Oxidative stress, antioxidant defenses and nitric oxide production following hyperoxic exposures

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ABSTRACT

Little data exist on the dose-response relationship between the partial pressure of inspired oxygen (PiO_2) and the cellular oxidative stress response in humans. The objective of this study was to determine the effects of PiO_2 on lipid peroxidation, antioxidant enzyme activity and nitric oxide (NO) production. Twelve healthy male divers breathed 100% O_2 in a hyperbaric chamber for two hours at 1 (101 kPa), 1.5 (152 kPa) and 2 (203 kPa) atmospheres absolute (atm abs). Venous blood was collected pre-, within 15 minutes post-, one and two hours post-hyperoxic exposures to determine changes in plasma and erythrocyte lipid peroxidation (thiobarbituric acid reactive substances –TBARS), antioxidant enzyme activity (superoxide dismutase-SOD), catalase-CAT, glutathione peroxidase-GPx), and plasma NO production (L-arginine [L-Arg], asymmetric dimethylarginine-ADMA, and nitrites). There were minor changes in TBARS and mixed responses in plasma and erythrocyte CAT and GPx activity. Plasma L-Arg increased following 1 and 1.5 atm abs exposures, yet ADMA and nitrites were unchanged. Only erythrocyte CAT and plasma GPx activity, and plasma L-Arg/ADMA demonstrated a significant PiO_2 dose-dependent relationship. Two-hour hyperoxic exposures at 1-2 atm abs of O_2 results in mixed oxidant-antioxidant responses and unaltered NO production. Moreover, there does not appear to be a strong systemic dose-dependent oxidative stress response at these hyperoxic exposures.

INTRODUCTION

Hyperbaric oxygen (HBO_2) therapy (breathing 100% oxygen (O_2) under pressures greater than 1 atmosphere absolute (atm abs), 101 kPa) is currently approved for the treatment of 14 conditions including decompression sickness, air or gas embolism, necrotizing soft tissue infections and crush injuries [1,2]. The therapeutic benefits of HBO_2 are well known and include a reduction in edema, impaired leukocyte adhesion, enhanced antibacterial mechanisms, fibroblast proliferation and angiogenesis [3,4]. However, HBO_2 can lead to oxygen toxicity, causing pulmonary damage, convulsions and death [5-7]. The basic mechanisms underlying the beneficial and negative effects of HBO_2 are not fully understood, but are believed to be attributed to the generation of reactive oxygen species (ROS) and the production of nitric oxide (NO) [8,9].

During normal metabolism, ~1-2% of the O_2 consumed by the electron transport system results in ROS production [10]. Under normobaric hyperoxia (NBO_2) and HBO_2, the generation of ROS is increased [11,12] with the degree of production dependent upon the partial pressure of oxygen (PO_2) [13]. The major ROS produced as a result of the incomplete reduction of O_2 include superoxide ($\cdot O_2$-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\cdot$OH) and peroxynitrite (ONOO-). The cellular response to oxidative stress is to scavenge ROS with antioxidants, including the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). However, if ROS production exceeds the cellular antioxidant defenses, lipid peroxidation, protein oxidation and nitration, and DNA damage ensues [10,14].

NO is a free radical and signaling molecule that plays a vital role in regulating blood flow, neuronal function, inhibition of platelet aggregation and smooth muscle cell proliferation, as well as in the regulation of cell adhesion, vascular permeability and erythrocyte deformability [15,16]. L-arginine (L-Arg) and O_2 are substrates for NO production, a reaction catalyzed

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by the family of nitric oxide synthase (NOS) enzymes [17]. NO production and function are altered with hyperoxic exposures, and can be both toxic and protective to cells depending upon the PO_2, tissue and NOS isoform. For instance, prolonged NBO_2 exposure results in oxidative pulmonary injury, possibly through the production of NO via the pro-inflammatory NOS isoform, inducible nitric oxide synthase (iNOS) [18]. The tissue damage is believed to be due to the inactivation of NO by $\cdot O_2$- and the formation of ONOO-, a process that can be attenuated by SOD [18,19]. HBO_2 exposure can result in central nervous system (CNS) O_2 toxicity, which is accelerated by the production of NO via endothelial nitric oxide synthase (eNOS) and the corresponding increased cerebral blood flow [20]. In contrast, eNOS production of NO in the lungs appears to protect against oxidative pulmonary injury [18]. A possible protective mechanism for preventing oxidative tissue injury is through NOS activity inhibition. Asymmetric dimethylarginine (ADMA) is derived from the catabolism of proteins containing methylated arginine residues, and is a potent inhibitor of NOS activity by displacing L-Arg from the NOS substrate binding site [21]. Thus, under hyperoxic exposure, ADMA concentrations may increase to inhibit NO production that may otherwise result in oxidative tissue injury [22].

Much of our understanding of the cellular mechanisms of HBO_2-induced oxidative stress and NO production is a result of research that employed protocols using O_2 tensions $\geq$ 2.4 atm abs; thus little is known of the effects at lower therapeutic PiO_2s. We, therefore, aimed to determine whether there is a predictable PiO_2 dose response to two hours of hyperoxic exposures at 1, 1.5 (152 kPa) and 2 (203 kPa) atm abs on oxidative stress and NO production in healthy, U.S. Navy-trained divers. Plasma and erythrocyte blood fractions were selected for assessment due to their susceptibility to oxidative-induced lipid peroxidation via ROS, and their antioxidant defense system [23,24]. Our hypothesis was that the degree of lipid peroxidation, activity of antioxidant enzymes and NO production would progressively increase with increasing PiO_2 level. To our knowledge, this is the first study investigating the dose-response relationship of hyperoxic induced oxidative stress on NO production and the antioxidant defense systems at lower hyperoxic levels of PiO_2 (1-2 atm abs) in healthy humans.

METHODS

Subjects

Twelve healthy male U.S. Navy-trained/certified divers (age 35 ± 13 years, range 20-59; height 180 ± 7 cm; weight, 86 ± 13 kg, mean ± SD) participated in this study. Of the 10 volunteers, two (20 and 24 years of age) were current smokers, with a smoking history of fewer than two pack-years at the time of the investigation. Although diving experience varied (from less than one year to more than 40 years), 10 of the 12 subjects had completed their U.S. Navy dive training within the previous 10 years.

Subjects were informed of the study purpose and requirements prior to providing written informed consent to participate. The study protocol (NSMRL. 2010.0002) was approved by the Naval Submarine Medical Research Laboratory Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects.

Design

On three occasions in a randomized manner, separated by a minimum of one week, subjects breathed 100% oxygen without air breaks for two hours at 1, 1.5 and 2 atm abs in a hyperbaric chamber (Genesis, NSMRL). All experiments started in the morning, with the hyperoxic exposures commencing between 08:30 and 09:30 a.m. Subjects did not perform any diving prior to or during the 3.5 weeks of testing. Prior to the start of the study, subjects were instructed by a registered dietitian (HGG) to maintain a consistent dietary pattern on each of the three testing days. The pattern was documented, and the subjects agreed to refrain from consuming foods high in nitrites/nitrates (*e.g.*, beef, pork, liver, leafy greens, avocados and cantaloupe) and L-Arg (*e.g.*, nuts and seeds). Food items were coded as being low, moderate or high in nitrites/nitrates and L-Arg. In addition, the volunteers were instructed to avoid/discontinue any dietary supplement use (*e.g.*, amino acids, protein powders and antioxidants) during the protocol, and to avoid exercise on experimental days until all of the post-exposure testing was completed. Smokers (n = 2) were instructed to avoid smoking on each testing day, and this was confirmed by measuring their exhaled CO with a MicroCO CO monitor (Micro Direct, Inc., Lewiston, Maine) prior to and following each exposure.

On four occasions during each of the three study days (pre-, 15 minutes post-, one hour and two hours

post-exposure), two aliquots of venous blood (approximately 8 mL each) were collected from the antecubital vein of the volunteers in EDTA containing vacutainers. After each collection point, one of the aliquots of blood was centrifuged at 2800 g for 15 min followed by removal and storage of plasma in 1.5 mL vials at -25°C. Erythrocytes were washed with ice cold HPLC grade water (1:4 ratio), centrifuged at 10000 g and stored in 1.5 mL vials at -25 °C. Plasma and erythrocytes were later analyzed for thiobarbituric acid reactive substances (TBARS), SOD, CAT and GPx, and plasma levels of L-Arg, ADMA, and nitrites. The second aliquot of blood was placed on ice until the end of the protocol and transported to the Naval Branch Health Clinic, Groton, Conn., for determination of erythrocytes, hemoglobin (Hb), hematocrit (Hct) and leukocytes using a Beckman Coulter LH 500 Hematology Analyzer (Beckman Coulter, Inc., Brea, Calif).

Biochemical analysis

Lipid peroxidation and antioxidant enzyme activity

Plasma and erythrocyte TBARS, SOD, CAT and GPx were assessed by commercially available assay kits (Cayman Chemicals, Ann Arbor, Mich.) in triplicates and analyzed with a DTX 880 Multimode Detector (Beckman Coulter Inc., Brea, Calif.). Plasma (0.1 mL) and erythrocyte (0.01 mL) TBARS, the malondialdehyde-thiobarbituric acid (MDA-TBA) adduct, was formed by reacting MDA and TBA at 100°C for one hour and measuring the absorbance at 540 nm. SOD (cytosolic-Cu/Zn, mitochondrial-Mn and extracellular-Fe) activity was determined in plasma (1:5, dilution) and erythrocytes (1:100) by the dismutation of $\cdot O_2$- (generated from xanthine oxidase and hypoxanthine) and measuring the absorbance at 460 nm. CAT activity was determined in plasma (1:4) and erythrocytes (1:2000) by measuring the formaldehyde produced with purpald at an absorbance of 540 nm. GPx activity was determined in plasma (1:2) and erythrocytes (1:200) from the oxidation of NADPH and measuring the decrease in absorbance over five minutes at 340 nm. The intra-assay and interassay coefficient of variations (CV) were 2% and 11% for TBARS, 9% and 9% for SOD, 6% and 11% for CAT, and 2% and 12% for GPx, respectively.

Nitric oxide production

Plasma L-Arg was determined in duplicates using ultra-performance liquid chromatography (UPLC-Acquity system®, Waters Corp., Milford, Mass.) after samples (0.05 mL) were prepared according to Buentello and Gatlin [25] and derivatized following manufacturer's instructions (MassTrak AAA Derivatization Kit, Waters Corp., Milford, Mass.). The between sample CV was 6%. Plasma ADMA (0.05 mL) was determined in triplicates by a commercial competitive ELISA kit (ALPCO Diagnostics, Salem, N.H.) with the absorbance measured at 450 nm on a DTX 880 Multimode Detector (Beckman Coulter Inc., Brea, Calif.). The intra-assay and interassay CV was 4% and 6%, respectively. Plasma nitrite was determined in triplicates by the chemiluminescence reaction of NO with ozone [26] using a Sievers Nitric Oxide Analyzer, model 280i (GE Analytical Instruments, Boulder, Colo.). Briefly, 100 µL of standard (15-1000 nM) or sample was injected into the purge vessel that contained ~50 mM NaI in glacial acetic acid and 200 µL of antifoam. Nitrite standards (15-1000 nM) were used to calculate the nitrite concentrations by recording the NO area under the curve and processing the data with the Sievers NOAnalysis™ software version 3.2. The between-sample CV was 7%.

STATISTICAL ANALYSIS

Data are presented as mean ± SD. Statistical analyses were carried out using SigmaPlot (version 11.0, Systat Software, Inc., San Jose, Calif.) and Statistica software (version 9.0, StatSoft, Tulsa, Okla.). A one-way repeated measures ANOVA was used to determine if there were differences between the baseline (pre-) for the three PiO_2 exposures, as well as to determine if there was a significant main effect of time, *i.e.*, changes between baseline and the post-exposure time points (15 minutes, one hour and two hours) for each hyperoxic exposure. If a test of normality or equal variance failed, the ANOVA was conducted on the transformed data (reciprocal or $\log_{10}$). When significant F ratios were present, a Holm-Sidak post hoc procedure was used to determine differences among group means. Statistical significance for the ANOVA and post hoc tests were set at $p<0.05$. When the ANOVA found significant changes in the post-exposure values from baseline, a within-subjects multiple regression analysis [27] was used to determine if the relative changes from baseline exhibited a significant relationship with the PiO_2 dose. In view of the multiple comparisons conducted for the multiple regression analysis, statistical significance for this analysis was set at $p<0.01$.

RESULTS

No differences were observed between baseline (pre-) 1, 1.5 and 2 atm abs exposures for hematological parameters, erythrocyte TBARS, plasma SOD and CAT activity, plasma L-Arg, ADMA and nitrites. Conversely, there were within-individual differences in pre-values for plasma TBARS (p=0.007), plasma GPx (p<0.001), erythrocyte SOD (p<0.001), CAT (p<0.001), and GPx activity (p=0.002). The baseline and post-hyperoxic antioxidant and oxidative responses for the two smokers all fell within 2 SD of the mean responses of the non-smokers. As a result of the data from the two smokers not being outliers and being consistent with the non-smokers, they were included in the group analysis.

The hematological parameters for the three PiO_2 exposures are listed in Table 1. There was no significant differences from pre-values for any of the conditions in erythrocytes and hemoglobin, but there was a significant increase of 1.9 ± 2.7% one hour post-1.5 atm abs exposure in hematocrit (p=0.039). Leukocytes displayed a significant increase of 6-9% following the 1.5 atm abs exposure (p=0.004), although the clinical magnitude of this change is negligible. There was no significant PiO_2 dose-dependent relationship at any of the post-exposure times for erythrocytes, hemoglobin, hematocrit or leukocytes.

Plasma and erythrocyte TBARS, an index of lipid peroxidation, are shown in Table 2. Plasma TBARS significantly increased 80 ± 79% one hour post- (p< 0.001) and 43 ± 44% two hours post- (p=0.021) 1 atm abs exposure. The significant post-exposure changes in TBARS at 1 atm abs *vs.* no changes following 1.5 and 2 atm abs exposures may be the result of the significantly lower pre- 1 atm abs condition values compared to the 1.5 and 2 atm abs baselines (p<0.01). In the erythrocytes, TBARS significantly decreased 13 ± 16% one hour post- 1 atm abs exposure (p=0.019), yet increased 18 ± 23% 15 minutes post- 1.5 atm abs exposure (p=0.018). There was no significant PiO_2 dose-dependent relationship for the changes in plasma or erythrocyte TBARS at any of the post-exposure time points.

Increasing PiO_2 resulted in variable changes in antioxidant enzyme activity shown in Table 3. The activity of plasma and erythrocyte SOD and plasma CAT was unchanged following all of the hyperoxic exposures. Erythrocyte CAT activity was unaffected by the 1 atm abs exposure, but decreased 18 ± 18% one hour post- 1.5 atm abs exposure (p=0.034), and consistently decreased following the 2 atm abs exposure (-18 ± 29% 15 minutes post, -37 ± 15% one hour post- and -27 ± 28% two hours post-, p<0.001).

TABLE 1 – Hematological parameters determined pre-, 15 minutes, 1 hour and 2 hours post- breathing 100% O_2 at 1, 1.5 and 2 atm abs

	PRE-	15 MIN POST-	1 H POST-	2 H POST-
Erythrocytes ($10^6 \cdot \mu L^{-1}$)				
1 atm abs	5.10 ± 0.50	5.10 ± 0.49	5.11 ± 0.47	5.01 ± 0.50
1.5 atm abs	5.04 ± 0.46	5.01 ± 0.42	5.12 ± 0.50	5.01 ± 0.45
2 atm abs	5.10 ± 0.46	5.04 ± 0.44	5.09 ± 0.49	5.04 ± 0.53
Hemoglobin ($g \cdot dL^{-1}$)				
1 atm abs	15.5 ± 1.1	15.5 ± 1.1	15.6 ± 1.1	15.4 ± 1.1
1.5 atm abs	15.3 ± 1.1	15.3 ± 1.0	15.4 ± 1.1	15.3 ± 1.0
2 atm abs	15.5 ± 1.2	15.3 ± 1.0	15.5 ± 1.1	15.3 ± 1.1
Hematocrit (%)				
1 atm abs	45.3 ± 2.7	45.4 ± 2.4	45.5 ± 2.5	44.5 ± 2.5
1.5 atm abs	44.7 ± 2.4	44.6 ± 2.1	45.4 ± 2.5*	44.5 ± 2.5
2 atm abs	45.2 ± 2.8	44.7 ± 2.5	45.4 ± 2.9	45.0 ± 2.9
Leukocytes ($10^3 \cdot \mu L^{-1}$)				
1 atm abs	6.5 ± 1.5	6.8 ± 2.2	6.9 ± 2.1	6.7 ± 2.3
1.5 atm abs	5.7 ± 1.2	5.9 ± 1.5†	6.1 ± 1.4†	6.0 ± 1.4*
2 atm abs	6.3 ± 2.1	6.1 ± 1.6	6.2 ± 1.6	6.0 ± 1.3

Values are means ± SD; n = 11-12 per exposure per group. Significantly different from baseline: *$p < 0.05$; † $p < 0.01$.

TABLE 2 – Plasma and erythrocyte thiobarbituric acid reactive substances (TBARS) determined pre-, 15 minutes, 1 hour and 2 hours post- breathing 100% O_2 at 1, 1.5 and 2 atm abs

	PRE-	15 MIN POST-	1 H POST-	2 H POST-
Plasma (µM)				
1 atm abs	6.4 ± 1.4	7.6 ± 2.7	10.9 ± 3.2‡	8.6 ± 1.6*
1.5 atm abs	9.0 ± 2.6	9.3 ± 1.5	10.1 ± 1.6	9.4 ± 2.6
2 atm abs	8.8 ± 1.7	9.4 ± 1.3	10.5 ± 3.0	8.2 ± 3.0
Erythrocytes ($\mu M \cdot g\ Hb^{-1}$)				
1 atm abs	0.17 ± 0.03	0.16 ± 0.02	0.14 ± 0.02*	0.15 ± 0.02
1.5 atm abs	0.16 ± 0.03	0.18 ± 0.04*	0.17 ± 0.01	0.17 ± 0.02
2 atm abs	0.17 ± 0.02	0.18 ± 0.03	0.17 ± 0.02	0.15 ± 0.02

Values are means ± SD; n = 12 per exposure per group. Significantly different from baseline: *$p < 0.05$; ‡ $p < 0.001$.

TABLE 3 – Plasma and erythrocyte antioxidant enzyme activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) determined pre-, 15 minutes, 1 hour and 2 hours post- breathing 100% O_2 at 1, 1.5 and 2 atm abs

	PRE-	15 MIN POST-	1 H POST-	2 H POST-
PLASMA				
SOD ($U \cdot mL^{-1}$)				
1 atm abs	11.6 ± 3.4	11.0 ± 3.6	12.7 ± 4.3	10.4 ± 3.6
1.5 atm abs	12.1 ± 2.9	11.4 ± 2.2	12.4 ± 2.9	13.1 ± 2.6
2 atm abs	11.9 ± 2.9	11.8 ± 2.1	12.8 ± 3.1	11.7 ± 2.4
CAT ($\mu mol \cdot min^{-1} \cdot mL^{-1}$)				
1 atm abs	0.047 ± 0.015	0.035 ± 0.018	0.060 ± 0.019	0.058 ± 0.022
1.5 atm abs	0.047 ± 0.018	0.066 ± 0.028	0.065 ± 0.033	0.064 ± 0.014
2.0 atm abs	0.059 ± 0.033	0.060 ± 0.030	0.068 ± 0.036	0.061 ± 0.032
GPx ($\mu mol \cdot min^{-1} \cdot mL^{-1}$)				
1 atm abs	0.126 ± 0.023	0.146 ± 0.022*	0.143 ± 0.022*	0.126 ± 0.019
1.5 atm abs	0.119 ± 0.020	0.138 ± 0.030	0.124 ± 0.023	0.121 ± 0.024
2 atm abs	0.155 ± 0.019	0.142 ± 0.018	0.142 ± 0.027	0.136 ± 0.023
ERYTHROCYTES				
SOD ($U \cdot g\ Hb^{-1}$)				
1 atm abs	1716 ± 368	2002 ± 481	2010 ± 502	1947 ± 348
1.5 atm abs	2376 ± 607	2297 ± 733	2179 ± 439	2194 ± 504
2 atm abs	2372 ± 530	2554 ± 458	2047 ± 486	2331 ± 683
CAT ($\mu mol \cdot min^{-1} \cdot g\ Hb^{-1}$)				
1 atm abs	172 ± 55	175 ± 44	176 ± 42	186 ± 52
1.5 atm abs	238 ± 52	279 ± 56	191 ± 49*	226 ± 57
2 atm abs	262 ± 73	198 ± 42†	161 ± 45‡	178 ± 47‡
GPX ($\mu mol \cdot min^{-1} \cdot g\ Hb^{-1}$)				
1 atm abs	11.8 ± 4.7	8.8 ± 5.4	11.3 ± 5.5	8.0 ± 5.3
1.5 atm abs	7.0 ± 3.6	13.2 ± 5.1‡	10.4 ± 3.2*	14.7 ± 3.0‡
2 atm abs	13.1 ± 5.4	7.5 ± 2.2†	4.9 ± 2.8‡	6.4 ± 3.5‡

Values are means ± SD; n = 12 per exposure per group. Significantly different from baseline: *$p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$.

Additionally, the relative changes in erythrocyte CAT activity were negatively correlated with PiO_2 at one hour post- (r = -0.66, p<0.001) and two hours post- (r = -0.64, p<0.001) exposures. Plasma GPx activity significantly increased 17 ± 18% 15 minutes post- and 16 ± 28% one hour post- 1 atm abs exposure (p<0.05), but did not change following the 1.5 and 2 atm abs exposures. The relative post-exposure changes in GPx activity were negatively correlated with PiO_2 at 15 minutes post- (r = -0.62, p=0.002) and one hour post- (r = -0.52, p=0.008) exposures. Erythrocyte GPx activity showed mixed changes following the different hyperoxic exposure levels. No changes were observed following 1 atm abs exposures, yet enzyme activity was significantly increased following 1.5 atm abs exposure (89 to 139%, p<0.05), and decreased following the 2 atm abs exposure (-35 to -60%, p< 0.01). There was no significant relationship between PiO_2 and the post-exposure changes in GPx activity. These disparate results likely reflect the low baseline erythrocyte GPx activity observed prior to the 1.5 atm abs exposures compared to the 1 and 2 atm abs exposures.

Plasma L-Arg, ADMA and nitrite levels are presented in Table 4. L-Arg was significantly increased one hour post- (95 ± 126%, p=0.047) and two hours post- (160 ± 95%, p<0.001) 1 atm abs exposure, and two hours following the 1.5 atm abs condition (96 ± 127%, p=0.029). Although not statistically significant, there was a trend for a negative correlation with PiO_2 for plasma L-Arg one hour post-exposure (r = -0.54, p=0.01). ADMA levels were unaffected by PiO_2, yet the ratio of L-Arg/ADMA (data not shown) displayed elevations one hour post- (162 ± 143%, p=0.027) and two hours post- (242 ± 267%, p<0.007) the 1 atm abs exposure. The only significant PiO_2 dose-dependent relationship for the changes in L-Arg/ADMA ratio occurred one hour following the exposures (r = -0.60, p=0.003). Plasma nitrites were unaffected by the three PiO_2 exposures.

DISCUSSION

There has been an increased interest in understanding the cellular responses to HBO_2 as the number of approved indications for HBO_2 therapy continues to expand. The commonly used therapeutic PiO_2 range is between 2-2.8 atm abs over the course of 60-90 minutes, yet the Undersea Hyperbaric Medical Society considers a PiO_2 of 1.4 atm abs the minimum beneficial dosage for HBO_2 therapy [1]. Unfortunately, only a limited number of investigations have assessed the effects of HBO_2 on oxidative stress and antioxidant responses [28,29], and NO production in healthy humans [30]. These previous investigations focused on the effects of higher therapeutic O_2 concentrations (> 2.0 atm abs), thus a gap exists in understanding the oxidative stress associated with hyperoxic exposures in the lower therapeutic range (≤ 2.0 atm abs). The present study was aimed at studying the acute effects of

TABLE 4 – Plasma arginine (Arg), asymmetric dimethylarginine (ADMA) and nitrite determined pre-, 15 minutes, 1 hour and 2 hours post- breathing 100% O_2 at 1, 1.5 and 2 atm abs

	PRE-	15 MIN POST-	1 H POST-	2 H POST-
Arg (µmol · L^{-1})				
1 atm abs	74 ± 31	70 ± 34	115 ± 39*	143 ± 48‡
1.5 atm abs	99 ± 43	118 ± 38	91 ± 32	142 ± 51*
2 atm abs	104 ± 33	102 ± 22	81 ± 15	103 ± 44
ADMA (µmol · L^{-1})				
1 atm abs	0.46 ± 0.08	0.39 ± 0.12	0.33 ± 0.11	0.41 ± 0.19
1.5 atm abs	0.43 ± 0.08	0.38 ± 0.07	0.43 ± 0.17	0.45 ± 0.12
2 atm abs	0.40 ± 0.06	0.35 ± 0.06	0.38 ± 0.07	0.40 ± 0.05
Nitrite (nmol · L^{-1})				
1 atm abs	104 ± 43	102 ± 31	89 ± 37	94 ± 36
1.5 atm abs	89 ± 35	95 ± 43	104 ± 57	126 ± 45
2 atm abs	124 ± 63	105 ± 47	101 ± 55	94 ± 49

Values are means ± SD; n = 9-12 per exposure per group. Significantly different from baseline: * p < 0.05; † p < 0.01; ‡ p < 0.001.

PiO_2 from 1 to 2 atm abs on the antioxidant response to oxidative stress and NO production in healthy males. The main observations from this research include minor changes in blood lipid peroxidation, mixed changes in antioxidant enzyme activity, and an unaltered production in NO following two-hour exposures to a PiO_2 from 1 to 2 atm abs. Moreover, there was not a consistent PiO_2 dose-dependent relationship with the oxidant-antioxidant responses and NO production.

In contrast to our hypothesis, TBARS did not consistently increase following the three PiO_2 exposures *(Table 2)*, nor was there a dose-dependent elevation in either the plasma or erythrocyte TBARS. These data are not in agreement with Oter *et al.* [13], who reported a PO_2 dose-dependent elevation in TBARS in the lung, brain and erythrocytes of rats at O_2 tensions from 1 to 3 atm abs. However, it should be noted that the level of TBARS reported by these investigators did not differ between the 1, 1.5 and 2 atm abs exposures. Interestingly, those that have examined the effects of HBO_2 (2.4-2.5 atm abs) on MDA levels in the blood of humans did not discover differences following the hyperoxic exposures [28,31]. A lack of change in the levels of MDA/TBARS could imply that the O_2 tension was either at an insufficient level or duration to induce a significant degree of lipid peroxidation in the blood. Alternatively, it could be that another index of oxidative stress (*e.g.*, DNA strand breaks or lipid peroxides) would have displayed a different pattern [28]. In the present investigation, the former explanation may be more plausible as, had there been significant oxidative damage, one would have expected a concomitant elevation in SOD activity to prevent $\cdot O_2$- accumulation. Furthermore, the concomitant generation of H_2O_2 from dismutation of $\cdot O_2$- would likely not exceed the cellular antioxidant capacity, thus there would be little changes in CAT and/or GPx activity. The activity of SOD was unaffected by elevated PiO_2 supporting this notion; however, the variable activity observed in CAT and GPx complicate interpretation *(Table 3)*. Specifically, there was a PiO_2 dose-dependent decrease on the relative changes following the hyperoxic exposures in erythrocyte CAT and plasma GPx activity, but not for plasma CAT and erythrocyte GPx activity. Elevation in $\cdot O_2$- can inhibit CAT and GPx activity [32], while elevated H_2O_2 depresses the activity of SOD [33]. Thus, the PiO_2 dose-dependent decrease in plasma GPx and erythrocyte CAT could indicate that $\cdot O_2$- was elevated; however, SOD would have likely shown an increase in activity due to increased substrate. Direct measurement of H_2O_2 would have aided in determining the enzyme activity patterns, which was not possible in this study. Taken together, these data suggest that hyperoxic exposures to 1-2 atm abs over two hours may be insufficient to cause significant and consistent dose dependent oxidant-antioxidant responses in plasma and erythrocytes of humans.

The fact that we observed mixed changes in antioxidant enzyme activity following hyperoxic exposures is not unique, as others have also reported variable responses in the activity of SOD, CAT and GPx (↔, ↑, ↓) to HBO_2 exposures ranging from 2.5 to 5 atm abs in rats and guinea pigs [7, 34-37], and humans [29, 31, 38, 39]. The disparate results from this study, as well as those reported by others, are likely attributed to the different protocols employed, primarily the PiO_2 level and duration of hyperoxic exposure, making direct comparisons difficult. In the lungs, brain and erythrocytes of rats, the changes in antioxidant enzyme activity has been reported to be directly proportional to the PiO_2 used [13], with the most evident changes occurring at 3 atm abs over 90- to 120-minute exposures [37], the recommended upper PiO_2 limit for HBO_2 treatment. When treating patients with HBO_2, the goal is to obtain the maximum therapeutic benefit while minimizing the potential negative consequences of high PO_2 administration (*i.e.*, pulmonary and CNS toxicity). At a lower O_2 dose (2.5 atm abs for 60 minutes interspersed with air breaks), previous studies have reported no changes in SOD, CAT or GPx activity in human plasma and erythrocytes [29,31]. The addition of intermittent air during HBO_2 (2.8 atm abs) has been previously reported to delay O_2 toxicity in guinea pigs and rats [7]. Although the mechanisms of the benefits of air intermittency are not firmly established, air breaks may provide temporary relief from hyperoxic-induced stress by allowing time for dismutation of $\cdot O_2$- by SOD and decomposition/reduction of H_2O_2 by CAT and GPx prior to the next phase of the treatment. Based on the aforementioned data, it seems that the PiO_2 level, duration and whether air breaks are included during HBO_2 treatment determine the degree of oxidant-antioxidant responses to the hyperoxic exposure.

Much less is known about the effects of elevated PiO_2 on NO production in healthy humans. Plasma NO is derived from several sources, including the endothelium and erythrocytes [40]. The lifetime of NO in the vasculature is short (2-3 milliseconds) as it is

rapidly scavenged by hemoglobin and oxidized to nitrite and nitrate [41]. Nitrite is generally accepted as being a reliable surrogate for assessing NO [42] and eNOS activity [43]. The fate of NO is influenced by the concentration of $\cdot O_2$-, such that if the concentration is increased it will react with NO to form ONOO-, a reaction that is much more rapid than the dismutation of $\cdot O_2$- by SOD [44]. Thus, the lack of changes in SOD activity observed herein could be a result of the reaction of $\cdot O_2$- with NO. However, this is questionable as plasma nitrites, which were within the suggested range for human plasma (55-210 nmol $\cdot$ L^{-1} [42]), were unaffected by the different PiO_2 exposures *(Table 4)*. Erythrocytes, which contain eNOS, are the primary storage site for nitrites/nitrates [40]. Although we did not measure erythrocyte nitrites, had there been an increased production one would have expected NO release, causing a corresponding elevation in plasma nitrites [39]. ADMA levels were also unchanged, suggesting that NOS activity was not impaired. Oddly, L-Arg was increased following the 1 and 1.5 atm abs exposures, which resulted in a similar increase in the ratio of L-Arg/ADMA. Moreover, there was a negative PiO_2 dose-response relationship on the relative changes in the ratio of L-Arg/ADMA one hour following the hyperoxic exposures. These data could imply, contrary to our hypothesis, that had the PiO_2 level been greater or the duration of exposure longer, an inhibition in NO production may have occurred. In support of this hypothesis, Akgul *et al.* [22] reported no changes in L-Arg, ADMA or NO in the brain of rats exposed to either room air, or a PiO_2 of 1 or 2 atm abs over two hours; however, at 3 atm abs they determined NO levels to be significantly reduced. Others who have examined the effects of HBO_2 therapy (2.4 atm abs over 75-90 minute) on wound healing also did not report significant changes in plasma L-Arg, and/or ADMA and NO [45,46]. Taken together, these data do not support an increased production of NO in the blood following two-hour hyperoxic exposures of 1-2 atm abs.

There were, however, limitations to the present investigation that warrant comment. First, the experimental design relied upon the pre-condition values to serve as a within- and between-group control. Although this is routinely practiced in human investigations it could be argued, based on the within-individual variability that was observed, that a room air control group would be preferential for establishing whether changes are attributable to either the PiO_2 level or time. Secondly, subjects underwent the different PiO_2 exposures separated by one week and were encouraged to maintain a similar lifestyle (*i.e.*, diet and activity) between successive treatments, and consume a diet low in L-Arg, nitrites and nitrates immediately before and during each test day. Upon review of the subjects' dietary intake, no differences were observed in L-Arg, nitrites and nitrates prior to each PiO_2 exposure, which was confirmed by a one-way repeated measures ANOVA ($p>0.05$). In addition, there was no significant correlation between pre-dietary intake of L-Arg, nitrites and nitrates and the post-exposure plasma L-Arg ($r = 0.035$, $p=0.73$) and nitrites ($r = 0.107$, $p=0.28$). Thus, it would appear that diet did not influence plasma L-Arg, the ratio of L-Arg/ADMA and nitrites. Finally, as a result of the diving experience of the subjects varying considerably, one could question whether the senior divers in our subject population may have developed an adaptation to elevated PiO_2s, similar to the exercise-induced oxidative stress hormesis theory [47]. This, however, is also unlikely, as Groger *et al.* [14] observed no differences in baseline blood antioxidant enzyme activity between non-diving non-endurance-trained and non-diving endurance-trained ($\dot{V}O_{2\ max}$, 74 $mL \cdot min^{-1} \cdot kg^{-1}$) controls, and Underwater Demolition Team (UDT) divers and combat swimmers. Therefore, the disparate oxidant-antioxidant responses between plasma and erythrocytes, and PiO_2 levels reported herein likely cannot be explained by diving experience alone.

In summary, a two-hour hyperoxic exposure to a PiO_2 from 1 to 2 atm abs results in minor elevations in lipid peroxidation, mixed responses in SOD, CAT and GPx activity, and an unaltered production of NO within blood. In addition, the majority of oxidative stress markers did not show a significant PiO_2 dose-dependent relationship at any of the post-exposure time points. In conclusion, a two-hour hyperoxic exposure at PiO_2 levels of 1, 1.5 and 2 atm abs causes subtle oxidant-antioxidant responses in plasma and erythrocytes of healthy males that is not strongly related to the hyperoxic dose. These data describe the changes in oxidative stress and antioxidant enzyme activity, as well as NO production, that occur from PiO_2 exposures of 1-2 atm abs in the blood of humans. However, it should be noted that enzyme activity in other tissues, e.g., lungs and brain, may respond differently to this level of hyperoxic stress. ■

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