Pressure-related effects of hyperbaric oxygen exposure on oxidation products and antioxidant enzymes in the rat lung

Kemal Simsek¹, Mehmet Ozler², Ergun Ucar³, Serdar Sadir², Seref Demirbas⁴, Bulent Uysal², Hakan Ay¹

Departments of ¹Undersea and Hyperbaric Medicine, ²Physiology, ³Pulmonary Medicine, ⁴Internal Medicine; Gulhane Military Medical Academy, Ankara, Turkey.

Summary
Objective: The aim of this study is to provide comprehensive information on the relationship between clinically used hyperbaric oxygen (HBO) protocols and its oxidative effects. In order to enlighten this issue, we investigated the effects of various HBO pressure modalities on oxidant and antioxidant parameters in the rat lung.

Methods: Sixty male Sprague-Dawley rats were divided into 6 groups. Group 1 was used as control. Groups 2 to 6 were subjected to 100% oxygen exposure at a pressure of 1, 1.5, 2, 2.5, and 3 ATA (atmosphere absolute) respectively for 2 hours. The lungs were taken immediately after exposure. Oxidation products of lipids (thiobarbituric acid reactive substances, TBARS) and proteins (carbonyl formation, PCO), and antioxidant enzyme levels (superoxide dismutase, SOD; catalase, CAT, glutathione peroxidase, GSH-Px) were determined.

Results: TBARS, PCO, SOD and CAT levels increased concordantly with pressure. Significant change of TBARS levels started from 100% oxygen exposure at 1 ATA (normobaric), but PCO and CAT levels were affected first after 1.5, and SOD activity after 2 ATA. A significant correlation exists between exposure pressure and oxidative parameters. GSH-Px activity was not affected significantly.

Conclusion: The oxidative effect of HBO in rat lung presents a positive correlation with increasing exposure pressure.

Key words: Antioxidant enzymes; Hyperbaric oxygen; Oxidative stress; Rat lung

Correspondence: K. Simsek Sualti ve Hiperbarik Tip AD, Gulhane Askeri Tip Akademi, 06010 Etilik, Ankara, Turkey. drkemsim@gmail.com

Received: December 27, 2010 Accepted: January 4, 2011 Published online: January 13, 2011

Introduction
Elemental oxygen is required to maintain cellular respiration but has a toxic potential due to the production of free radicals [1, 2]. Oxygen-induced tissue damage is reported for most organs [3, 4] and this has to be considered when patients are exposed to an elevated oxygen concentration and pressure. Exposure to oxygen is highest for lung tissue because partial oxygen pressure (PaO₂) is higher in the alveoli than in the arteries [5]. Hyperbaric oxygen (HBO) therapy, i.e. breathing of 100% oxygen while inside a pressurized treatment chamber, is used in the management of different diseases and conditions. The efficacy of the treatment depends on either increased dissolved oxygen in the blood (e.g. ischemia-reperfusion damage, soft tissue infections, radiation necrosis, impaired wound healing) or to the high exposure pressure (e.g. decompression sickness, air embolism). However, oxygen under pressure behaves as a drug causing both beneficial and toxic effects. Edema reduction, impairment of leucocyte adhesion, enhancement of antibacterial mechanisms and stimulation of fibroblast proliferation and neovascularization constitute the majority of the beneficial pharmacological effects of HBO therapy but the basic mechanisms are not well established [6]. The cellular formation of reactive oxygen species (ROS), which increases during exposure to hyperoxia, is considered to play a key role in the toxicity of oxygen [7].

Many investigators have been interested in the oxidative stress and/or oxygen toxicity causing effects of HBO and many of such studies concentrated on the lung as an important target organ. It was reported that HBO exposure to animals in excess of 1.5 ATA (atmosphere absolute) induces marked injury to the lung [7-10]. Lungs from animals exposed to normobaric hyperoxia have increased superoxide ion (•O₂⁻), hydrogen peroxide (H₂O₂), and lipid peroxides [10]. The increased production of oxygen radicals in lung cells has been postulated to be a major factor in the etiology of lung damage during hyperoxia [1].

In fact, hyperbaric medicine is considered safe under appropriate supervision and utility. Toxic effects of oxygen are observed at extremely high doses over prolonged periods. HBO treatment increases the relative dose of oxygen; thus
susceptible patients need to be recognized and modifications made to prevent the manifestations of oxygen toxicity. Damaging or toxic effects of oxygen therapy are probably related to the unbridled formation and release of ROS, such as •O₂ and H₂O₂. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) keep the formation of these radicals in check until the oxygen load overwhells the enzymes, leading to the detrimental effects structural molecules of the cell [11].

Most studies investigating HBO’s toxic effects have been established with relatively high values of HBO exposure for a long time and/or excessive pressure, which are not used in clinical treatments. The approved maximum pressure and duration of HBO used clinically are 3 ATA and 120 minutes, and the commonly used therapeutic pressure range from 1.8 to 2.8 ATA for 60-90 min [6]. The purpose of the present study was to clarify the oxidative effect of HBO sessions from normobaric pressure to 3 ATA, the therapeutically used maximum pressure, with a standard duration of 2 hours on rat lung, the primary affected organ. Thiobarbituric acid reactive substances (TBARS) and protein carbonyl content (PCO), indicators of lipid peroxidation and protein oxidation, were measured in lung tissues to determine the degree of oxidative stress, and SOD, CAT and GSH-Px activity were measured to reflect the antioxidant status as antioxidant enzymes fighting against ROS, namely •O₂ and H₂O₂.

**Materials and methods**

**Animals and groups**

A total of 60 adult, male Sprague-Dawley rats bred in our laboratory were used for the experiment. The rats were 12 weeks old and weighed 200-250 g. Housing was at 22-24°C with light from 08.00 AM to 08.00 PM and free access to water. All animals were fed a commercial diet during the experiment. They were randomly distributed into six equal groups (n=10 for each group):

- Group 1; control group (without any exposure)
- Group 2; normobaric oxygen group (exposed to 1 ATA 100% O₂)
- Group 3; exposed to 1.5 ATA 100% O₂
- Group 4; exposed to 2 ATA 100% O₂
- Group 5; exposed to 2.5 ATA 100% O₂
- Group 6; exposed to 3 ATA 100% O₂

**HBO exposure**

The exposure duration was set at 2 hours for all experiments. An animal hyperbaric chamber (made in Etimesgut Military Equipment Factory, Ankara, Turkey) was used for O₂ and air exposure. The ventilation rate was 3-4 l/min. All administrations were initiated at the same hour (8 AM) to avoid biological rhythm differences.

**Chemicals**

All chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) and all organic solvents from Merck KGaA (Darmstadt, Germany). All reagents were of analytical grade and were prepared each day (except the phosphate buffer) and stored in the refrigerator at +4°C. The reagents were equilibrated at room temperature for 0.5 h before use when the analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at +4°C for 1 month.

**Tissue preparation**

Immediately after the exposure period, the animals were anesthetized with ketamine (85 mg/kg) and xylazine (12.5 mg/kg) and their chest was opened by median sternotomy. The lung parenchyma was flushed with 10 ml of ice-cold physiological saline (PS) via a right ventricular puncture to the heart to remove blood from tissues and to slow tissue metabolism [12]. Then the lungs were removed immediately, washed with PS to remove residual blood, put into tubes, frozen with liquid nitrogen and stored at -70°C. The frozen lung tissues were homogenized in phosphate buffer (pH 7.4) by means of a homogenizator (Heidolph Diax 900; Heidolph Elektro GmbH, Kelhaim, Germany). The supernatant was divided into 2-3 parts, put in separate tubes, and stored at -70°C again.

**Biochemical analysis**

First of all, the protein content of lung homogenates was measured by the method of Lowry [13] with bovine serum albumin as the standard. The carbonyl contents of lung tissue was determined spectrophotometrically by the method of Levine [14], based on the reaction of the carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone. The results were given as nmol carbonyl per mg-protein. Lipid peroxidation levels were measured with the thiobarbituric acid (TBA) reaction by the method of Ohkawa [15]. Estimated TBARS levels were expressed as nmol/g-protein. SOD activity was assayed using the nitroblue tetrazolium (NBT).
method of Sun [16]. NBT reduction to blue formazan is monitored by absorbance at 560 nm. One unit (U) of SOD is defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%. The calculated SOD activity was expressed as U/mg-protein. CAT activity was assayed according to the modified method of Aebi [17]. In this assay the rate of disappearance of H₂O₂ was measured spectrophotometrically at 240 nm. One U of CAT activity was defined as the amount of enzyme that decomposed 1 mmol H₂O₂ per minute under the assay conditions. CAT activity was expressed as U/g-protein. GSH-Px activity was measured using the method described by Paglia & Valentine [18] in which GSH-Px activity was coupled to the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was spectrophotometrically followed at 340 nm. GSH-Px activity was presented as U/g-protein. A Shimadzu spectrophotometer (UV-1601; Kyoto, Japan) was used for all these assays.

Statistical analysis
All results were expressed as the mean and the standard error of the mean (SEM). Numeric data were analyzed first using the Kruskal-Wallis test to determine differences between the groups and the Mann-Whitney U test was employed to analyze two groups consecutively. Furthermore, Pearson Correlation analysis was performed to elucidate whether there was a relation between the degree of exposure pressure and the oxidative parameters. P values of less then 0.05 were regarded as significant. All analyses were performed with the SPSS software (version 11.0, SPSS Inc. Chicago, IL, USA).

Results
All numeric data is summarized in Table 1. To provide a clearer overview, oxidative parameters, PCO and TBARS, are demonstrated in Fig.1 and antioxidant enzymes are presented in Fig.2. Generally, TBARS, PCO, SOD and CAT levels increased in a pressure-dependent manner. Correlation analysis indicated a statistically significant relation between incremental exposure pressures and the increase of these three parameters (p<0.05 for all). GSH-Px activity was not affected significantly.

Oxidation products
TBARS levels of the rat lung tissue increased significantly (p<0.05) starting from normobaric O₂ exposed group (group 2). Significance became more important as exposure pressure increased (p<0.01; group 6 vs. control group). Beginning at exposure of 1.5 ATA 100% O₂, the PCO content presented significantly increased levels (p<0.05; groups 3, 4, 5, and 6 vs. control).

Antioxidant enzymes
SOD activity was also found to be increased in all treatment groups. The increase became significant starting at 2 ATA of 100% O₂ exposure (p<0.05; groups 4, 5, and 6 vs. control). Starting at 1.5 ATA HBO exposure, CAT activity was also found to be significantly increased when compared with control animals (p<0.05; groups 3, 4, 5, and 6 vs. group 1). Although a slight increase of GSH-Px activity seems to appear in HBO exposed groups, none of these values was found to be significant when compared with each other group (p>0.05).

| Table 1. Summary of measured data (Mean±SEM) in the rat lung tissue. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Groups | PCO (nmol/mg-prot) | TBARS (nmol/g-prot) | SOD (U/mg-prot) | CAT (U/g-prot) | GSH-Px (U/g prot) |
| 1 Control | 0.045 ± 0.008 | 0.58 ± 0.05 | 8.01 ± 0.85 | 30.21 ± 1.31 | 15.17 ± 0.63 |
| 2 1 ATA O₂ | 0.061 ± 0.010 | 1.11 ± 0.04* | 10.06 ± 0.82 | 34.85 ± 3.72 | 16.28 ± 0.46 |
| 3 1.5 ATA O₂ | 0.094 ± 0.015* | 1.25 ± 0.05* | 10.72 ± 0.91 | 42.54 ± 2.83* | 16.81 ± 0.70 |
| 4 2 ATA O₂ | 0.110 ± 0.017* | 1.38 ± 0.04* | 13.07 ± 0.86* | 44.94 ± 1.62* | 17.48 ± 0.64 |
| 5 2.5 ATA O₂ | 0.129 ± 0.022* | 1.44 ± 0.07* | 14.76 ± 0.67* | 49.20 ± 2.11* | 17.21 ± 0.87 |
| 6 3 ATA O₂ | 0.158 ± 0.027** | 1.57 ± 0.10** | 15.56 ± 0.62* | 54.38 ± 3.22* | 17.18 ± 0.52 |

PCO, TBARS, SOD and CAT levels (*p<0.05, and **p<0.01 vs. the control group), but not GSH-Px activity (p>0.05), increased in treatment groups.
Şimşek et al: Pressure-related oxidative effect of HBO

Discussion

The lung is the primary affected organ of oxygen toxicity. In 1899, Lorraine Smith reported the pathologic effects of increased oxygen tension on the respiratory system for the first time [19]. The Lorraine Smith effect became known as a clinical syndrome of decreased vital capacity, sternal chest pain, and patchy atelectasis. Dry cough, substernal chest pain bronchitis, shortness of breath, and pulmonary edema and fibrosis were arranged as the major signs and symptoms of pulmonary oxygen toxicity [3]. Due to this fact, clinicians should be careful when patients receive oxygen therapy in either normobaric or hyperbaric conditions.

Some studies reported reduction in pulmonary function after chronic HBO treatment, but the authors notified these findings not considerable to be of clinical significance [5]. Thus, the most important reason for the restriction of HBO usage seems to be the potential risk of oxygen toxicity from the 100% oxygen breathed during the session. Although HBO has been used clinically since 1960s, there are no reports of serious oxygen toxicity as a result of HBO treatment; only few clinical works exists regarding the oxygen toxicity of HBO treatment [20]. Experimental studies on the subject have observed direct toxic effects of HBO with relatively higher atmospheric pressures such as 4-5 ATA and longer than 2 hour durations [8-10]. It is impossible to adapt these results to approved therapeutic limits (maximum 3 ATA pressure and 2 hours duration). In this work, we planned to study the effect of HBO exposure with clinically used pressures and duration on oxidative parameters. In previous studies, a collaborating team of our institution reported that 2 hours of HBO exposure at 3 ATA elevated lung TBARS, SOD and CAT [20], and brain TBARS, SOD and GSH-Px levels [22]. Then, in a study to test the hypothesis whether there may be relationship between HBO administration pressures and its oxidative effects, it was shown that SOD and TBARS levels are rising up with HBO exposure in a pressure-dependent manner in rat brain, lung and erythrocytes [23]. The present study was performed to provide more comprehensive information on the relationship between incremental HBO exposure pressure at a fixed duration (2 hours) and its oxidative effects by measuring additional parameters of the oxidant/antioxidant system, i.e. CAT, GSH-Px and PCO. Since ambient air exposure in high pressures seems to be ineffective in the earlier studies, we didn’t arrange such a group in the present one. A wide range of parameters was determined to reflect

Figure 1. Pressure-dependent increase of TBARS and PCO in the rat lung. Correlation analysis indicates significance for both (p<0.01 for both; Pearson Correlation value = 0.549 for TBARS, 0.667 for PCO).

Figure 2. Effect of HBO exposure with incremental pressure on SOD, CAT and GSH-Px activity in the rat lung. Correlation analysis indicates significance for SOD and CAT (p<0.001 for both; Pearson Correlation value = 0.791 for SOD, 0.849 for CAT), but not for GSH-Px activity (p>0.05).
the oxidative status; PCO as specific measurement of tissue injury caused by oxygen radicals, TBARS as the commonly measured product of lipid peroxidation, and antioxidant enzymes SOD, CAT and GSH-Px. Consequently, all measured parameters except GSH-Px appeared directly proportional to HBO administration pressure and statistical correlation supported these findings. TBARS and SOD findings were similar to the previously prepared work [23]; taken together, each of these two studies are supporting the other.

Antioxidant enzymes SOD, GSH-Px, and CAT are critical to the cellular defenses against ROS. SOD reduces $\bullet O_2$ to $H_2O_2$ that is in turn reduced by GSH-Px and CAT to water. SOD has 3 isoforms, namely EC-SOD (extracellular), Cu,Zn-SOD, and Mn-SOD [24]. Since Cu,Zn-SOD resides in both the cytoplasm and mitochondria and accounts for the majority of cellular lung SOD activity [25], we choose it from among the other isoforms to determine. In contrast to the present, some work reported decrease in antioxidant enzyme activity nearby increased lipid peroxidation levels [10, 11]. This may due to exhaustion of antioxidant defense systems since such studies were performed under toxic HBO exposure conditions. Our present work indicates that HBO exposure under clinically approved conditions cause antioxidant enzyme induction. But with increasing exposure pressure the oxidative effect of HBO, indicated by TBARS and PCO levels, may lead to an imbalance in the ratio of oxidants to antioxidants and the endogen antioxidant system may be insufficient to fight against ROS injury. This suggestion is especially supported by the meaningful increase of TBARS levels starting just from normobaric 100% $O_2$ exposure, while statistical differences of SOD levels started to appear with 2, and CAT levels with 1.5 ATA.

There is no doubt that glutathione (GSH) is one of the most important cellular antioxidants, also for the lung. This importance was emphasized by several studies focused on HBO toxicity and GSH metabolism [9], but most of them were performed with excessive HBO exposure. Although slightly increased, the statistically unaffected GSH-Px levels of our study were an interesting finding. Thus, we claimed that in clinically approved HBO administration conditions CAT may be the first activated enzyme in order to scavenge $H_2O_2$, and GSH-Px would take part in antioxidant defense mechanisms if severe oxygen toxicity shows up. Nevertheless, this suggestion must be supported by further studies. In addition, studies focused on how mRNA expression of antioxidant enzymes are affected by HBO administration will more elucidate the molecular actions of HBO.

In conclusion, HBO causes oxidative stress which is mainly due to pure oxygen exposure. The frequently preferred HBO pressure range is 2-2.5 ATA [6] and oxidative stress in this range is relatively low as compared with that seen at 3 ATA. Successful effects of well-established HBO-protocols have been proved in a considerable number of clinical [26, 27] and experimental [28-30] studies in literature. Nevertheless, due to the pressure-dependent oxidative effect, clinicians may be advised to adjust exposure pressures high enough for sufficiency but as low as possible to avoid from undesired influence. Since antioxidants are already shown to be effective against HBO-induced oxidative reactions [8, 21, 22], antioxidants may be added to standard HBO therapy protocols especially higher exposure pressures are used.

References
19. Smith LJ. The pathological effects due to increase of oxygen tension in the air breathed. J Physiol 1899; 24:19-35.