Research Article

Spirometry and oxidative stress after rebreather diving in warm water

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ABSTRACT

Introduction: Hyperbaric oxygen (HBO_2) therapy and use of enriched air can result in oxidative injury affecting the brain, lungs and eyes. HBO_2 exposure during diving can lead to a decrease in respiratory parameters. However, the possible effects of acute exposure to oxygen-enriched diving on subsequent spirometric performance and oxidative state in humans have not been recently described recently. We aim to investigate possible effects of acute (i) hyperbaric and (ii) hyperbaric hyperoxic exposure using scuba or closed-circuit rebreather (CCR) on subsequent spirometry and to assess the role of oxidative state after hyperoxic diving.

Methods: Spirometry and urine samples were obtained from six well-trained divers (males, mean ± SD, age: 43.33 ± 9.16 years; weight: 79.00 ± 4.90 kg; height: 1.77 ± 0.07 meters) before (CTRL) and after a dive breathing air, and after a dive using CCR (PO_2 1.4). In the crossover design (two dives separated by six hours) each subject performed a 20-minute session of light underwater exercise at a depth of 15 meters in warm water (31-32°C). We measured urinary 8-isoprostane and 8-OH-2-deoxyguanosine evaluating lipid and DNA oxidative damages.

Results: Different breathing conditions (air vs. CCR) did not significantly affect spirometry. A significant increase of 8-OH-dG (1.85 ± 0.66 vs. 4.35 ± 2.12; $P < 0.05$) and 8-isoprostane (1.35 ± 0.20 vs. 2.59 ± 0.61; $P < 0.05$) levels after CCR dive with respect to the CTRL was observed. Subjects didn't have any ill effects during diving.

Conclusions: Subjects using CCR showed elevated oxidative stress, but this did not correlate with a reduction in pulmonary function.

KEYWORDS: oxidative stress; respiratory mechanics; exercise; rebreathing

INTRODUCTION

Hyperbaric oxygen (HBO_2) is a widely applied therapeutic approach in which an individual breathes near 100% oxygen intermittently while inside a hyperbaric chamber with a pressure equal or exceeding 1.4 atmospheres absolute (ATA). It is used for the treatment of diseases such as: decompression illness, ischemia-reperfusion injury, necrotizing infections, chronic non-healing wounds, gas gangrene and others [1,2]. Hyperbaric oxygen therapy as well as the use of oxygen-enriched air or closed-circuit rebreather (CCR) during diving can potentially result in oxidative injury, which affects the brain, lungs and eyes, mainly due to the toxic effects of oxygen free radicals [3-5]. Central nervous system oxygen toxicity (CNS-OT) and pulmonary oxygen toxicity are the most concerning effects of breathing an enriched air mixture [6-8]. As previously reported [9], exposure to HBO_2 may lead to temporary reductions in pulmonary function. Experiments specifically designed to investigate the possible effects of acute exposure to enriched-air diving on subsequent spirometric performance and oxidative state in humans have not been recently described in the literature. Although the design of this diving project is highly conservative respect to unit pulmonary toxic dose per depth and diving duration, the authors attempt to speculate on the possible link between spirometry and oxidative state adaptations in asymptomatic healthy volunteers after an acute enriched-air exposure in warm water. It has been shown that exposure to high ambient pressure (hyperbarism) may affect the mechanical characteristics and synthesis of elastin and collagen [10].

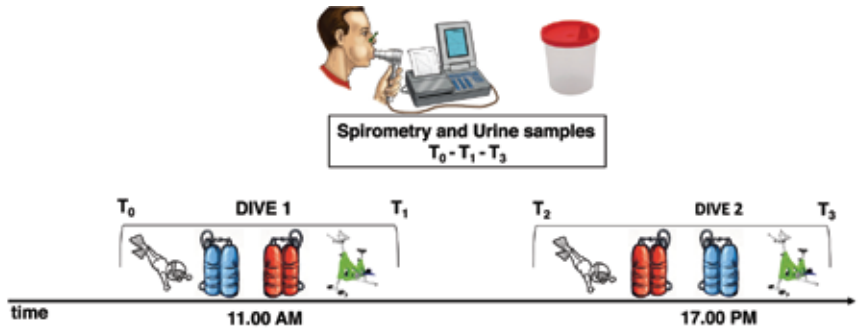

FIGURE 1. Experimental design: T_0: spirometry and urine sampling at beginning of the protocol (control); T_1: spirometry and urine sampling after immersion using air or CCR; T_3: spirometry and urine sampling after dive using air and CCR at final time point of the study

While literature reports some respiratory measurements taken during hyperbaric exposure [11,12], there is a substantial lack of data about possible alterations in respiratory tissue mechanics measured after safe and conservative immersion. A spirometric investigation revealed a long-lasting impairment of the conducting function of the small airways in humans accustomed to perform deep dives [13]. Interestingly, these results were observed in subjects using oxygen but also in subjects using air as breathing gas [13]. In addition, a small but significant reduction in the forced vital capacity (FVC) was observed 24 hours after a single dive to a depth of 50 meters while breathing air [14], suggesting that an increment in airway resistance occurred and persisted long after the dive.

Recent experimental work was performed on animals (rats) to investigate the possible effects of previous hyperbaric exposure on the mechanics of respiratory parameters such as respiratory system resistance and elastance, but the authors found no significant effect [15].

The aim of our preliminary study focuses on the hypothesis that oxidative stress could induce the mechanics of respiratory impairment during hyperoxic diving. The experiment described below was undertaken to investigate possible effects of a single conservative dive using self contained underwater apparatus (scuba) or CCR on post-dive spirometric performance in healthy human subjects. To correlate the possible reductions in pulmonary function with the oxidative state, we explored the effects of both scuba diving (hyperbarism) and CCR diving on the urinary concentration of 8-isoprostane (8-iso-PGF2α), an index of lipid peroxidation, and of 8-OH-2-deoxyguanosine (8-OH-dG), an index of oxidative DNA damage.

METHODS

Experimental design

The experimental protocol received the approval of our local ethical institution (no HEC-DSB 06/16) and adhered to the principles of the Declaration of Helsinki. This study was a controlled, intrasubject experimental design, with repeated measures. Fourteen days before the beginning of the testing sessions, to ensure that all the divers knew the protocol and could complete the amount of work required, a familiarization meeting was organized. Divers were instructed to refrain from heavy physical activity and alcohol consumption in the two days preceding the immersions. Throughout each dive subjects performed a 20-minute-long mild exercise session on an underwater bike (OKEO, Genoa, Italy). The dive depth was set at 15 meters, where subjects performed an activity guided by the Borg CR-10 scale at intensity level 3 (25 rpm) [16]. The ascent rate was set at 10 meters/minute, with a decompression stop at 5 meters for three minutes. All divers answered "no" to questions about (a) consuming medications and (b) diving or flying 48 hours prior to the immersions. Each subject conducted two dives in one day, separated by six hours. In detail, according to the crossover design during the first immersion (11:00 AM), three subjects performed a dive breathing air (T_0-T_1, Figure 1) and three subjects performed a dive using CCR with a partial pressure of inspired oxygen (PiO_2) of 1.4 breath-

TABLE 1. Demographic and anthropometric parameters

subject	gender	age (years)	height (m)	mass (kg)
1	M	33	1.79	85
2	M	45	1.65	70
3	M	34	1.73	80
4	M	46	1.82	80
5	M	58	1.76	79
6	M	44	1.85	80
mean ± SD	M	43.33 ± 9.16	1.77 ± 0.07	79.00 ± 4.90

The mean values ± SD, (n= 6) for mass (kg), height (m) and age (year) are indicated.

ing an enriched-air mixture (maximum fraction of inspired O_2 reached = 56%). While during the second immersion (17:00 PM), the two groups exchanged the mixture breathed (T_2-T_3, Figure 1). Spirometry and urine samplings were taken before the first dive (T_0, Figure 1) in order to establish the baseline condition (CTRL) for each parameter investigated. The experimental setting for the trials was the world's deepest pool "Y-40, the Deep Joy," with a water temperature of 31-32°C in Montegrotto Terme (Padua, Italy).

Subjects

We recruited 10 diving instructors and after obtaining medical clearance, screened them. All were non-smokers. After considering subjects' adherence to the inclusion criteria – to have no history of orthopedic, cardiovascular, renal or metabolic disorders – six male volunteers (Table 1) were selected for the study. All participants gave their informed consent, and every precaution was taken to protect their privacy.

Testing sessions and samples collection

Every testing session included a dive in which a light underwater exercise was performed. Moreover, each diver was brought to a mobile laboratory before (T_0) the first immersion and after each immersion (post; T_1 and T_3) for spirometry and urine sampling collection (Figure 1). In detail, we measured FVC, or forced expiratory volume, in the first second (FEV1) FEV1/FVC%; peak expiratory flow (PEF); forced expiratory flow (FEF25-75%); and maximal expiratory flow (MEF) in the usual intervals (MEF75%, MEF50%, MEF25%). Spirometric parameters were recorded two minutes after the immersion and outside the water by a turbine sensor of a portable spirometer (Pony FX; Cosmed; Rome, Italy), recently validated [17,18], and whose technical characteristics accomplished recent international standards [19]: flowmeter, bidirectional digital turbine Ø 28 mm; flow range, 0.08-20 liters/second; volume range, 12 liters; accuracy of reading, ± 2%; resistance, < 0.6 $cmH_2O/l/s$; temperature sensor, 0-50° C. The equipment received the flow data in real time, at a frequency of 100 Hz, recording and exporting the data to the computer. The system calibration was done by the manufacturer. Obtained spirometric data were compared with those expected on the basis of recent standard corrected for age and height [20].

Urine samples collected were stored at -80°C until analyses were performed. Samples were thawed only for the analyses, which were performed within two weeks from collection.

A competitive immunoassay was used for the determination of 8-isoprostane (8-iso-PGF2α) concentration, a marker of lipid peroxidation, in urine (Cayman Chemical, U.S.). Urine was purified using solid-phase extraction cartridges. The purification and subsequent enzyme-linked immunosorbent assay (EIA) were performed following the manufacturer's recommendations. The EIA employs 8-iso-PGF2α tracer and 8-iso-PGF2α antiserum. The sample 8-iso-PGF2α concentration was determined using a standard curve. Samples and standards were read at a wavelength of 412 nm. 8-OH-dG has been established as a marker of oxidative DNA damage. This compound was quantified in excreted urine using a commercially available EIA kit (Cayman Chemical, U.S.). The EIA employs an anti-mouse IgG-coated plate and a tracer consisting of an 8-OH-dG-enzyme conjugate. The sample 8-OH-dG concentration was determined using an standard curve. Samples and standards were read at a wavelength of 412 nm. Urinary concentrations of 8-iso-PGF2α and 8-OH-dG, as any urinary marker, vary considerably; therefore, the urinary parameters are usually standardized basing on the amount of creatinine excreted in the urine when the collection of the 24- hour urine sample is not possible. Indeed, in the absence of renal disease, the excretion rate of creatinine in an individual is relatively constant. Thus, urinary creatinine levels may be used as an index

TABLE 2. Spirometric indexes

	FVC [l]	FEV1 [l]	FEV1/FVC% [l]	PEF [l/s]	FEF25-75% [l/s]	MEF75% [l/s]	MEF50% [l/s]	MEF25% [l/s][l/s]
T_0 (Ctrl)	4.8±0.7 (102.7 ±11.3%)	3.9±0.6 (101.9±7%)	80.8±6.9 (101.7±8.3%)	9.4±1.1 (103.8±13.5%)	3.9±1 (92.2±18.7%)	8.7±1.4 110.1±18.2%)	4.7±1.0 (95.6±19.6%)	1.7±0.6 (79.4±20.1%)
T_1 (Air)	4.8±0.8 (103.5±12.5%)	3.8±0.5 (100.3±8.6%)	78.5±6.2 (98.9±8.3%)	8.9±1.5 (98.1±19.3%)	3.74±0.84 (87.95±19.58%)	8±1.5 (102±20.8%)	4.7±1.1 (94.7±23.6%)	1.6±0.4 (77.6±17.2%)
T_3 (CCR)	4.8±0.8 (105.1±11.8%)	3.84±0.56 (102.3±7.3%)	79.8±5.7 (100.2±8.5%)	9.1±1.1 (101.5±14.7%)	3.7±0.8 (89.3±21.6%)	7.8±1.3 (97±14.1%)	4.4±0.9 (90.3±19.6%)	1.7±0.4 (82.1±22.6%)
P	ns	ns	ns	ns	ns	ns	ns	ns

The mean values (± SD, n= 6) of spirometric indexes measured before (CTRL, T_0) and after each immersion (T_1 and T_3) diving are indicated.
The percentage of expected values according to international standards, and the statistical significance ($P > 0.05$) of the differences are also reported.

of standardization. A creatinine assay kit (Cayman Chemical, U.S.) was used to measure creatinine levels in urine samples. Creatinine concentration was determined using a creatinine standard curve.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism package (GraphPad Prism 6, Graphpad Software Inc., San Diego, California). Data are presented as mean ± SD. Obtained data did not pass the Shapiro-Wilk normality test; hence we analyzed them with the Friedman non-parametric test for multiple comparisons and Dunn's post-hoc to determine differences between dives and the control condition for both oxidative stress biomarkers and spirometry. A P-value < 0.05 was considered statistically significant.

RESULTS

Spirometry indexes

We did not observe any adverse reaction in our subjects during diving. In particular, no sign of CNS-OT and/or convulsions were detected. Indexes measured before immersions (T_0) after dive breathing air (T_1) and using CCR (T_3) are reported in Table 2. Values are expressed as mean ± standard deviation. Different breathing conditions during diving do not appear to have significant effect on spirometric indexes ($P > 0.05$).

Oxidative damage biomarkers

Figure 2 shows oxidative damage through 8-OH-dG (DNA damage) and 8-isoPGF2α (lipid peroxidation) tested at each urine collection time (T_0, T_1, T_3). Our results (Figure 2a) show an increase of 8-OH-dG levels after both dives, whether breathing air or using CCR; in T_3 the increment is statistically significant ($P < 0.05$) [T_0 (CTRL): 1.85 ± 0.66; T_1 (Air): 3.90 ± 1.55; T_3 (CCR): 4.35 ± 2.12]. Also, lipids peroxidation (Figure 2b) shows an increment of 8-isoprostane levels after each dive. Similar to 8-OH-dG, measured values are significantly higher in T_3 than in the control condition T_0 ($P < 0.05$) [T_0 (CTRL): 1.35 ± 0.20; T_1 (Air): 2.42 ± 0.89; T_3 (CCR): 2.59 ± 0.61].

DISCUSSION

The absolute values of spirometric indexes we obtained are very similar to the predicted ones (Table 2). It was found that neither hyperbarism (air breathing) nor CCR in the tested conditions significantly affected spirometric performance in healthy subjects. In contrast, increments in oxidative damage indexes were detected both for T_1 and T_3, which was a statistically significant result for T_3.

Hyperbarism

It may be estimated that the PiO_2 in T_0 and T_1 was about 400 mmHg. This PiO_2 value induced no significant alteration of after-dive spirometric performance in our subjects, suggesting negligible effects on airway resistance.

Significant effects of hyperbaric environments on airway resistance have been previously reported, but measurements were taken during the exposure [11, 21]; hence, results were attributed to increased inspiratory gas density and incidence of turbulent flow [12,22], effects which were obviously not present during the experimental procedure in our investigation.

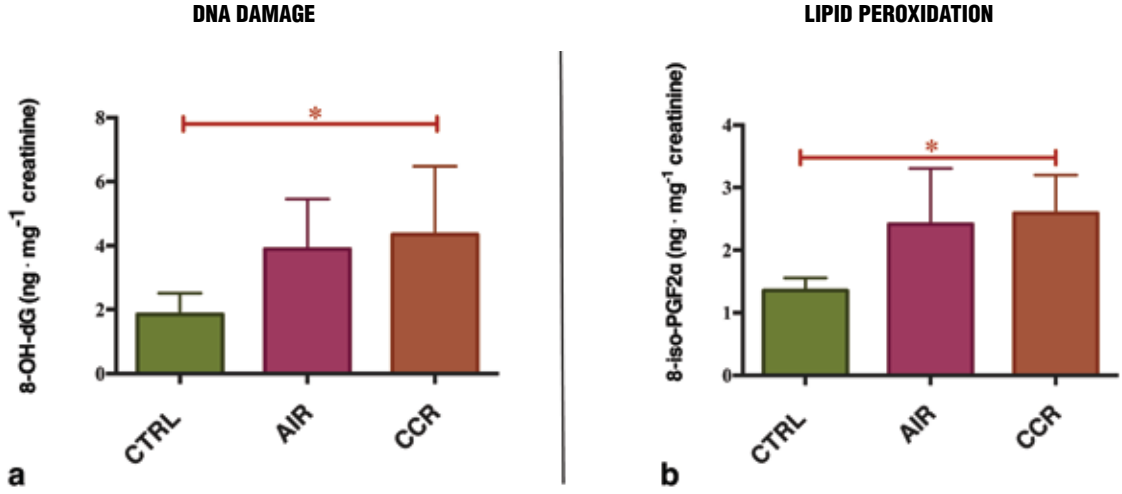

FIGURE 2. Oxidative damage biomarkers results: (a) 8-OH-dG urinary levels at T_0 (CTRL) and after dives at T_1 (air) and T_3 (CCR); **(b)** 8-Isoprostane urinary levels at TT_0 (CTRL) and after dives at T_1 (air) and T_3 (CCR). * significantly different ($P < 0.05$)

Scant data are available in the literature describing possible effects on respiratory mechanics lasting after exposure to hyperbarism. Tetzlaff, et al. [14] described a 3% reduction of forced vital capacity in control subjects breathing compressed air. However, this occurred 24 hours after a 50-meter dive, while the same was not observed three hours later. An effect on the parameters of respiratory mechanics, such as some indexes of expiratory flow limitation, was observed in both air and oxygen divers, but only as a chronic, long-term consequence of diving [13]. We were not able to monitor respiratory mechanics over time after the exposure, so our results pertain to the acute effects only. They are, however, in keeping with results reported by Tetzlaff, et al. three hours after hyperbarism [14].

Interestingly, recent experimental work on rats previously exposed to hyperbarism with a PO_2 similar to that presently tested revealed no significant acute alteration in the parameters of respiratory mechanics [15], which confirms the present results.

It may be concluded that, at least when PO_2 is kept near or below 400 mmHg, the increased production of reactive oxygen and nitrogen species is not sufficient to induce significant lung and/or airway damage, at least in the present experimental conditions and as revealed by spirometric investigation. This conclusion is also supported by the results indicating that the tested urinary indexes of oxidative damage did not show a significant increase.

Hyperbaric hyperoxia

It may be estimated that the PiO_2 in T2 and T3 was about 1063 mmHg (PiO_2 1.4). This PiO_2 value induced no significant alteration of spirometric performance in our subjects, suggesting negligible effects on airway resistance.

In an older paper [23], HBO_2 was described to have insignificant effects on respiratory system elastic properties and alveolar surfactant, while a reduction in the airway resistance to air flow was detected. Similarly, the absence of effects on respiratory system compliance has been more recently confirmed (24).

Nevertheless, more recent findings suggested that HBO_2 may affect respiratory mechanics. For example, prolonged HBO_2 has been described to reduce exhaled nitric oxide concentration [25], possibly inducing bronchoconstriction [26] and a related increment in airway resistance. This increment is also suggested by data showing that prolonged HBO_2 affects spirometric performance of healthy individuals, inducing a decrement in the value of the maximum expiratory flow at 50% of the vital capacity (MEF50) [27] or of MEF50 and MEF25

[13]. Indeed, Adamiec [28] for prolonged and Dewar, et al. [29] for acute HBO_2 exposure were able to demonstrate increased values of airway resistance in humans.

A role for oxygen radicals in causing this effect was proposed later by Katsumata, et al. [30], and more recently confirmed from data taken on experimental animals [31]. Recent work on the rat, performed by applying the end-inflation occlusion method [15,26, 31,32] confirmed that acute HBO_2 induces significant deleterious effects on respiratory mechanics, increasing both airway resistance and respiratory system elastance [32]. These results may appear in contrast with present data, but it has to be noted that the PiO_2 in the present experiments was much lower than that used in the rats (about 1063 mmHg instead of 1900 mmHg), and also lower with respect to that used in humans by Dewar, et al. [29], (about 1500 mmHg).

Oxidative damage in hyperbaric hyperoxia

Hyperoxic exposure induces an increase of free radical generation in biological tissues [33] and disturbs the redox balance. The oxidative stress may affect the ventilatory control by influencing not only central chemoreceptors but also many other elements including non-respiratory systems that modulate the chemoreflexes [34].

Our data confirm that hyperoxia in divers can compromise redox status. Urinary lipid peroxidation (8-isoprostanes) significantly increased likewise in air and CCR (+79% and +92% respectively), probably originating from an increased oxygen concentration amplified by additional variables such as muscular exercise and cold water [35]. Moreover, hyperoxia can induce DNA damage, as reported in experimental animals exposed to oxygen concentrations of > 80%, where the free radical can be produced by the interaction of inhaled oxygen with the mitochondrial electron transport chain or membrane-bound NADPH oxidase [36]. After experimental sessions in air and CCR, the DNA damage concentration increased significantly (+110% and +133% respectively). These data are in agreement with Witte J, et al. [37] who report that the DNA damage increased dose-dependently with the partial pressure of oxygen and exposure time. These results are important, as DNA damage is reportedly implicated in pulmonary disorders [36]. In this regard, it has to be noted that our experiments were performed on male subjects, who have been recently reported to present a reduced risk of hyperoxia-induced convulsions versus female subjects [38]. Also, the imposed workload during diving was not heavy. It may be presumed that our subjects worked below their anaerobic threshold so that increased convulsion risk because of increased $PaCO_2$ was avoided

CONCLUSIONS

In our experimental conditions, significant increments in urinary indexes of oxidative damage were detected after CCR (HBO). Taken together, available results indicate that the toxic effect of HBO_2, due to the production of oxygen radicals, is directly related to the oxygen partial pressure of the inspired gas. From a practical point of view, it may be suggested that CCR may be safely applied with respect to respiratory mechanics alterations when the PiO_2 can be maintained at relatively low values (for example, below 1063 mmHg, 1.4 PiO_2). Indeed, CCR did induce increased oxidative stress, but this was not high enough to significantly affect airway physiology, at least as judged by standard spirometric performance evaluation.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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