
### Results

The levels of 8-isoprostane, AOPPs and MDA in patients before and after HBO exposure on the days of first and fifteenth therapy are shown in Figures 1-3, respectively.

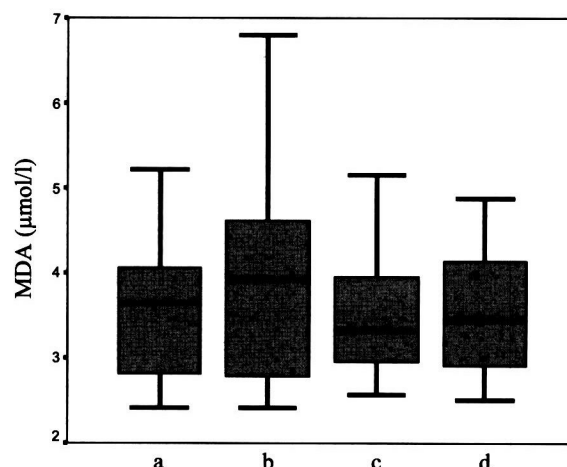
**Within-day variations:** After the first HBO therapy, 8-isoPGF $_{2\alpha}$  and AOPP levels did not differ significantly when compared to the baseline values in diabetic patients. The baseline values were  $67.8 \pm 42.8$  pg/ml for 8-isoPGF $_{2\alpha}$  and  $289 \pm 206$   $\mu\text{mol/l}$  for AOPPs.

On the day of 15th session, 8-isoprostane levels were increased in 69 % and AOPP levels were increased in 75 % of the patients after HBO therapy. On the fifteenth day, mean levels of 8-isoprostane were  $68.9 \pm 38.3$  pg/ml before the HBO treatment, and  $96.4 \pm 78.7$  pg/ml after the HBO therapy ( $p < 0.05$ ; Fig. 1). Similarly, AOPPs exhibited a slight increase after HBO therapy on the fifteenth day ( $176 \pm 122$  vs.  $256 \pm 185$   $\mu\text{mol/l}$ ,  $p < 0.05$ ; Fig. 3).

MDA concentration was significantly increased after the first HBO therapy with an increase in 75 % of the patients ( $3.56 \pm 0.79$  vs.  $3.95 \pm 1.22$   $\mu\text{mol/l}$ ,  $p < 0.05$ ); but



**Fig. 2.** Distribution of AOPP levels in patients (n=20) before and after HBO therapy. Characteristics of the diagram are similar to Figures 1 and 3. **(a)** 1<sup>st</sup> day before HBOT, **(b)** 1<sup>st</sup> day after HBOT, **(c)** 15<sup>th</sup> day before HBOT, **(d)** 15<sup>th</sup> day after HBOT. **c versus a and d:**  $p < 0.05$ .



**Fig. 3.** Distribution of MDA levels in 20 patients. Boxes and lines represent the same characteristics as in Figures 1 and 2. **(a)** 1<sup>st</sup> day before HBOT, **(b)** 1<sup>st</sup> day after HBOT, **(c)** 15<sup>th</sup> day before HBOT, **(d)** 15<sup>th</sup> day after HBOT. **b versus a:**  $p < 0.05$ .

it showed no within-day variation on the day of 15th HBO treatment (Fig. 3).

*Inter-day variations:* Following fourteen consecutive HBO therapies, plasma AOPP levels were significantly lower prior to the fifteenth session in comparison to the basal level on the first day ( $176 \pm 122$   $\mu\text{mol/l}$  vs.  $289 \pm 206$   $\mu\text{mol/l}$ ,  $p < 0.05$ , Fig. 2). This decrement was observed in 75 % of the patients, whereas MDA and 8-isoprostane levels did not exhibit any between-day variations.

There were no significant correlations among the three parameters of oxidative stress.

## Discussion

In the pathogenesis of diabetic complications, there is an increasing evidence for the role of oxidative stress, which is manifested by enhanced lipid peroxidation, increased  $F_2$  isoprostanes and superoxides, and increased protein and DNA damage (Davi *et al* 2005). HBO therapy is an exogenous factor that may cause an additional oxidative stress in the patients undergoing the treatment of diabetic foot ulcers. In the present study, oxidative stress was detected through the measurements of three markers – isoprostanes, malondialdehyde, and AOPPs – in diabetic patients before and after hyperbaric oxygen therapy. Isoprostane levels are known to vary depending on the availability of arachidonic acid, oxygen concentration, and generation of the ROS (Basu and Helmersson 2005). Apart from being

a sensitive marker of oxidative stress, 8-isoprostanes are mediators of important biological effects. By contrast with MDA, 8-isoprostane acts through receptors able to elicit distinct signal transmission pathways (Comporti *et al* 2005). The ongoing increase of isoprostane concentration after fifteen sessions of HBO therapy suggest that isoprostane formation is independent of the adaptive response to oxidative stress. Since isoprostanes may affect the tissues as mediators of signal transmission, their association with some other clinical parameters needs to be investigated in detail.

It was previously shown that an initial HBO treatment of human subjects leads to the induction of adaptive response that protects cells against the induction of DNA damage by a second HBO treatment (Rothfuss *et al* 1998). It is also evident that ROS generated by HBO triggers the upregulation of antioxidant enzyme activities, thereby induces tolerance against ischemia in the tissues (Nie *et al*. 2006). In the present study, MDA was increased following the initial treatment of HBO, while it remained unchanged on the day of the fifteenth HBO session. This finding is in accordance with the above mentioned reports as well as with our previous experiment that revealed significant increases in plasma MDA levels in rats following the first HBO treatment, but no change on the day of the 15th session (Korpinar and Cimsit, unpublished observations). Our current findings in diabetic patients indicate the upregulation of antioxidant enzymes by the initial HBOT. Although we did not measure the activities of antioxidant defense enzymes in this study, there is ample evidence to confirm

this hypothesis (Kim *et al.* 2001, Harabin *et al.* 1990, Gregorevic *et al.* 2001).

AOPPs are elevated significantly in diabetes, and accumulation of AOPPs and AGEs in tissues play an important role in the diabetic long-term complications, including endothelial damage (Gillery 2001, De Cristofaro *et al.* 2003). Plasma AOPP levels have been reported to correlate with concentrations of dityrosine and advanced glycation end-products as indices of oxidative protein damage, but not with thiobarbituric reactive substances as lipid peroxidation markers (Witko-Sarsat *et al.* 1996, Gil-del Valle *et al.* 2005). In agreement with above mentioned studies, we did not find any correlation between MDA and AOPP levels in diabetics. AOPP levels are tightly correlated with AGEs in both types of diabetes (Kalousova *et al.* 2002). In our diabetic subjects, decreased AOPP level after successive HBO treatments may be related to the ROS-induced cellular signaling and protection. Furthermore, it is known that AOPP activates polymorphonuclear neutrophils and monocytes (Witko-Sarsat *et al.* 2003), and activation of these cells aggravates the inflammatory reactions. Oxygen under pressure behaves as a drug causing both beneficial and toxic effects (Hink and Jansen 2001). HBO improves the oxygen delivery to the tissues, accelerates the rate of healing, and also has anti-infectious properties against various microorganisms (Mathieu *et al.* 2006). It has been proved by randomized, controlled clinical trials that HBOT is effective in diabetic wound healing (Abidia *et al.* 2003, Kalani *et al.* 2002). In our study, all the wounds were found in a better condition on the day of 15th session compared to the initial state. A decreased

formation of AOPPs, which is indicative of decreased protein damage, may be one of the consequences/contributors of the healing process. Furthermore, some exogenous factors that alter antioxidant status of the human body (e.g. smoking, use of statins) may significantly interact with the effects of HBOT on wound healing. However, our studied population was not large enough to determine the differences between subgroups.

In conclusion, this study supports the view that HBO triggers and upregulates the defense mechanisms against oxidative stress. Increased oxygenation of tissues due to HBOT may also activate other endogenous factors that prevent hazardous effects of the disease itself. In addition, it is now evident that drugs such as statins and ACE inhibitors have a strong intracellular antioxidant activity, and may therefore exert beneficial effects on diabetic vascular complications (Tsiara *et al.* 2003, Da Ros *et al.* 2004). The contribution of these drugs to the healing effect of HBO needs to be elucidated by further studies.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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