

RESPONSE OF RAT ERYTHROCYTE OXIDATIVE STRESS MARKERS TO REPETITIVE HYPERBARIC OXYGEN EXPOSURES UP TO 40 DAILY SESSIONS

ODGOVOR MARKERA OKSIDATIVNOG STRESA U ERITROCITIMA PACOVA NA VIŠESTRUKO IZLAGANJE HIPERBARIČNOM KISEONIKU DO 40 SEANSI DNEVNO

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Summary

Background: Studies with single-session hyperbaric oxygen exposures have shown that HBO-induced oxidative stress is proportional to exposure pressure and duration. Since the efficacy of hyperbaric oxygen mainly depends on repetitive exposures, this study aimed to investigate the oxidative effect of hyperbaric oxygen administered for 5 to 40 sessions.

Methods: Sixty rats were divided into one control and 6 study groups. Study groups were exposed to 5, 10, 15, 20, 30, and 40 daily consecutive 2.8 atm/90 min hyperbaric oxygen sessions. Animals were sacrificed 24 h after the last hyperbaric oxygen administration. Malondialdehyde and carbonylated protein levels as well as superoxide dismutase activities were determined in isolated rat erythrocytes.

Results: Carbonylated protein levels increased significantly after just 5 hyperbaric oxygen exposures; reached a peak level with 10 exposures; were still significantly higher than controls after 15 sessions; and decreased to normal limits after 20 exposures. Malondialdehyde levels were found to be significantly increased in the 10 to 30, but not in the 5 and 40-session groups. Superoxide dismutase activity showed elevated levels only in the 5 and 10 times hyperbarical oxygen-exposed groups.

Conclusions: The suppressed oxidative stress level after 40 exposures suggests an effective endogenous antioxidant defense in repetitive HBO administrations.

Keywords: adaptive response, antioxidant defense, free radicals, oxidation products, reactive oxygen species

Kratka sadržaj

Uvod: Studije o jednostrukom izlaganju hiperbaričnom kiseoniku pokazale su da je oksidativni stres izazvan izlaganjem hiperbaričnom kiseoniku srazmeran pritisku i trajanju izlaganja. Pošto efikasnost izlaganja hiperbaričnom kiseoniku uglavnom potiče od višestrukog izlaganja, cilj ove studije bio je da se istraži oksidativni efekat izlaganja hiperbaričnom kiseoniku u trajanju od 5 do 40 seansi.

Metode: Šezdeset pacova podeljeno je na jednu kontrolnu i 6 eksperimentalnih grupa. Eksperimentalne grupe podvrgnute su uzastopnim seansama udisanja hiperbaričnog kiseonika (2,8 atm/90 min) po 5, 10, 15, 20, 30 i 40 puta dnevno. Životinje su žrtvovane 24 časa posle poslednje seanse. U izolovanim eritrocitima pacova određeni su nivoi malondialdehida i karboniliranog proteina kao i aktivnosti superoksid-dizmutaze.

Rezultati: Nivoi karboniliranog proteina značajno su porasli posle samo 5 izlaganja hiperbaričnom kiseoniku; dostigli vrhunac nakon 10 izlaganja; ostali značajno povišeni u odnosu na kontrolu posle 15 seansi; i vratili se u normalne okvire posle 20 izlaganja. Nivoi malondialdehida bili su značajno povišeni u grupama sa 10 do 30 izlaganja, ali ne i sa 5 i 40 seansi. Aktivnost superoksid-dizmutaze pokazala je povišene nivoie samo u grupama sa 5 i 10 izlaganja hiperbaričnom kiseoniku.

Zaključak: Sniženi nivo oksidativnog stresa nakon 40 izlaganja ukazuje na efikasnu endogenu antioksidantnu odbranu kod višestruke primene hiperbaričnog kiseonika.

Ključne reči: adaptivni odgovor, antioksidantna odbrana, slobodni radikali, proizvodi oksidacije, reaktivne kiseonične vrste

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List of abbreviations: HBO, hyperbaric oxygen; MDA, malondialdehyde; NBT, nitroblue tetrazolium; PCC, protein carbonyl content; SOD, superoxide dismutase

Introduction

It is widely known that (hyperbaric) hyperoxia can lead to an excessive production of reactive molecules that can trigger oxidation/peroxidation cascades of several functional and structural biomolecules (1). On the other hand, reactive molecules are now known not only to lead to cellular injury but also to act as signaling agents. In this way they play a role in a number of physiological functions in living organisms (2). Hyperbaric oxygen (HBO) treatment, a therapeutic modality depending on the inhalation of 100% oxygen under a pressure exceeding that of the atmospheric pressure, has been used for decades as a life saving application in critical cases including carbon monoxide poisoning, air/gas embolism and decompression illness as well as acute traumatic wounds, crush injuries, burns, gas gangrene and compartment syndrome (3). However, 'oxygen toxicity' was an important scientific issue in the former medical literature and it was often pointed out as a potential risk of HBO administrations (4).

From another point of view, more recent reports suggest that the enhanced level of reactive molecules, e.g., the superoxide radical and hydrogen peroxide, may play an important role for the beneficial actions of HBO therapy (5). Nowadays, it is not certain, apart from the well-explained oxygen supplying/enriching and bubble reducing effects, whether both oxygen and nitrogen derived reactive species may take part in the therapeutic mechanisms of HBO (6–8). Through the last decade, our laboratory has been concentrated on defining the oxidative action of HBO within therapeutically applied limits. In a series of experimental studies conducted in rats, we found that HBO-induced oxidative stress is directly proportional to the exposure pressure (9, 10) and duration (11, 12). Another experimental set revealed an important finding that the enhanced levels of oxidation products declined to their baseline values at one hour later following a single HBO exposure (13, 14). Finally, in our most recent studies, different from the abovementioned one-session HBO exposure procedures, after exposure to daily HBO sessions for up to 8 weeks, clear rises in lipid and protein oxidation products along with the antioxidant enzyme superoxide dismutase (SOD) were detected in the rats' lung (15), but not in their brain tissue (16).

The present work was established as a complementary issue to previous studies in order to elucidate the HBO-induced oxidative interactions in longer administration periods one step forward. Erythrocytes, the oxygen-carrying cells and one of the main targets of hyperoxia-related oxidative action (9, 11, 14), were chosen for this purpose.

Material and Methods

Study design

The Experimentation Ethics Committee of our institution approved the experimental procedures of the study (issue 08/75K). Sixty adult male Sprague-Dawley rats bred at the Gulhane Military Medical Academy Research and Progress Center were used for the study. Rats were 12 weeks old and weighed 200–250 g at the beginning of the experiment. Housing was at 22–24 °C with light from 08.00 a.m. to 08.00 p.m. including free access to water. All animals were fed with a standard commercial rat chow during the experiment.

Rats were divided into 6 study groups (n=8 for each) which were exposed to HBO for 1, 2, 3, 4, 6 and 8 weeks. HBO administrations were set as 5 daily consecutive exposures followed by 2-day intervals. All animals in the study groups were sacrificed 24 h after their final HBO treatment. Separate control groups consisting of 6–8 animals for each time point were forbidden by our institutional Ethics Committee. In order to evaluate the possible effects of aging, 2 control animals were sacrificed at the same time with the 6 study groups; thus, the control group of the study consisted of 12 (6 × 2) animals. A detailed schedule of the study design is given in *Table 1*.

Table 1 Detailed experimental schedule.

	Day 1 to 5	Day 6 (Sacrificing)	Day 7
Week 1	5 HBO sessions (48 animals)	8 study + 2 control rats	Nothing applied
Week 2	5 HBO sessions (40 animals)	8 study + 2 control rats	
Week 3	5 HBO sessions (32 animals)	8 study + 2 control rats	
Week 4	5 HBO sessions (24 animals)	8 study + 2 control rats	
Week 5	5 HBO sessions (16 animals)	No sacrificing	
Week 6	5 HBO sessions (16 animals)	8 study + 2 control rats	
Week 7	5 HBO sessions (8 animals)	No sacrificing	
Week 8	5 HBO sessions (8 animals)	8 study + 2 control rats	

Note that all HBO administrations and animal sacrifice were performed at 10.00 a.m.

HBO Exposure

An animal hyperbaric chamber (made in Etimesgut Military Equipment Factory; Ankara, Turkey) was used for HBO exposure. The HBO sessions were set as 2.8 atm pressure for 90 min in all study groups. Compression and decompression of the chamber were completed gradually in 5–10 min; continuous 100% O₂ ventilation at a rate of 3–4 L/min was maintained throughout the 90-min exposure periods in the chamber. All administrations were started at the same hour in the morning (10.00 a.m.) to avoid the possible effects of circadian rhythm (17).

Tissue preparation

Animals were kept for one day after the last HBO session to exclude interference of the acute actions of HBO exposure. They were then anesthetized via an intraperitoneal injection of ketamine (85 mg/kg) plus xylazine (12.5 mg/kg). Their chests were opened and 4–6 mL blood specimens were obtained from the inferior vena cava. The rats were sacrificed by bleeding and hypovolemic shock under anesthesia.

Blood samples were separated into plasma and erythrocytes by centrifugation (for 10 min) at +4 °C (Hermle Z323K; Gosheim, Germany). Erythrocyte samples were washed three times with cold physiological saline and then hemolyzed by adding a 4-fold volume of distilled water. The final hemolysates were divided into three parts, put into eppendorf tubes and stored at –80 °C until assay.

Biochemical analysis

In the erythrocyte hemolysates, lipid peroxidation levels were measured using the thiobarbituric acid reaction by the method of Ohkawa et al. (18). This method was used to obtain a spectrophotometric (Helios epsilon, USA) measurement of the color produced during the reaction to thiobarbituric acid with malondialdehyde (MDA) at 535 nm. The calculated intra-assay coefficient of variation (CV%) and inter-assay CV% for MDA were 4.4% and 5.5%, respectively.

The carbonylated protein content (PCC) was determined with the method described by Levine et al. (19). MDA and PCC levels were expressed as millimoles per gram protein. The intra- and inter-assay CV% for PCC measurements were calculated as 4.8% and 6.1%, respectively.

The activity of the antioxidant enzyme SOD was assayed using the nitroblue tetrazolium (NBT) method of Sun et al. (20). Briefly, NBT was reduced to blue formazan by the superoxide anion radical, which has strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that

inhibits the rate of NBT reduction by 50%. The estimated SOD activities were expressed as units per gram protein. Intra- and inter-assay CV% values for SOD activities were estimated to be 3.6% and 4.1%, respectively.

Finally, in order to normalize the measured data, the protein content of the hemolysates was measured according to the method of Lowry et al. (21) with bovine serum albumin as the standard.

Statistical analyses

Normality analyses were performed by using the Shapiro-Wilk test and the entire data set of the study was found to be normally distributed. Thus, parametric statistics were used for the evaluation of the results. Since the One Way Analysis of Variance (ANOVA) indicated intergroup significance, *post hoc* Bonferroni test was performed for group to group comparisons. P values less than 0.05 were considered as significant. All analyses were performed using the SPSS software (Version 15.0; SPSS, Chicago, IL, USA).

Results

According to general observations such as symptoms for barotraumas, hyperoxic convulsions, weight gain or loss, no unexpected effect due to HBO exposure was observed throughout the study. All animals survived until the scheduled time of sacrifice. The outcome is presented in the box-plot graphics (Figures 1–3) enabling one to see the median, minimum, maximum and quartile values at a glance.

Lipid peroxidation

Erythrocyte MDA levels tended to increase with the first measure point after 5 HBO sessions. However, statistical significance was noted initially after 10 HBO exposures and continued up to the end of the 6th week (30 HBO administrations). Twenty-four hours after the 40th HBO session, MDA levels were found to have declined to nearly control values. The values after 20 and 30 HBO administrations were also recorded as being significantly higher than the 5-session group's MDA levels. Detailed P values are given with the graphical demonstration of the erythrocyte MDA levels in Figure 1.

Protein oxidation

The erythrocytes' PCC values were found to be significantly higher than the controls just with the 5- and 10-session HBO exposure groups. After 15 HBO administrations a decline in erythrocyte PCC values was recorded but the degree was still significantly

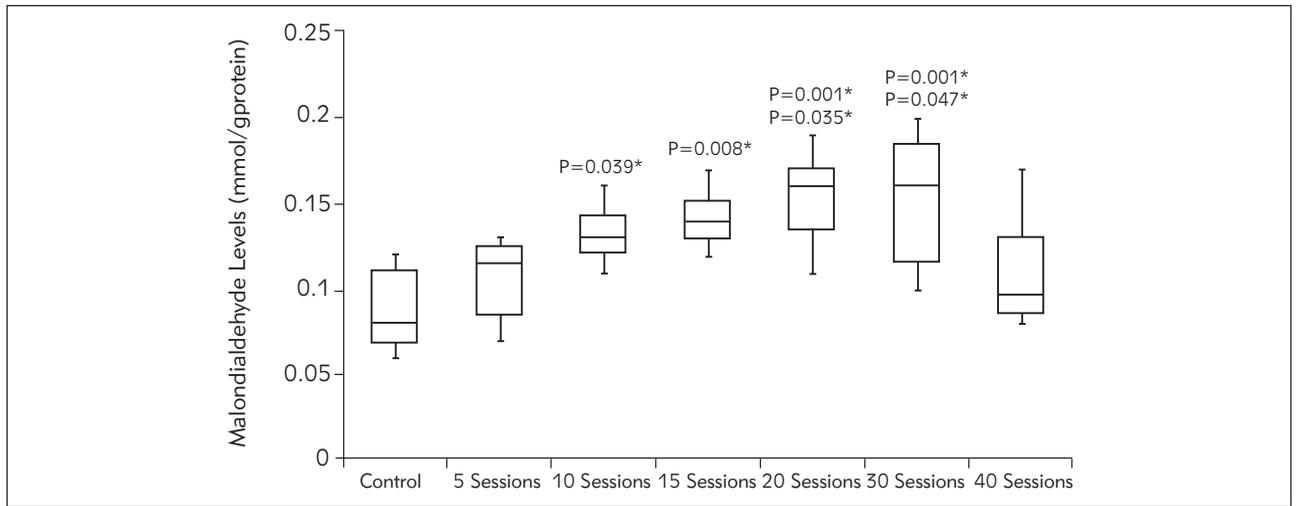


Figure 1 Erythrocyte MDA levels were found to be significantly higher compared with *control, and **5-session HBO groups.

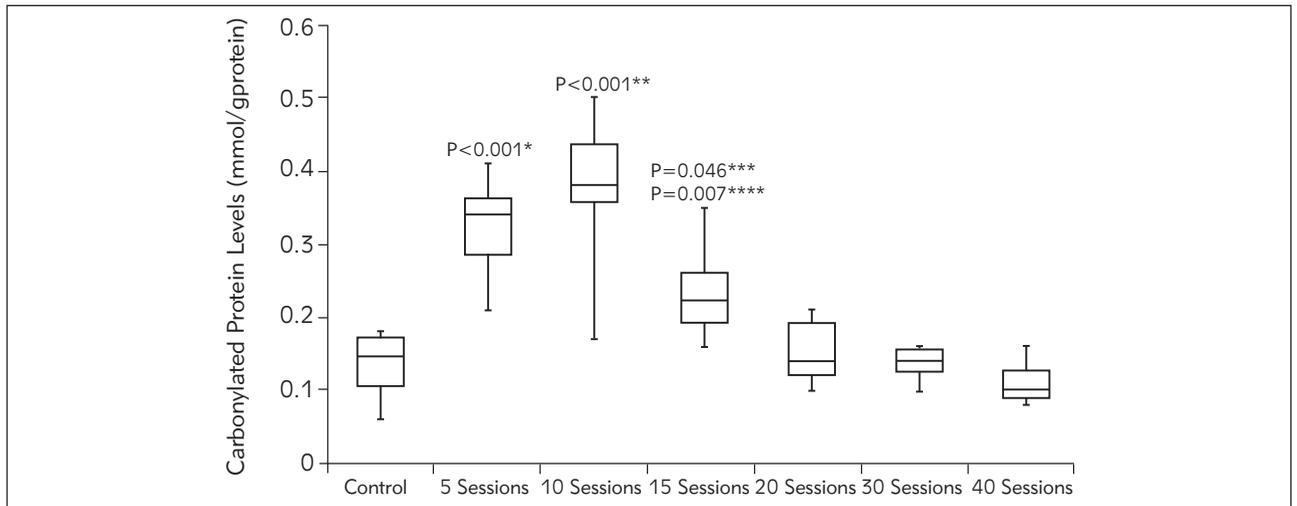


Figure 2 Carbonylated protein amounts of erythrocytes were significantly higher than *all groups except the 10- and 15-session HBO groups, **all groups apart from the 5-session HBO group, ***the control group, and ****the 40-session HBO group.

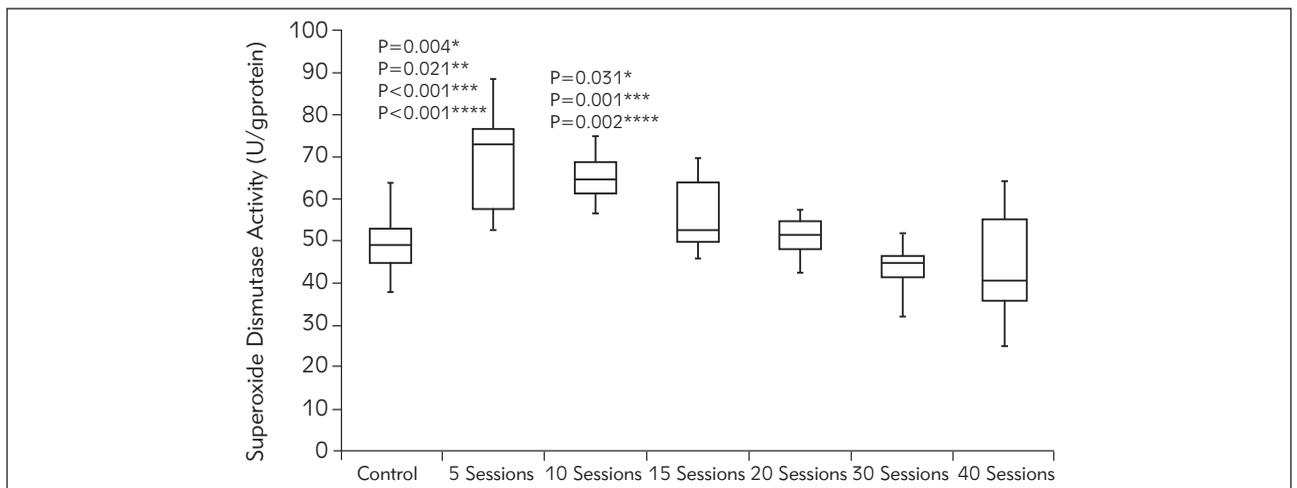


Figure 3 The SOD enzyme activities were increased significantly in the 5- and 10-session HBO groups as indicated by *vs. control values, **vs. the 20-session HBO group, ***vs. the 30-session HBO group, and ****vs. the 40-session HBO group.

higher than the control group as well as than the 40-session HBO group. With 20 and more HBO sessions carbonylated protein levels were recorded within similar ranges to controls. More detailed group to group comparisons and exact P values for PCC levels are shown in *Figure 2*.

Superoxide dismutase enzyme activity

The antioxidant enzyme SOD immediately responded via significant elevation of enzymatic activity to HBO exposure in the groups sacrificed earlier, i.e. the 5- and 10-session HBO groups. With longer HBO exposure periods, SOD activities tended to decline; thus, the recorded SOD activities of the 5- and 10-session HBO groups were also significantly higher than in the most of the other groups (*Figure 3*).

Discussion

In this study we investigated the potential oxidative effect of repetitive HBO treatments for up to 40 sessions on rat erythrocytes. The levels of lipid peroxidation and protein oxidation were recorded in order to reflect the oxidative status, and meanwhile the activity of the antioxidant enzyme SOD was detected. Key findings of the study were: (i) a rise in both of the measured oxidation products, i.e. MDA and PCC, in the earlier stages with 5 to 15 HBO exposures, which were simultaneously accompanied by elevated SOD activities; (ii) gradual increment of MDA but not PCC and SOD activity levels up to 30 HBO sessions; and finally (iii) normalized values for all 3 measured parameters with no difference from control levels after 40 HBO administrations.

Former studies clearly demonstrated that single HBO exposures cause oxidative reactions in cultured cell lines (22), experimental animals (9) and healthy human volunteers (23). Interestingly, this oxidative effect was shown to discontinue with following HBO exposures (24) along with an HBO-triggered adaptive protective mechanism (25). On the other hand, in a more recent study, the isolated lymphocytes obtained from combat divers, who were repetitively exposed to 100% oxygen breathing under pressure due to their jobs, represented an enhanced sensitivity to oxidative damage (26). Thus, more research is needed in this field in order to elucidate the oxidative interactions with clinically relevant multiple-HBO exposures.

Studies focused on oxidative actions of repetitive HBO exposure were generally performed with patients undergoing HBO therapy for different reasons (27). It is difficult to distinguish whether the oxidative stress levels measured in these studies depend on HBO or the pathology for which the patient was treated. Other studies performed on healthy volunteers were mostly ceased after the first

or an additional second HBO exposure (23). However, the use of HBO in clinical conditions depends mainly on at least 10 treatments and may exceed 30 sessions in cases of refractory pathologies (28). Indeed, exposing healthy human beings unnecessarily to HBO for more than 10 times will be ethically disputable and animal studies remain important.

In our previous work, an increasing oxidative effect of repetitive HBO exposure was demonstrated in the rat's lung tissue (15). Briefly, with 20 daily exposures to HBO, significantly increased MDA and PCC levels were detected in the lung tissues and remained as high values after 30 and 40 exposures. The good news was that increased activities of antioxidant enzymes accompanied the rise of these oxidation products. On the other hand, the brain tissue specimens of rats did not reflect any significant change for oxidant and antioxidant indices in the same experimental set (16). With regard to hyperoxia-induced oxidative injury, the lung tissue is the first target, since it is the entering site of oxygen; then, oxygen passes into the blood and the oxygen-carrier cells, erythrocytes, represent an ideal secondary target for detecting hyperoxic oxidative interactions (9). Therefore, in the present work, we focused on the erythrocytes in order to investigate possible similarities and/or differences with the abovementioned lung and brain studies (15, 16). As a result, both MDA and PCC levels were detected to increase significantly at earlier time-points than previously seen in the lung (15). The activity of SOD also increased with a similar course to the oxidation products.

Among the many biological targets of oxidative stress, lipids are the most involved class of biomolecules (29–31). Lipid oxidation gives rise to a number of secondary products and MDA is the principal and most studied product of polyunsaturated fatty acid peroxidation (32). In our current study, erythrocyte MDA levels significantly increased at the time-point of 10 HBO administrations and remained high for up to 30 sessions (*Figure 1*) reflecting the presence of oxidative stress within this time interval. Then, after 40 HBO exposures, a decline to insignificant values was detected indicating sufficient endogenous repair action of the organism.

Protein carbonylation is a type of protein oxidation and a well-established marker for oxidative stress (33). In the present study, erythrocytes' PCC levels increased significantly at the earliest stage with 5 HBO exposures and this continued after 10 and 15 sessions (*Figure 2*). This finding is interesting since, in the lung tissue, the first significant increase of PCC levels was recorded with 20 HBO administrations and continued up to 40 exposures (15). Again, similar to the final result for MDA values, the near-to-control PCC levels of the 20-, 30- and 40-times HBO

exposed groups provide evidence for a successful defense or regulatory mechanism.

The hyperoxic state during HBO treatments causes primarily an increased production of the superoxide radical (26) and, as a response, upregulation of the antioxidant enzyme SOD was reported for several times (34). Almost all of our previous one-session HBO exposure studies also resulted in increased SOD activities in rat erythrocytes (9, 11, 14). Similarly, we recorded significant elevation of SOD activities in the present work, but only in the early 5 and 10 times HBO administered groups. Then, SOD activities tended to decline and resulted in totally normalized values at the end-stage of the study (Figure 3).

Taken together, at the last measure point after 40 HBO administrations, all three measured parameters, i.e. MDA, PCC and SOD, were found to be within their normal ranges. Due to the short half-life of the enzyme SOD (35), but the relatively longer half-life of the oxidation end products such as MDA (36), the earlier decline of SOD in the present experimental set is an expected result. Hence, although MDA and PCC levels remained significantly higher for a longer period than SOD, their final normalized values supported the previous suggestions on adaptive protection against oxidative stress with repetitive HBO treatments (25). The sole measurement of SOD as a marker for the antioxidant systems has to be emphasized as a limitation of the present study. Normally, we planned to investigate the members of the glutathione system, but during the biochemical analyses unforeseen problems occurred and hindered further widening of our parameter spectrum.

In conclusion, when compared with the previous lung study (15), the main similarity is the relatively

synchronous elevation of the oxidant and antioxidant system markers. The main difference, however, is the timing for these increased values. The harmony between the measured oxidation products and the antioxidant enzymes can be interpreted as evidence for a controlled level of oxidative stress recruiting reactive molecules into signaling pathways instead of harming biomolecules. The earlier increases of oxidation products in erythrocytes, or, from another standpoint, their postponed increases in the lung tissue may stand for different sensitivity ranges of diverse body cells. Antioxidant enzyme activities were not found to be depressed and no sign of exhaustion of the antioxidant system appeared. Thus, the overall results prove the safety of long-term HBO treatments. More detailed studies, including detections of the previously suggested molecules heme oxygenase-1 (25) and hypoxia inducible factor- α (37), warrant more precise knowledge in order to elucidate the underlying mechanisms of HBO-related oxidant/antioxidant interactions.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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