



Oxidative stress assessment in breath-hold diving

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Abstract

Purpose Breath-hold diving results in significant changes in blood gases' levels. Challenging variations in oxygen partial pressures may induce reactive oxygen species (ROS) production that exacerbate oxidative stress and, consequently, affect endothelial function. The aim of this study was to investigate the effects of breath-hold diving on oxidative stress damage, assessing ROS production. Nitric oxide metabolites, inducible nitric oxide synthase (iNOS), aminothiols, and renal function were evaluated too as markers of redox status and renal damage.

Methods ROS production was assessed with electron paramagnetic resonance. Oxidative status values were measured at pre- and post-40 m dive in a deep swimming pool (Y-40) from six divers (mean age 46.6 ± 9.3 years; height 176 ± 4 cm; BMI 25 ± 2.9 kg/m²).

Results Significant ($p < 0.05$) increases at post-dive of ROS production rate (0.158 ± 0.003 vs 0.195 ± 0.006 $\mu\text{mol min}^{-1}$), lipid peroxidation (8-isoprostane: 375.67 ± 195.62 vs 420.49 ± 232.31 pg mg⁻¹ creatinine), nitrate (27.91 ± 19.71 vs 30.80 ± 20.44 μM), iNOS (31.30 ± 4.52 vs 35.68 ± 6.72 IU mL⁻¹) and neopterin concentration (96.20 ± 40.41 vs 118.76 ± 27.84 $\mu\text{mol mol}^{-1}$ creatinine) were recorded. Conversely, the antioxidant capacity significantly decreased (3.423 ± 0.089 vs 3.015 ± 0.284 mM) after immersion.

Conclusion Overproduction of ROS and consequent oxidative damage to lipids of membrane and antioxidant capacity decreasing reflect also a hypoxic condition, which in the breath-hold diving typically occurs in the last few meters below the surface. iNOS produces NO in large quantities under the examined extreme conditions. Neopterin and creatinine concentration level increased, suggesting an “impairment of renal function” as a likely physiological response to PaO₂ variations during dive activity.

Keywords Reactive oxygen species · Nitric oxide · Electron paramagnetic resonance · Breath holding

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Abbreviations

ABD-F	4-Fluoro-7-sulfamoylbenzofurazan
EPR	Electron paramagnetic resonance
HPLC	High-performance liquid chromatography
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
ROS	Reactive oxygen species
TAC	Total antioxidant capacity
TCEP	Tris-(2-carboxyethyl)-phosphine hydrochloride

Introduction

During breath-hold diving, the human body is subjected to extreme environmental conditions, thus resulting in significant changes in blood gases' levels (Lindholm and Lundgren 2008; Pendergast et al. 2015; Bosco et al. 2018a). First, the

descent phase is associated with a transient hyperoxia due to the increase in hydrostatic pressure, the compression of the chest wall, and the consequent reduction of intra-pulmonary gas volume (Bosco et al. 2007a, 2018b; Fitz-Clarke 2018). On the contrary, the ascent phase is characterized by several reverse hemodynamic changes, resulting in hypoxia and a build-up of CO₂ (Bosco et al. 2003, 2004, 2007b, 2018a).

As suggested by previous works on hyperbaric hyperoxic exposure in animals and humans (Bosco et al. 2007b, 2018c, d; Morabito et al. 2011), such challenging variations in oxygen partial pressures are known to induce inflammation (Bosco et al. 2010, 2018c) increase of reactive oxygen species (ROS) production that exacerbate oxidative stress and, consequently, the damage of cellular lipids, proteins, and DNA, and may also affect endothelial function. Nevertheless, ROS are known to act as important signaling molecules, essential to cell viability, playing various regulatory roles.

In addition, Theunissen et al. (2013a) showed that circulating nitric oxide (NO) increased after a series of breath-hold dives and hypothesized that also ROS increased excessively. The reaction of ROS with NO produces peroxynitrites (ONOO⁻), thus reducing the ability of NO to achieve vasodilation and negatively affecting endothelial function. Moreover, the generation of peroxynitrites causes additional oxidative stress by increasing oxidase activity and inactivating antioxidant capacity (Thom 2011; Morabito et al. 2011).

Thiols are extremely efficient antioxidants, which are able to protect cellular lipids, proteins, and nucleic acids against peroxidative damage owing to their strong reductive capacity and their ability to react with free radicals (Ellman and Lysko 1979; Theunissen et al. 2013a; Vezzoli et al. 2016). Therefore, human plasma thiols redox status can be used as index of a peripheral response of the organism and the detection and quantification of reduced and oxidized forms of amino thiols are important in the investigation of oxidative stress-related risk factors and diseases (Herrmann 2001). Erythrocytes have been used as a simple model to study the cellular effects of ROS and appropriate for intracellular redox status analysis (Vezzoli et al. 2016).

Endothelial dysfunction and oxidative stress have been widely investigated in scuba diving (Thom et al. 2013; Brubakk et al. 2014; Cialoni et al. 2019), but only few similar works are published in literature about breath-hold diving and oxidative stress (Jouliia et al. 2003; Theunissen et al. 2013a, b). Moreover, several correlations have been hypothesized but not showed yet, between repeated breath-hold dives and organ damage, such as renal function impairments (Kjeld et al. 2015; Oh et al. 2017) or brain subclinical injuries (Kohshi et al. 2014). However, no investigations have been performed on a single wet dive regarding oxidative stress and, to date, the pathogenesis of organ damage in breath-hold diving remains unclear.

Physical exercise is known to increase the generation of ROS in response to increased oxygen utilization causing a disturbance in the prooxidant/antioxidant balance in favor of the former which results in oxidative stress. In our experimental protocol, all divers performed a sled-assisted breath-hold dive to 40 m to minimize the effects of physical exercise. The experiment was held at the world's deepest pool "Y-40 THE DEEP JOY" with a constant water temperature of 31.5 ± 0.5 °C.

The aim of this study was to investigate the effects of breath-hold diving on oxidative stress biomarkers by an experimental setting that provided the opportunity to get an insight into the pathways in the absence of other (ambient/exercise) disturbing factors.

Moreover, we planned to assess ROS production and the determination of antioxidant activity by electron paramagnetic resonance (EPR), the only unique tool that allows direct measurements of free radical species (Mrakic-Sposta et al. 2017; Dikalov et al. 2018) and the changes of the intensity of the EPR spectrum of stable radicals, which results from their interaction with antioxidants. EPR technique using the spin probe cyclic hydroxylamine was adopted for the sensitive quantification of ROS (Dikalov et al. 2007; Mrakic-Sposta et al. 2012) and stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used for assessing the antioxidant capacity in samples (Kozik et al. 2015; Zang et al. 2017). In addition, nitric oxide metabolites, inducible nitric oxide synthase (iNOS), amino thiols, and renal function were evaluated too as markers of redox status and renal damage.

Materials and methods

Subjects and experimental design

Eight well-trained healthy breath-hold divers were enrolled for the trials held at "Y-40 THE DEEP JOY" pool (Montegrotto Terme, Padova, Italy), but only six divers completed the dive at 40 m (mean age 46.6 ± 9.3 years; height 176 ± 4 cm; BMI 25 ± 2.9 kg/m²). The experimental protocol was approved (n. HEC-DSB/03-18) by the Human Ethical Committee of the Department of Biomedical Science of the University of Padova (Italy), and all volunteers signed an informed consent according to the Declaration of Helsinki.

All divers, accompanied by a professional instructor, performed a sled-assisted breath-hold dive to 40 m. Prior to submersion, an arterial cannula was inserted in the radial artery of the non-dominant limb. To ensure the safety of the divers, a second professional diver was stationed during the descending and ascending phases at 20 m depth. More details on subjects, experimental procedures, timing of the descent (45.1 ± 2.7 s), time on the bottom (46.8 ± 4.8 s) and

ascent (38.8 ± 5.1 s) have been previously reported (Bosco et al. 2018a).

Blood and urine sample collection

Blood samples were collected to determine plasma levels of ROS, antioxidant capacity, nitrite/nitrate, iNOS enzyme, and erythrocyte aminothiols concentrations. Lipid peroxidation was assessed by 8-isoprostane concentration determination in urine. Blood and urine samplings were carried out 10 min before submersion at 40-m depth (pre-dive) and 2 min after diver's surfacing (post-dive).

Approximately 6 mL of arterial blood was drawn from the radial artery. The samples were collected in heparinized and EDTA Vacutainer tube (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), plasma and erythrocytes were immediately separated by centrifuge (5702R, Eppendorf, Hamburg, Germany) at $1000 \times g$ for 10 min at 4 °C. Urine samples were collected by voluntary voiding in a sterile container provided to the subjects. All samples were stored in multiple aliquots at -80 °C until assayed and thawed only once before analysis.

ROS and antioxidant capacity by electron paramagnetic resonance

Electron Paramagnetic Resonance, X-band (9.3 GHz) (E-Scan-Bruker BioSpin, Billerica, MA, USA) was used to assess the ROS production and total antioxidant capacity (TAC) in plasma. Sample temperatures were stabilized at 37 °C by Temperature and Gas Controller "Bio III" unit (Noxigen Science Transfer & Diagnostics GmbH, Germany), interfaced with E-Scan. Bruker software was adopted for spectra acquisition and handling (Win EPR System, V. 2.11). Each sample was analyzed in triplicate.

ROS production assessment method was previously described (Mrakic-Sposta et al. 2012, 2014). Briefly, 50 μ L of plasma was treated with CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) probe solution (1:1). Absolute production rate of ROS (μ mol min^{-1}) was obtained converting relative quantitative determination using the stable radical CP (3-carboxy 2,2,5,5-tetramethyl-1-pyrrolidinyloxy) as external reference.

Antioxidant capacity was measured using DPPH \cdot (2,2-diphenyl-1-picrylhydrazyl), a free radical compound soluble and stable in ethanol. Briefly, 5 μ L of plasma was added to 45 μ L of buffer solution (5 mM potassium phosphate, pH 7.4 containing 0.9% sodium chloride) then reactions were initiated by the addition of 50 μ L of DPPH \cdot as a source of free radicals, as previously indicated (Kozik et al. 2015; Zang et al. 2017). A concentration of 1 mM DPPH \cdot prepared in absolute ethanol was chosen by preliminary experiments because it was much in excess to

the concentration of antioxidants in the plasma samples, thus allowing all the antioxidants to react with the radical. Reaction mixtures were incubated for 30 min at room temperature and then 50 μ L of the obtained solution was put in the glass EPR capillary tube. All operations were performed in the dark to avoid photochemical effect on DPPH \cdot . A linear calibration curve was computed from pure Trolox-containing reactions. The regression equation for the linear relationship between the percentage inhibition of the EPR signal intensity and the mol number of Trolox was equal to:

$$y = -2038.4x + 366.89,$$

where y is the amplitude of DPPH \cdot signal and x is the Trolox concentration. This equation was used to calculate the antioxidant capacity expressed in terms of Trolox equivalent antioxidant capacity (TAC, mM).

Nitrite and nitrate plasma levels (NO_2/NO_3)

EDTA plasma samples were ultra-filtered through a 30 kDa molecular weight cutoff filter (AmiconUltra; Millipore, EMD Millipore Corporation, Billerica, MA, USA) using an ultracentrifuge (4237R, ALC, Milan, Italy) at $4000 \times g$ for 60 min at 4 °C to reduce background absorbance due to the presence of hemoglobin which is known to interfere with subsequent spectrophotometric measurements. The ultra-filtered material was recovered and used to measure nitrite and nitrate concentrations by colorimetry based on the Griess reaction (Green et al. 1982), using a commercial kit (Cayman, BertinPharma, Montigny le Bretonneux, France). Samples were read at 545 nm. A linear calibration curve was computed from pure nitrite and nitrate standards.

All samples were determined by a microplate reader spectrophotometer (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland) in duplicate, and the inter-assay coefficient of variation was in the range indicated by the manufacturer.

Inducible nitric oxide synthase (iNOS) (protein synthesis determination) expression

To assess inducible nitric oxide synthase (iNOS) expression, protein synthesis in plasma, a human NOS_2/iNOS ELISA kit (cat no EH0556; FineTest, Wuhan China) was used. This assay was based on sandwich enzyme-linked immune-sorbent assay technology. The analysis was carried out according to the manufacturer's instructions. NOS_2/iNOS protein synthesis was determined using a standard curve. Samples and standards were read at a wavelength of 450 nm.

Determination of thiols

Total (tot), and reduced (red) aminothiols (Cys = cysteine, CysGly = cysteinylglycine, Hcty = homocysteine, and GSH = glutathione) were measured in the erythrocytes according to previously validated methods (Dellanoce et al. 2014; Vezzoli et al. 2016). Briefly, Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) and 4-fluoro-7-sulfamoylbenzofurazan (ABD-F) were used as reducing and derivatizing agents, respectively; reduced aminothiols were assessed by erythrocytes with 10% trichloroacetic acid (1:1 v/v). NaOH (0.4 M, 10 μ L), borate buffers (1 M, pH 11, 70 μ L as well as 1 M, pH 9.5, 30 μ L), each of them containing 4 mM EDTA, and ABD-F (10 g/L, 10 μ L, in borate buffer pH 9.5) were added to 100 μ L of each of the obtained supernatants. Samples were incubated at 4 $^{\circ}$ C for 90 min and then 10 μ L was injected into a high-performance liquid chromatography (HPLC) system for analysis.

Thiol separation was performed at room temperature by isocratic HPLC analysis on a Discovery C-18 column (250 \times 4.6 mm I.D, Supelco, Sigma-Aldrich, St. Louis, MO, USA), eluted with a solution of 0.1 M acetate buffer, pH 4.0: methanol, 81:19 (v/v), at a flow rate of 1 mL/min. Fluorescence intensities were measured with an excitation wavelength at 390 nm and an emission wavelength at 510 nm, using a fluorescence spectrophotometer (Jasco, Japan). A standard calibration curve was used.

8-Isoprostane (lipid damage)

Lipid peroxidation was assessed in urine by competitive immunoassay of 8-isoprostane concentration (8-iso-PGF 2α), (Cayman Chemical, USA). Urine was purified using the solid phase extraction cartridges. The purification and the subsequent EIA assay were performed following the manufacturer's recommendations. The EIA employs 8-iso-PGF 2α tracer and 8-iso-PGF 2α antiserum. 8-iso-PGF 2α concentrations were determined using a standard curve. Samples and standards were read at a wavelength of 412 nm.

Creatinine and neopterin concentration

Creatinine and neopterin concentrations were measured by an isocratic high-pressure liquid chromatography (HPLC) method. Briefly, urine samples were thawed and centrifuged at 13,000 rpm for 5 min at 4 $^{\circ}$ C; the supernatant was then adequately diluted with chromatographic mobile phase (15 mM of K $_2$ HPO $_4$, pH 3.0). Neopterin and creatinine levels were measured using a Varian pump (240, auto sampler ProStar 410) coupled to a fluorometric detector (JASCO FP-1520, λ_{ex} = 355 nm and at λ_{em} = 450 nm) for neopterin and to a UV-Vis detector (Shimadzu SPD 10-AV, λ = 240 nm) for creatinine determinations. Neopterin and

creatinine separations were performed at 50 $^{\circ}$ C on a 5 μ m Discovery C18 analytical column (250 \times 4.6 mm I.D., Supelco, Sigma-Aldrich) at a flow rate of 0.9 mL min $^{-1}$. The calibration curves were found to be linear over a concentration of 0.125 $^{-1}$ mmol L $^{-1}$ and 1.25 $^{-10}$ mmol L $^{-1}$ for neopterin and creatinine (the score in μ mol L $^{-1}$ was divided by 88.4 to get mg dL $^{-1}$ creatinine), respectively. Inter-assay and intra-assay coefficients of variation were less than the 5%.

Statistical analysis

All data are presented as mean \pm SD. Statistical analysis was performed using the GraphPad Prism package (GraphPad Prism 8.2, GraphPad Software Inc., San Diego, CA, USA). After a normality test (Kolmogorov–Smirnov), data were analyzed with a non-parametric test. Wilcoxon matched-pair test was used to compare values of assessed biomarkers of redox status and renal function at Pre- versus Post-session in divers. p < 0.05 was considered statistically significant.

Results

Oxidative status values were obtained at pre- and post-dive from all divers examined. Significant (p < 0.05) increases at post-dive of ROS production rate (0.158 ± 0.003 vs 0.195 ± 0.006 μ mol min $^{-1}$) (Fig. 1a) and lipid peroxidation (8-isoprostane: 375.67 ± 195.62 vs 420.49 ± 232.31 pg mg $^{-1}$ creatinine) (Fig. 2c) were measured. Conversely, the antioxidant capacity (TAC) significantly decreases (3.423 ± 0.089 vs 3.015 ± 0.284 mM) after

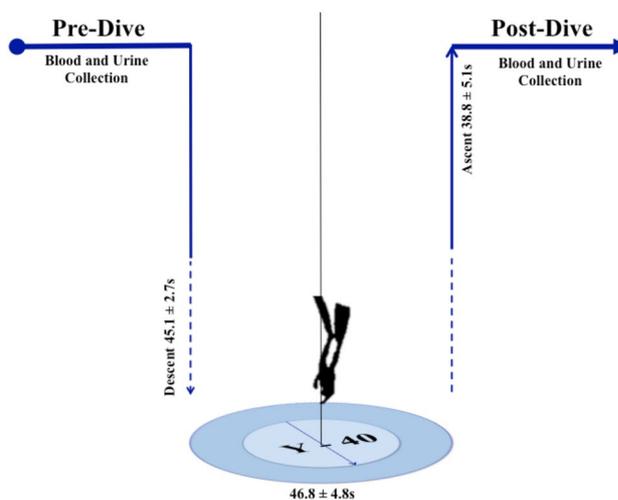
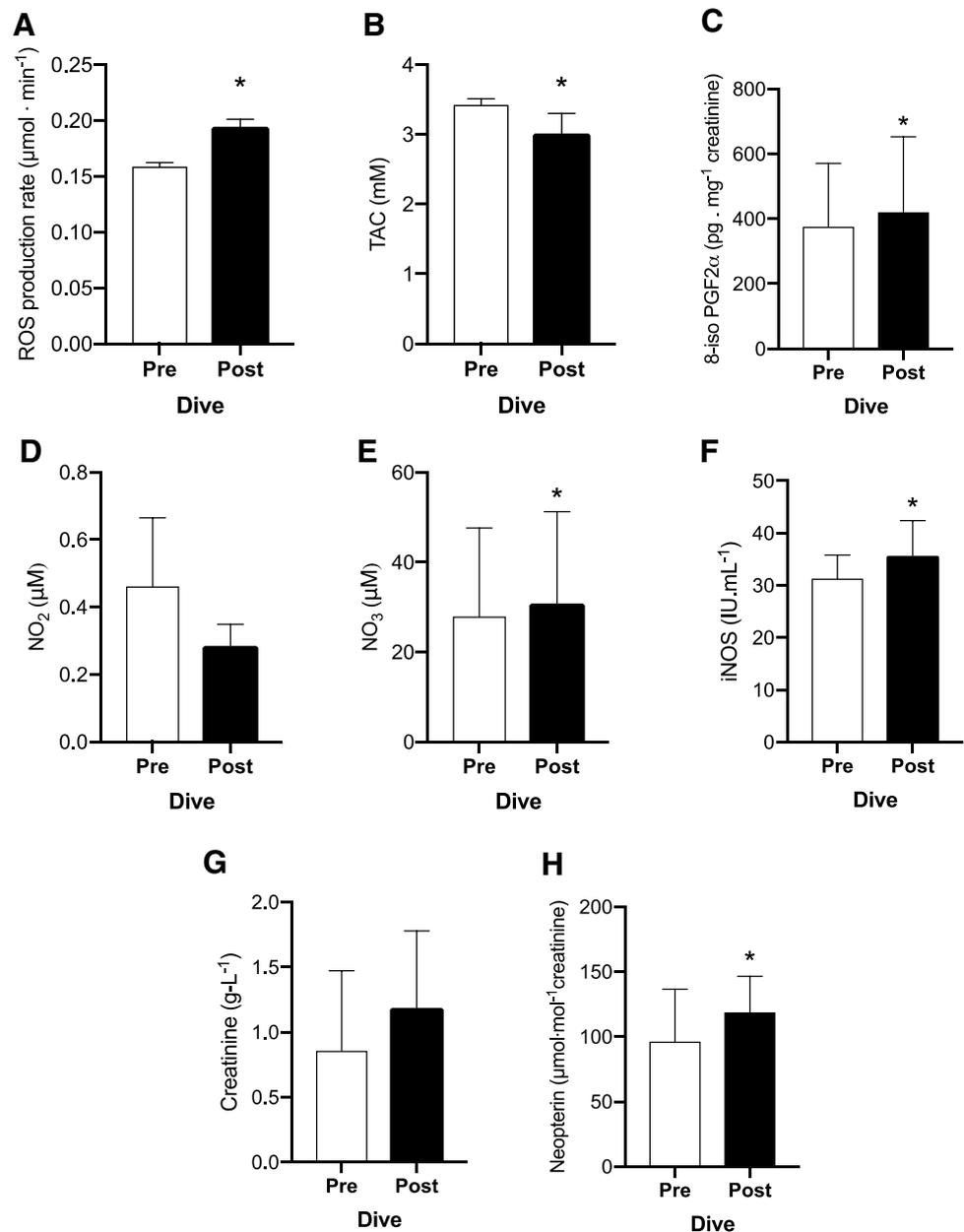


Fig. 1 Experimental study design. Pre-dive blood and urine sampling. Then, divers performed a sled-assisted breath-hold dive to 40 m. Intermediate times for each diving phase, are reported. After dive surfacing (post-dive), blood and urine sampling were collected within 2 min

Fig. 2 Biomarker in breath-hold diving. Histogram plot (mean \pm SD) of **a** ROS production rate **b** TAC, **c** 8-isoprostane, **d** nitrite, **e** nitrate, **f** inducible nitric oxide synthase, **g** Creatinine and **h** neopterin concentration collected at pre- and post-dive in breath hold divers. * $p < 0.05$, significantly different



immersion (Fig. 2b). Concentration of cysteine tot. significantly ($p < 0.05$) decreased, while the concentration of other aminothiols (Table 1) did not significantly change in erythrocytes post-dive.

We showed a significant ($p < 0.05$) increase in nitrate (NO₃) (27.91 ± 19.71 vs 30.80 ± 20.44 μM) (Fig. 1e) and iNOS protein level (31.30 ± 4.52 vs 35.68 ± 6.72 IU mL^{-1}) (Fig. 1f) concentrations post-dive. No significant difference was recorded with regard to nitrite (NO₂) levels (0.37 ± 0.20 vs 0.25 ± 0.06 μM) (Fig. 1d).

The histogram panels of Fig. 1g, h (creatinine and neopterin/creatinine levels, respectively) showed a non-significant increase of creatinine level and a significant ($p < 0.05$)

increase in neopterin concentration (96.20 ± 40.41 vs 118.76 ± 27.84 $\mu\text{mol mol}^{-1}$ creatinine) post-dive.

Discussion

The deep swimming pool (Y-40) represents a very interesting and particular dive site. Moreover, it is exceptionally helpful as a test environment, but it is anyway different as compared with real diving conditions (i.e., sea or lake) as per water temperature and density. However, our results suggest that breath-hold diving exposes the human body to environmental stress, implying an increase in ROS production,

Table 1 Redox status in erythrocytes

	Divers' aminothiols		<i>p</i>
	Pre-dive	Post-dive	
Cys tot ± SD	62.92 ± 22.68	50.92 ± 16.91	0.031*
Cys red ± SD	12.05 ± 0.71	11.44 ± 1.00	0.062
CysGly tot ± SD	3.44 ± 1.72	2.65 ± 0.72	0.437
CysGly red ± SD	3.44 ± 1.72	0.58 ± 0.11	0.156
Hcy tot ± SD	2.56 ± 0.43	2.43 ± 0.25	0.437
Hcy red ± SD	0.68 ± 0.01	0.68 ± 0.02	> 0.999
GSH tot ± SD	2048.88 ± 346.64	2154.99 ± 294.80	0.437
GSH red ± SD	1539.16 ± 320.34	1530.42 ± 405.57	0.843

Mean (± SD) aminothiol values in divers at pre- and post-immersion. The concentrations of the various forms are expressed as $\mu\text{mol L}^{-1}$.

p value across samples are reported

Cys cysteine, CysGly cysteinylglycine, Hcty homocysteine, GSH glutathione, red reduced, tot total

*Significantly different ($p < 0.05$)

NO levels, lipid peroxidation, renal damage and decrease in antioxidant capacity.

As widely reported, physical exercise and hyperoxia/hypoxia increase ROS concentration in the whole body (Mrakic-Sposta et al. 2015; McLeay et al. 2017; Tillmans et al. 2019). In the present study, overproduction of ROS (about +27%) with a consequent exacerbation of oxidative damage to lipid membrane (about +12%) at post-immersion was observed. Correlated to the increase of the ROS production rate, the antioxidant capacity decreased (−12%) too. Since in this experiment, the influence of physical exercise was minimized using a sled, we believe that the increase in oxidative stress during breath-hold diving reflects the hyperoxic and hypoxic conditions that typically occur at depth and in the last few meters below the surface, respectively (Fitz-Clarke 2018; Bosco et al. 2018a).

Aminothiols play a central role in redox-sensitive cell signaling mechanisms (Vezzoli et al. 2016). Therefore, they are reliable measures of the systemic oxidative status and cysteine pools reflecting extracellular oxidative burden (Jones 2006). After a 40-m breath-hold dive, data showed a constant concentration of GSH, and a decrease of total cysteine level (−19%) as a compensatory mechanism, while other analyzed aminothiols—namely cysteinylglycine and homocysteine—did not change significantly. It is noteworthy that the altered concentration of one aminothiol species may cause complex changes in overall thiol dynamic equilibrium even to the disruption of the redox-regulated signaling mechanisms. Erythrocytes are much more vulnerable to oxidative damage because of their continuous exposure to changes in oxygen fluxes and their high concentrations of polyunsaturated fatty acids and heme iron (Smith 1995). The decrease of total cysteine level in erythrocytes

is thereafter a mechanism apt to preserve redox status in cellular compartments.

We want to underline that the examined divers are characterized by high basal aminothiol levels compared to normal population (Dellanoce et al. 2014) and consequently higher values of antioxidant capacity were recorded. A similar observation is related to the anti-oxidative benefits associated with exercise training, with overexpression of the antioxidant system (Margaritelis et al. 2017). In professional divers, this phenomenon might be probably linked to adaptive pathways to the ROS production during repeated immersions.

The present results confirm the increase in circulating NO level after breath-hold diving as previously reported by Theunissen et al. (2013a). Otherwise in our experimental study, the increase of circulating NO metabolites (NO_3 about +10%) and iNOS protein level are probably due rather to modifications in PaO_2 (hyperoxic and hypoxic) condition than to physical exercise because of the sled-assisted diving.

iNOS produces NO in large quantities under more extreme conditions and it is associated with the regulation of peripheral vascular tone and blood flow (Theunissen et al. 2013b). Our data seem to confirm this hypothesis; indeed, we observed an increase (+14%) of iNOS levels after hyperoxia and hypoxia/re-oxygenation exposure.

An increase in systemic oxidative stress may also be associated with altered biochemical parameters measured in urine and in particular to neopterin and creatinine concentration level (Murr et al. 2002; Shao et al. 2014; Mrakic-Sposta et al. 2015).

Increase of urinary neopterin concentration (+23%) was observed in the present study immediately after immersion, and was associated with a rise in ROS levels, along with a decrease in antioxidant capacity.

Higher creatinine levels (+40%) were recorded in Post-Dive (Fig. 2), suggesting a possible early urinary dysfunction at this stage. Even if not significant—probably due to the high variability recorded among the subjects—these data are consistent with those obtained by Oh et al. (2017).

Despite our study did not evaluate the long-term effects of breath-hold diving on kidney function, our divers apparently showed a temporary “impairment of renal function” as a likely physiological and adaptive response to hypoxia.

Limitations

This study represents a pilot study and suffers from some limitations. Further investigations are needed to confirm our preliminary results. Certainly, the low number and the high interindividual variability of investigated subjects is a limit, as well as the lack of data at 40-m depth and after at least 2 h from the surfacing (i.e., follow-up). Anyway, the strength consists in measuring for the first time the oxidative

biomarkers (i.e., ROS production and antioxidant capacity by EPR and aminothiols redox status) after a breath-hold dive at 40 m in an experimental setting limiting disturbing factors. To assess endothelial function, measurement was limited to the listed NO metabolites due to feasibility needs. In the future, other experiments could replicate this wet dive and assess other endothelial function markers.

Conclusion

After breath-hold diving, the increase in ROS production and NO metabolites was detected. Consequently, an increase in oxidative damage and a decrease in antioxidant capacity are generated after breath-hold diving. Moreover, a transient damage in renal function is showed in breath-hold diving. Future studies are required to investigate the long-term effects during or after single or repetitive dives.

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Author contributions All experiments were performed at the deepest swimming pool Y-40 THE DEEP JOY, (Montegrotto Terme—Padua Italy), at the university of Padova (Padova, Italy) and National Research Council (Milano, Italy). SMS contributed to the study design, data analysis, interpretation and drafting of the manuscript. AV, CDN and MM contributed to the data analysis and interpretation, critical review of the manuscript; AR, MP, SM and PC contributed to the data collection and critical review of the manuscript; GB contributed to the study design, data collection and data interpretation, and critical review of the manuscript. GB confirms the study objectives and procedures are honestly disclosed. All the authors approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest None declared.

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