Competitive apnea diving sessions induces an adaptative antioxidant response in mononucleated blood cells

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Competitive apnea diving sessions induces an adaptative antioxidant response in mononucleated blood cells

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Abstract The aim was evaluating the effects of hypoxia/reoxygenation repetitive episodes during 5 days of apnea diving (3-day training/2-day competition) on peripheral blood mononuclear cells (PBMCs) antioxidant defenses, oxidative damage, and plasma xanthine oxidase activity. Blood samples, from seven professional apnea divers, were taken under basal conditions the previous morning to the first training session (pre-diving basal), 4 h after ending the competition (4 h post-diving) and the following morning (15 h after last dive) in basal conditions (post-diving basal). Glucose levels significantly decreased whereas triglycerides increased at 4 h post-diving, both returning to basal values at post-diving basal. Glutathione reductase and catalase activity significantly increased after 4 h post-diving remaining elevated at post-diving basal. Glutathione peroxidase and superoxide dismutase activities and catalase protein levels progressively increased after diving with significant differences respect to initial values at post-diving basal. No significant differences were observed in circulating PBMCs and oxidative damage markers. Plasma xanthine oxidase activity and nitrite levels, but not the inducible nitric oxide synthetase, significantly increased 4 h post-diving, returning to the basal values after 15 h. In conclusion, chronic and repetitive episodes of diving apnea during five consecutive days increased plasma xanthine oxidase activity and nitric oxide production which could enhance the signalling role of reactive oxygen and nitrogen species for PBMCs antioxidant adaptation against hypoxia/reoxygenation.

Keywords Dive · Exercise · Hypoxia · Oxidative stress · Xanthine oxidase

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Introduction

Diving apnea subjects the organism to successive, intermittent episodes of hypoxia and reoxygenation. The succession of episodes of reduced oxygen supply to tissues and reoxygenation may be responsible for an enhanced production of oxygen free radicals, resulting in cellular damage [8]. Accordingly, cellular hypoxia and reoxygenation participate in injury caused by ischemia/reperfusion and it has been related to pathogenesis in human diseases such as sleep apnea, circulatory shock, myocardial ischemia, stroke, and organ transplantation [23, 25]. Cellular antioxidant systems have demonstrated a great adaptation to chronic exercise to avoid the oxidative damage caused by reactive oxygen species (ROS) produced during exercise [13, 24]. In

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a previous study, we evidenced that chronic episodes of diving apnea produce an adaptation in neutrophils characterized by a delay in the oxidative burst response and by an increased thioredoxin reductase and glutathione reductase in order to facilitate protein reduction [30]. Moreover, other studies reported that blood acidosis and plasma oxidative stress were reduced after 3 months of apnea training [17]. The lymphocyte antioxidant defenses have shown adaptations to exercise-induced oxidative stress [34]. It has been reported that the expression of the antioxidant enzymes in lymphocytes is induced and regulated by ROS [20, 33]. However, this adaptative response of lymphocyte antioxidant defenses did not avoid the oxidative damage in cellular components when the exercise is exhaustive [37].

Oxygen availability to the hypoxic tissues is mediated by the vasodilator effects of nitric oxide (NO) [18]. NO is a major signalling and effector molecule mediating the response to hypoxia, because of its characteristics of vasodilatation (improving blood flow and oxygen supply) and modulation of energetic metabolism (reducing oxygen consumption and promoting utilization of alternative pathways) [39]. NO mainly synthesized in endothelial cells by NO synthetases (NOS) diffuses to the lumen where it executes its signalling function, and it also could be eliminated by its oxidation by oxygen, resulting in the formation of nitrite [35]. Nitrite can be reduced to NO by heme- or pterin-based enzymes, and NO also can be released from stores in hypoxic conditions [39]. This NO generation along the physiological oxygen gradient suggests a central role in vasodilatation responses to hypoxia [5, 12]. Peripheral vasodilatation could reduce the generation of gas bubbles in the main vessels minimizing the pernicious effects of the decompressive sickness. It was evidenced that pre-dive application of NO donor protects against bubble formation in rats and in experienced divers [9, 41].

Skeletal muscle is the major source of ROS production during exercise. However, other tissues such as the heart, lungs, or blood cells also contribute to the total body generation of ROS during exercise. Xanthine oxidase oxidizes hypoxanthine to produce xanthine and generate superoxide radicals [26]. Additionally, xanthine oxidase is able to reduce nitrite to NO in acidic pH contributing to peripheral vasodilatation [4]. The activation of xanthine oxidase could also be important in the generation of free radicals during exhaustive physical exercise in a way similar to that occurs in episodes of ischemia–reperfusion [40]. Moreover, it was reported that allopurinol (an inhibitor of xanthine oxidase) reduces cellular damage and inhibits gene expression associated with exhaustive exercise [14, 15].

We hypothesize that the repetitive episodes of hypoxia/reoxygenation in elite divers could activate plasma xanthine oxidase and consequently induce an adaptation in peripheral blood mononuclear cells (PBMCs), attenuating the post-dive oxidative stress. The aim of the present study was to evaluate the effects of repetitive sessions of diving apnea during a competition on PBMCs antioxidant defenses and nitric oxide production as well as on markers of cellular oxidation.

Materials and methods

Subjects and experimental procedure

Seven voluntary male professional fish hunters volunteered to participate in this study (age 30.0± 2.1 years old, weight 72.8±1.9 kg, and height 173± 4.9 cm). The divers also reported a systolic blood pressure of 122 mmHg and a diastolic blood pressure of 75 mmHg and a mean heart rate of 65 beats/min in basal conditions. The years of training ranged between 5 and 20 years, with 3–4-h diving five times a week. Four of the participants were winners of the Balearic Islands in spearfishing; three were European winners and two world winners. Subjects were informed of the purpose of this study and the possible risks involved before giving their written consent to participate. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by the Ethical Committee of Clinical Investigation of the Balearic Islands. The participants performed 3 days of hard training and 2 days of competition (Spanish Championship of Fish-hunters, Pollença, Spain). The characteristics of a training day and the competition days monitored with a dive computer are reported in Table 1. Briefly, the training sessions were performed at lower depth and with low immersion rate than the competition in order to avoid an excess of fatigue. The first day of competition consisted of 4-h fishing where divers expended more than 2 h in apnea, performing 105 immersions. The second day of competition was shortened because of the bad weather and only lasted 2 h with 43 immersions. All together, the competition plus the training days submitted the divers to about 6.5 h of apnea and more than 325 immersions.

Table 1 Characteristics of the diving apnea sessions

	Training day	Competition day 1	Competition day 2
Total apnea session time (min)	194±24	250±11	118±6
Number of immersions	58.4±8.8	105±24	43.0±11.0
Mean immersion depth (m)	19.6±0.7	25.2±2.2	26.0±4.6
Total immersion time (min)	75.4±4.3	121±7	44.6±6.5
Total surface time (min)	119±21	128±9	73.0±9.2

Characteristics of one representative training day and the two competition days. Means±SEM

Venous blood samples were taken from the antecubital vein with suitable vacutainers with EDTA as anticoagulant. Samples were taken the morning previous to the beginning of the training sessions (basal pre-diving competition) after overnight fasting, 4 h after ending the competition (4-h post-diving competition), and 15 h after ending competition in the following morning in basal conditions (basal post-diving competition). Blood samples were used to purify PBMCs and to obtain plasma [35]. Hematological parameters were determined in an automatic flow cytometer analyzer Technicon H2 (Bayer) VCS system. Biochemical parameters were determined by standardized clinical methods using an autoanalyzer DAX-72 (Technicon, Bayer). Antioxidant enzyme activities, markers of oxidative damage, and nitrite production were determined in PBMCs. Xanthine oxidase activity was determined in plasma.

PBMCs enzyme activities

Antioxidant enzyme activities were determined in PBMCs with a Shimadzu UV-2100 spectrophotometer at 37 °C. Catalase activity was measured by the spectrophotometric method of Aebi using H_2O_2 as substrate [1]. Glutathione peroxidase (GPx) activity was measured using the spectrophotometric method of Flohé and Gunzler [10]. This assay required H_2O_2 and NADPH as substrates and glutathione reductase as an enzyme indicator. Glutathione reductase (GRd) activity was measured by the Goldberg and Spooner (1984) spectrophotometric method using oxidized glutathione as the substrate. Superoxide dismutase (SOD) activity was measured using a xanthine/xanthine oxidase system to generate the superoxide anion [11]. This anion produced the reduction of cytochrome C, which was monitored at 550 nm. The superoxide dismutase in the sample removed the superoxide anion and produced an inhibition of the cytochrome c reduction.

PBMCs oxidative stress markers

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed in PBMCs using a colorimetric assay specific for MDA determination. Briefly, samples and standards were placed in glass tubes containing the chromogenic agent *n*-methyl-2-phenylindole (10.3 mM) in acetonitrile/methanol (3:1). HCl (12 N) was added, and the samples were incubated for 1 h at 45 °C. The condensation of one molecule of MDA with two molecules of this chromogen yields a stable chromophore with maximal absorbance at 586 nm. MDA concentration was calculated using a standard curve of known concentration.

Protein carbonyl derivates were measured by an adaptation of the method of Levine [22]. Samples deproteinized with trichloroacetic acid were resuspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM and incubated for 60 min at 37 °C. Then, samples were precipitated with 20 % trichloroacetic acid and centrifuged for 10 min at 1000×*g* at 4 °C. The precipitate was washed twice with ethanol–ethyl acetate (1:1; *v*/*v*) to remove free DNPH. Guanidine 6 M, in phosphate buffer 2 mM, pH 2.3, was added to the precipitate, and samples were incubated for 40 min at 37 °C and absorbance was measured at 360 nm. The molar absorption of 22,000 M^{-1} cm^{-1} was used to quantify the levels of protein carbonyl.

PBMCs nitrite determination

Nitrite levels were determined in PBMCs by the acidic Griess reaction using a spectrophotometric method [35]. Cell samples were centrifuged for 10 min at 900×*g* at

4 °C. Supernatants were collected and deproteinized with acetone and kept overnight at −20 °C. Samples were centrifuged for 10 min at 15,000×*g* at 4 °C, and supernatants were recovered. A 96-well plate was loaded with the samples or standard nitrite solutions (100 μl) in duplicate. Fifty microliters sulfanilamide (2 %*w*/*v*) in 5 % HCl was added to each well, and 50 μl *N*-(1-napthyl)-ethylenediamine (0.1 % *w*/*v*) in water was then added. Absorbance was measured at 540 nm following an incubation of 30 min. Nitrite concentration was calculated with a standard curve of known concentration.

iNOS and catalase protein determination

Inducible nitric oxide synthase (iNOS) and catalase protein levels were determined in PBMCs by ELISA using polyclonal antibody anti-human iNOS (Stressgen) and anti-human catalase (Calbiochem) [35]. Suitable dilutions of the PBMCs suspensions and iNOS or catalase standard were placed in each well of the plate per duplicate (polystyrene assay plate, Costar). The plate was incubated at 37 °C for 3 h. A solution of 1 % bovine albumin was added into each well, and the plate was incubated (37 °C for 3 h) to saturate all binding protein sites. The plate was then washed four times with NaCl 0.9 %–Tween 20. The commercial antibody (diluted 1000-fold) was placed into each well, and the plate was newly incubated for 3 h at 37 °C. The plate was then washed as above. The secondary antibody against the IgG chain, conjugated to alkaline phosphatase (diluted 500-fold), was placed into each well and the plate was incubated in the same conditions as above. The wells were newly washed and the phosphatase substrate solution was added. Finally, absorbance was measured at 405 nm.

Determination of xanthine oxidase activity

Amplex® Red Xanthine/Xanthine Oxidase Assay Kit (Molecular Probes) was used to determine xanthine oxidase activity in human plasma as described by the manufacturer.

Statistical analysis

Statistical analyses were carried out using a statistical package for social sciences (SPSS 19.0 for windows). Results were expressed as means±SEM and $p<0.05$ was considered statistically significant. Kolmogorov–Smirnov normality test revealed that the data for all the variables was normally distributed. One-way ANOVA was then used to determine the significance of changes in all parameters measured.

Results

The effects of diving apnea on hematological and biochemical parameters are reported in Table 2. The only parameters significantly affected by scuba diving were the levels of glucose and triglycerides. Glucose levels significantly decreased whereas triglycerides increased at 4 h post-diving. Both metabolites returned to basal values at basal 15 h post-diving.

Changes in PBMCs counts, antioxidant enzymes, and oxidative damage markers are presented in Table 3. No significant differences were observed in circulating PBMCs and markers of oxidative damage (MDA and protein carbonyl derivates). All enzymatic activities as well as catalase protein levels progressively increased during the competition. The differences were significant at 4 h post-diving competition for catalase and GRd, while all measured activities and catalase protein levels were significantly increased respect to initial values at basal 15 h post-diving.

PBMCs iNOS protein levels and nitrite production are reported in Table 4. No significant effects induced by scuba diving were evidenced in PBMCs iNOS protein levels. Nitrite levels, as a marker of nitric oxide production, significantly increased after diving, returning to the basal values at 15-h recovery in the following day after end the competition.

Plasma xanthine oxidase activity is presented in Fig. 1. The enzymatic activity was significantly increased at 4 h post-diving (98 % increase, $p=0.006$). The xanthine oxidase activity returned to basal values at basal 15 h post-dive.

Discussion

Repetitive sessions of diving apnea during consecutive 5 days combine successive episodes of hypoxia and reoxygenation and the practice of physical activity. This activity results in increased nitrite levels and plasma xanthine oxidase activity and in the activation of PBMCs antioxidant defenses. It is well established that splenic contraction associated with apnea is a

Table 2 Effects of diving apnea on hematological and biochemical parameters

	Basal pre-diving competition	4-h post-diving competition	Basal 15-h post-diving competition	*p* value
Erythrocytes (10^6/μl)	4.93±0.07	5.02±0.09	4.86±0.13	p=0.561
Hemoglobin (g/dl)	14.7±0.1	14.9±0.2	14.3±0.3	p=0.365
Hematocrit (%)	42.8±0.4	43.5±0.6	42.3±0.8	p=0.471
Glucose (mg/dl)	92.0±2.8	76.0±8.3*	94.4±2.9	p=0.045
Triglycerides (mg/dl)	68.7±8.8	101±16*	50.2±6.9	p=0.033
Total cholesterol (mg/dl)	180±6	175±8	174±9	p=0.810
HDL-C (mg/dl)	60.0±3.1	55.1±2.0	56.8±2.5	p=0.490
LDL-C (mg/dl)	106±5	99.1±8.1	107±9	p=0.713

Hematological and biochemical parameters in plasma samples of professional divers (n=7) before (basal) and 4 and 15 h after apnea diving sessions (one-way ANOVA). $p<0.05$; asterisks indicate differences with respect to the basal pre-diving competition

physiological response that causes an increase in hemoglobin concentration and hematocrit, promoting a prolonged breath-holding [27]. In the present results, the increment in both parameters was not statistically significant. The divers that participate in the study are professionally well trained and perform many dives along the year, and this could minimize the hematological response to apnea diving even the time when the blood samples were taken after diving (4 and 15 h after diving) could also be responsible of the lack of differences. Glucose levels were significantly reduced whereas triglycerides were increased after continuous apnea diving in order to mobilize the energetic reserves to maintain the performance all over the competition day.

Xanthine oxidase activity was increased after diving suggesting a role in the instauration of oxidative stress during the apnea diving. However, xanthine oxidase-derived ROS formed during exhaustive exercise such as a marathon race are also responsible for nuclear factor-κβ (NFκβ) activation because the treatment with allopurinol prevents NFκβ activation and the subsequent upregulation of the expression of antioxidant enzymes [15]. In accordance, this xanthine oxidase activation could participate in the PBMCs antioxidant adaptation induced by repetitive sessions of apnea diving. Moreover, it was reported that the prevention of ROS formation by inhibition of xanthine oxidase

Table 3 Effects of diving apnea on peripheral blood mononuclear cell counts, antioxidant enzymes, and oxidative damage markers

	Basal pre-diving competition	4-h post-diving competition	Basal 15-h post-diving competition	*p* value
PBMCs (10^3/μl)	2.22±0.16	2.51±0.14	2.40±0.18	p=0.094
Oxidative damage markers				
MDA (μmol/10^9 cell)	2.77±0.24	3.37±0.22	2.68±0.25	p=0.116
Carbonyl derivates (μmol/10^9 cell)	20.8±2.1	23.4±3.4	23.3±2.6	p=0.299
Antioxidant enzyme activities				
Catalase (K/10^9 cell)	14.0±1.2	17.8±1.1*	20.4±1.0*	p=0.002
GPx (nKat/10^9 cell)	34.6±1.7	38.0±1.6	41.9±0.9*	p=0.007
GRd (nKat/10^9 cell)	29.9±2.4	40.4±4.7*	39.8±3.1*	P<0.000
SOD (pKat/10^9 cell)	15.9±1.2	17.5±1.2	19.6±1.4*	p=0.004
Antioxidant enzyme protein levels				
Catalase (ng/10^6 cell)	36.5±1.8	41.3±2.3	46.6±1.9*	p=0.004

Effects of diving apnea on peripheral blood mononuclear cell counts, antioxidant enzymes, and oxidative damage markers in plasma samples of professional divers (n=7) before (basal) and 4 and 15 h after apnea diving sessions (one-way ANOVA). $p<0.05$; asterisks indicate differences with respect to the basal pre-diving competition

Table 4 Effects of diving apnea on lymphocyte iNOS levels and nitrite production

	Basal pre-diving competition	4-h post-diving competition	Basal 15-h post-diving competition	*p* value
iNOS (ng/10^6 cells)	142±16	150±11	146±15	p=0.934
Nitrite (nmol/10^9 cells)	37.8±1.3	46.9±3.0*	39.6±2.2	p=0.028

Effects of diving apnea on lymphocyte iNOS levels and nitrite production in plasma samples of professional divers (n=7) before (basal) and 4 and 15 h after apnea diving sessions (one-way ANOVA). $p<0.05$; asterisk indicates differences with respect to the basal pre-diving competition

with allopurinol protects against cell damage caused by exhaustive exercise [15]. ROS are produced during exercise and also during apnea diving work as signals that regulate cellular adaptations to exercise but also could be responsible of causing cause oxidative damage acting as a double-edged sword.

The increased ROS production associated to repetitive episodes of hypoxia-reoxygenation could induce an antioxidant response in PBMCs in order to avoid oxidative damage. It was reported that increased ROS production induces the synthesis of antioxidant enzymes such as SOD in lymphocytes [21, 36]. In a previous study, we evidenced that an acute session of apnea diving during 4 h is able to increase SOD activity in lymphocyte after 1 h of recovery [32]. Changes in antioxidant system were also obtained in previous studies after static and dynamic apneas [3, 16, 17, 28]. In addition, scuba diving which is characterized by exercising in hyperoxia increases the mRNA levels of genes related with the inflammatory and immune response in neutrophils [31]. However, in the present study the stimulus is greater as the divers performed around 300 immersions during the 5 days monitored, resulting in a significant antioxidant response. Catalase activity was increased 4 h post-diving competition, and this increase continued raising 15 h post-diving competition. Moreover, the increase in the enzymatic activity was supported by an increased catalase protein levels indicating the activation of the biosynthetic machinery in response to the repetitive apnea dives. GRd also reported a significant increase evidenced at 4- and 15-h post-diving competition. GRd is important for maintaining the viability of cells because it recycles glutathione in order to maintain the cellular redox status. GSH is the most abundant cellular thiol, and it is involved in several metabolic processes including a central role in the detoxification of ROS [7].

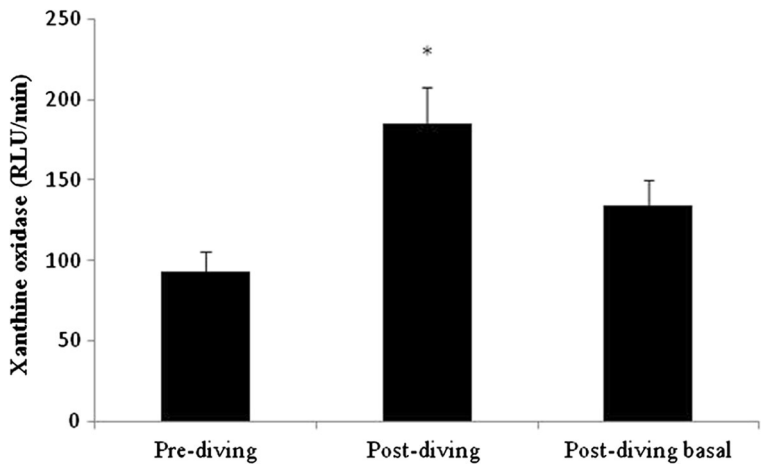

Fig. 1 Effects of diving apnea on xanthine oxidase activity in plasma samples of professional divers (n=7) before (pre-diving) and 4 (post-diving) and 15 (post-diving basal) hours after apnea diving sessions (one-way ANOVA). $p<0.05$; *asterisk* indicates differences respect to the basal pre-diving competition

The present results showed that PBMCs antioxidant response is enough to avoid cellular damage as it was evidenced with the lack of changes in MDA and protein carbonyl derivates.

Cellular nitrite concentration has been used as an indicator of NO production [6]. Nitric oxide synthesized by endothelial NOS plays an essential role in the regulation of vascular tone and blood flow. Nitric oxide could be also synthesized from nitrite through its reduction by enzymes such as xanthine oxidase or hemoglobin in hypoxic conditions [4]. PBMCs present the inducible isoform of NOS (iNOS) which produces high levels of NO when cells are activated. NO produced by PBMCs has a role as a toxic defense molecule against infectious organisms but also regulates the functional activity, growth, and death of many immune cells. In accordance, it has been demonstrated that NO upregulates SOD expression and activity and induced anti-apoptotic effects in human neutrophils [19, 30]. In a previous study, diving to 20-m depth to a cumulative breath-hold time of approximately 20 min over an hour resulted in significant increases of NO and peroxynitrite, whereas thiols (R-SH) were significantly reduced [38]. The increased NO production after diving could be related to the PBMCs antioxidant response in order to minimize oxidative damage which was evidenced with a significant increase in antioxidant enzymes at basal post-diving. Moreover, it was reported that NO not only exerts cytotoxic effects but also mediates gene expression by modulating several signal transduction cascades increasing the expression of antioxidant enzymes. [19, 29]. However, the iNOS protein levels were unchanged at post-diving indicating that the increased NO production derivates from a direct activation of the enzyme or from other source such as the nitrate reduction to nitrite by buccal anaerobic bacteria [2] and posterior reduction of nitrite by xanthine oxidase.

In conclusion, repetitive episodes of diving apnea during five consecutive days result in PBMC-enhanced antioxidant defense adaptation mainly evidenced 15 h after the last dive that could protect these cells from the oxidative stress generated by chronic hypoxia/reoxygenation. Xanthine oxidase could play a role in increasing reactive species during apnea diving and in promoting the activation of cellular antioxidant mechanisms during the recovery period.

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