Original articles

Oxidative stress in breath-hold divers after repetitive dives

Sigrid Theunissen, Nicola Sponsiello, Miroslav Rozloznik, Peter Germonpré, François Guerrero, Danilo Cialoni and Costantino Balestra

Abstract

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Introduction: Hyperoxia causes oxidative stress. Breath-hold diving is associated with transient hyperoxia followed by hypoxia and a build-up of carbon dioxide (CO_2), chest-wall compression and significant haemodynamic changes. This study analyses variations in plasma oxidative stress markers after a series of repetitive breath-hold dives.

Methods: Thirteen breath-hold divers were asked to perform repetitive breath-hold dives to 20 metres' depth to a cumulative breath-hold time of approximately 20 minutes over an hour in the open sea. Plasma nitric oxide (NO), peroxinitrites ($ONOO^-$) and thiols (R-SH) were measured before and after the dive sequence.

Results: Circulating NO significantly increased after successive breath-hold dives (169.1 ± 58.26% of pre-dive values; P = 0.0002). Peroxinitrites doubled after the dives (207.2 ± 78.31% of pre-dive values; P = 0.0012). Thiols were significantly reduced (69.88 ± 19.23% of pre-dive values; P = 0.0002).

Conclusion: NO may be produced by physical effort during breath-hold diving. Physical exercise, the transient hyperoxia followed by hypoxia and CO_2 accumulation would all contribute to the increased levels of superoxide anions (O_2^{2-}). Since interaction of O_2^{2-} with NO forms $ONOO^-$, this reaction is favoured and the production of thiol groups is reduced. Oxidative stress is, thus, present in breath-hold diving.

Key words

Freediving, breath-hold diving, hyperoxia, free radicals, nitric oxide, exercise

Introduction

At the end of the dive, breath-hold diving may result in hypoxia/hypercapnia, where alveolar oxygen partial pressure can be as low as 20–30 mmHg and arterial oxygen saturation around 50%.[1] Moreover, in order to increase the maximal breath-hold time, some divers perform hyperventilation, which reduces the partial pressure of carbon dioxide (PCO_2) before breath-holding and delays the urge to breathe. This procedure increases the risk of a hypoxic event during the dive. Whilst breath-hold divers may experience hypoxia during the ascent, the compression phase of the dive is associated with the reverse, hyperoxic conditions, because of increasing hydrostatic pressure leading to reduction of the intra-pulmonary gas volume and compression of the chest wall (Boyle's Law).[2] This, in turn, induces a reduction of cardiac output during the dive.[3] Oxidative stress has also been involved in cardiovascular pathologies and hypertension.[4–6]

In a previous study, decreased flow-mediated dilatation (FMD) of the brachial artery was observed after a series of breath-hold dives.[6] Interestingly, circulating nitric oxide (NO) was increased in these conditions. It was hypothesised that the production of reactive oxygen species (ROS) was increased during breath-hold diving, and that these ROS react with nitric oxide (NO) to form peroxinitrites ($ONOO^-$) which, in turn, reduce the ability of NO to achieve vasodilation.[6] The increased NO production could be due to physical exercise.[7] Different factors can be involved in the increased oxidative stress, including transient hyperoxia followed by hypoxia and CO_2 build up and/or an impaired tetrahydrobiopterin bioactivity, as found in hypertension-induced endothelial dysfunction.[8] Alternatively, in the absence of oxidative stress, a decreased FMD could result from alterations in cardiovascular function and autonomic control.[9]

The aim of this study was to observe whether oxidative stress markers are increased in breath-hold diving in order to confirm/refute the hypothesis that NO reacts with ROS to form $ONOO^-$ leading to a decrease in its bioactivity.

Methods

STUDY POPULATION

After written informed consent and institutional ethics committee approval (B200-2009-39), 13 non-smoking, experienced (at least four years of experience) male breath-hold divers volunteered for the study. Prior to entering the study, they were assessed as fit to dive by a qualified diving physician. None of the subjects had a history of previous cardiac abnormalities and none of them was on any cardio-active medication. All participants were asked to refrain from performing strenuous exercise for 48 hours before testing. For all the subjects, the daily diet was controlled by a medical nutritionist (author NS), avoiding nitrate-rich

food. All blood samples were drawn from an antecubital fossa vein during the morning and the last meal was dinner more than 10 hours before. The study was conducted in accordance with the Helsinki Declaration.

DIVE PROFILE AND TIMELINE OF MEASUREMENTS

Each breath-hold diver performed successive dives to a depth of 20 metres' sea water (msw) for a cumulative breath-hold time of approximately 20 minutes. Dives were organised in pairs allowing each diver to act as the safety buddy for the other (the thirteenth diver buddied with a safety diver). The total time in the water was approximately one hour. All dives were performed (after breathing air) in the open sea off Santa Maria di Leuca, Italy. Water temperature at the surface was approximately 30°C, and 18°C at depth. Air temperature was 35°C. A 3 mm wetsuit was used by all divers.

MEASUREMENTS

Blood samples were collected on-shore one hour before diving and soon after the dives, on returning to the dive centre 15 minutes away from the dive site by boat. The samples were drawn into an EDTA tube and centrifuged according to the protocol (1,000 rpm for 15 min for the NO, 3,500 rpm for 10 min for the $ONOO^-$ and 13,400 rpm for 15 min for the R-SH at 4°C) in order to separate blood cells and plasma. The plasma was then stored at -80°C in a fridge installed specifically for this purpose in the diving centre. All analyses were performed within the following six months on the same microplate (one for each test) in order to analyse all the samples at the same time and avoid variance bias.

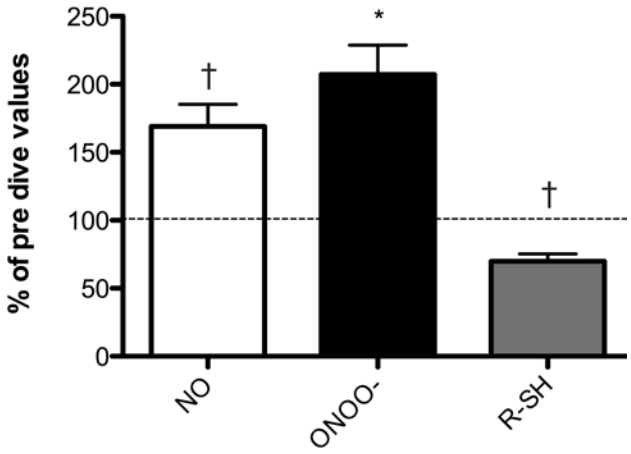
Plasma levels of nitrite and nitrate, NO metabolites, were determined by a colorimetric method (Fluka, Industriestrasse 25CH-9471, Buchs, Switzerland) according to the manufacturer's instructions. Peroxinitrites were measured using the OxiSelect™ Nitrotyrosine ELISA kit. The plasma samples or nitrated bovine serum albumin (BSA) standards were first added to a nitrated BSA preabsorbed EIA plate. After a brief incubation, an anti-nitrotyrosine antibody was added, followed by a horseradish peroxidase-conjugated secondary antibody. The protein nitrotyrosine content in the samples was determined by comparing with a standard curve prepared from predetermined nitrated BSA standards.

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.[10] The assessment of thiols (R-SH) in plasma was performed by the microanalysis method of Ellman.[11]

STATISTICAL ANALYSIS

Statistical analyses were conducted using GraphPad Prism 5 (La Jolla, CA, USA) on a PC. Data are expressed as a percentage of pre-dive values. The difference between the percentage of pre-dive values and 100% was compared by a two-tailed one-sample t-test when normality of the sample was reached as assessed by the Kolmogorov-Smirnov test. Otherwise, the Wilcoxon Signed Rank test was used. Statistical significance level was set at $P < 0.05$.

Figure 1
Evolution of nitric oxide (NO), peroxynitrite ($ONOO^-$) and thiols (R-SH) after repetitive breath-hold dives to 20 msw with a cumulative submergence time of 20.6 +/- 1.5 min; results are expressed in percentage of pre-dive values
(* P = 0.0012; † P = 0.0002)



Results

The divers (age 29 ± 4.7 years; height 176.5 ± 4.4 cm; weight 73.9 ± 4.6 kg) performed an average of 9 ± 2 dives with a mean cumulative breath-hold time of 20.6 ± 1.5 minutes. The mean individual duration time in the water was 61.47 ± 8.17 minutes. The mean surface interval between successive breath-hold dives was 4.5 ± 1.45 min. Variations in recovery time were dependent on the breath-hold times, which varied amongst the pairs of divers. All divers completed the study and no one developed symptoms of decompression sickness.

The plasma concentration of nitrate and nitrite, a marker of circulating NO, significantly increased after the dives (169.1 ± 58.26% of pre-dive values; P = 0.0002; Figure 1).

Nitrotyrosine, a marker of peroxynitrite level, doubled after the dives. (207.2 ± 78.31% of pre-dive values; P = 0.0012; Figure 1). Thiols were significantly reduced after the dives (69.88 ± 19.23% of pre-dive values; P = 0.0002; Figure 1).

Discussion

We observed an increase in circulating NO and peroxinitrites ($ONOO^-$) concommittant with a decrease in thiols (R-SH). NO derives from the transformation of L-arginine into L-citrulline and NO by nitric oxide synthase. This reaction needs five electrons and is dependent on NADPH

as the electron donor. It is the most important vasoprotector identified thus far. Some of the NO formed in endothelial cells is released into the circulation. Because of its affinity for erythrocytic haemoglobin, circulating NO undergoes multiple reactions with haemoglobin that lead to a decrease in its bioavailability in the vascular compartment.[12]

NO reacts and interacts with ROS, and this crosstalk can also have important effects on cardiac function.[13] To our knowledge, there are limited data on the time course of vascular alterations after breath-hold diving.[14] In scuba diving, decreases in intravascular volume and cardiac preload have been reported commonly after diving.[15] This is concomitant with a moderate increase in vascular resistance and may be the result of an inactivation of NO, probably through oxidative stress.[16] Indeed, plasma nitrite and oxidative stress markers remain altered during the 15-min recovery phase after hyperoxia.[17]

The present results confirm our previous data, which showed an increased circulating level of NO after a series of breath-hold dives.[6] This might be explained by acute moderate physical exercise, which is known to increase NO production.[7] Breath-hold divers have positive buoyancy at the surface and in the first metres of descent; physical effort is required to overcome this. When they ascend at the end of the dive, breath-hold divers have to fin upwards until the depth at which they recover their positive buoyancy.

Oxidative stress is associated with the generation of ROS, including superoxide anion (O_2^{2-}). This free radical reacts with NO to generate peroxinitrite ($ONOO^-$), which causes additional oxidative stress by increasing oxidase activity and inactivating antioxidant enzymes.[18] In this study, more $ONOO^-$ and less thiol (R-SH) were observed, which shows that oxidative stress is present during breath-hold diving.

To our knowledge, this is the first time these changes (in $ONOO^-$ and R-SH) have been reported in breath-hold divers. Hyperoxia found during the deep phase of breath-hold diving enhances the production rate of superoxide anion which is converted into hydrogen peroxide (H_2O_2) or reacts with NO to form $ONOO^-$.[19,20] Reacting rapidly with it, extracellular superoxide anions can decrease the bioavailability of NO.[21] The reduced availability of NO caused by O_2^{2-} leads to vasoconstriction and impairs NO-dependent vasodilation, which is consistent with recent findings on breath-hold divers.[6,22] Although H_2O_2 is a weak oxidant and relatively inert with most molecules, it oxidizes cysteine thiole (R-SH) groups within the protein molecule. This reversible reaction modifies their structure and function.[23] When ROS are present at physiological levels, NO reacts with proteins to form R-SH. But an increased level of O_2^{2-} facilitates its interaction with NO to form $ONOO^-$, instead of R-SH.[24] The total amount of R-SH is therefore reduced, as observed in this study. Superoxide anion also tends to react with itself. This phenomenon is termed 'dismutation' and leads to the production of water and oxygen through the action of superoxide dismutase (SOD).

Trained free divers increase SOD activity during breath-hold diving, and acute changes in antioxidant enzyme activities suggest that they may be protected from excessive antioxidant depletion and oxidative stress during breath-hold diving.[25] Suppression of the post-apnea oxidative stress by an increased concentration of thiobarbituric acid reactive substances after three months of breath-hold training has been reported.[26] In parallel, an activation of the plasma antioxidant system against oxidative stress has been reported in seals and in scuba diving.[27,28]

The increase in PO_2 during breath-hold immersion is so short-lived that it is difficult to believe that it could play a significant role. Nevertheless, it has been shown that for breath-hold divers even short hyperoxic or hypoxic periods act as a powerful trigger for physiological responses with successive breath-hold dives.[29,30] Thus, it seems that $ONOO^-$ is generated during such diving, even if hyperoxia is intermittent. This production leads to an inactivation of NO reducing the bioavailability of NO to participate in vasodilation. This may be a factor contributing to the endothelial dysfunction observed after breath-hold diving.[6]

Conclusion

After breath-hold diving, more circulating NO is observed with an increase in $ONOO^-$ and a reduction of R-SH. NO may be produced by the physical effort of breath-hold diving. Physical exercise, the transient hyperoxia followed by hypoxia and accumulation of CO_2 increase the level of superoxide anion (O_2^{2-}). This facilitates interaction of O_2^{2-} with NO to form $ONOO^-$, opposed to a production of R-SH. Oxidative stress is thus present in breath-hold diving.

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S. Theunissen[1,2], N. Sponsiello[3,4], M. Rozloznik[1], P. Germonpré[3,5], F. Guerrero[2], D. Cialoni[1,3,4], C. Balestra[1,3,4]

[1] Haute Ecole Paul-Henri Spaak, Environmental & Occupational Physiology Lab., Brussels, Belgium
[2] Université de Bretagne Occidentale, UFR Sciences et Techniques, 6 avenue Le Gorgeu, CS 93837, Brest
[3] DAN Europe Research, Brussels, Belgium
[4] DAN Europe, Apnea Task Force, Contrada PADUNE, Roseto, Italy
[5] Center for Hyperbaric Oxygen Therapy, Military Hospital Queen Astrid, Brussels, Belgium.

Address for correspondence:
Sigrid Theunissen
Environmental Occupational & Ageing Physiology Department
Pôle Universitaire de Bruxelles Wallonie
Haute Ecole Paul Henri Spaak
91 Av. C. Schaller
B 1160 Brussels, Belgium
E-mail: *<sigtheunissen@gmail.com>*