Nitric oxide-related endothelial changes in breath-hold and scuba divers

S. Theunissen1,2, F. Guerrero2, N. Sponsiello1,3,4, D. Cialoni1,3,4, M. Pieri3, P. Germonpré1,5, G. Obeid5, F. Tillmans1, V. Papadopoulou1,6, W. Hemelryck1, A. Marroni3, D. De Bels1,7, C. Balestra1,3,4

1 Haute Ecole Paul Henri Spaak, Environmental, Occupational & Aging Physiology Lab., Brussels, Belgium; 2 Université de Bretagne Occidentale, UFR Sciences et Techniques, Brest, France; 3 DAN Europe Research, Brussels, Belgium; 4 DAN Europe, Apnea Task Force, Roseto, Italy; 5 Center for Hyperbaric Oxygen Therapy, Military Hospital Queen Astrid, Brussels, Belgium; 6 Department of Bioengineering, Imperial College London, London, UK; 7 Intensive Care Department, Brugmann University Hospital, Brussels, Belgium

CORRESPONDING AUTHOR: Sigrid Theunissen M.Sc. – sigtheunissen@gmail.com

ABSTRACT

Objective: Scuba and breath-hold divers are compared to investigate whether endothelial response changes are similar despite different exposure(s) to hyperoxia.

Design: 14 divers (nine scuba and five breath-holding) performed either one scuba dive (25m/25 minutes) or successive breath-hold dives at a depth of 20 meters, adding up to 25 minutes of immersion time in a diving pool. Flow-mediated dilation (FMD) was measured using echography. Peripheral post-occlusion reactive hyperemia (PORH) was assessed by digital plethysmography and plasmatic nitric oxide (NO) concentration using a nitrate/nitrite colorimetric assay kit.

Results: The FMD decreased in both groups. PORH was reduced in scuba divers but increased in breath-hold divers. No difference in circulating NO was observed for the scuba group. Opposingly, an increase in circulating NO was observed for the breath-hold group.

Conclusion: Some cardiovascular effects can be explained by interaction between NO and superoxide anion during both types of diving ending to less NO availability and reducing FMD. The increased circulating NO in the breath-hold group can be caused by physical exercise. The opposite effects found between FMD and PORH in the breath-hold group can be assimilated to a greater responsiveness to circulating NO in small arteries than in large arteries.

INTRODUCTION

During a self-contained underwater breathing apparatus (scuba) dive, divers are exposed to various external influences, which may affect cardiovascular function. Indeed, previous studies have shown that scuba diving is associated with an increased pulmonary artery pressure [1], reduced cardiac output [2] as well as right ventricular overload, impaired left ventricular diastolic performance [3] and arterial endothelial function [4,5]. Most of these changes are still present post-dive. Several hypotheses have been proposed to explain the post-dive endothelial dysfunction such as venous gas emboli (VGE) formation acting on endothelial cells [6,7] or presence of reactive oxygen species (ROS) [8] related to the increased oxygen partial pressure. Intravascular bubbles are treated as a foreign body which has been shown to activate the complement pathway in vitro [9,10] and may lead to endothelial damage [6,7,11]. VGE are often observed during decompression following a dive, be it wet [12] or dry [13]. Brubakk et al. [5] reported that a single air dive produces endothelial dysfunction. The production of venous gas emboli has been questioned for breath-hold divers since they dive on a single breath and are not supplied with pressurized gas throughout the dive as are scuba divers [14]. It could thus be expected that in the case of very few and possibly no bubble formation while breath-hold diving, there would be no or limited endothelial dysfunction.

Sustained hyperoxia during scuba diving leads to an
increased presence of ROS which are known to have an effect on endothelial function. This can be reduced by four weeks of oral antioxidant supplementation [15]. Hyperoxia also leads to alterations in cardiovascular function and autonomic control during the exposure and after the return to normoxic breathing [16].

On the contrary, during breath-hold diving, the oxygen partial pressure increases during the deep phase, but for a limited period of time. Breath-hold diving is then associated with transient hyperoxia followed by hypoxia and a build-up of CO₂, chest-wall compression and significant hemodynamic changes.

Therefore the aim of the study is to compare scuba and breath-hold divers to investigate the effect of breath-hold diving (intermittent hyperoxia) on endothelial-dependent vasodilation, especially since a vasoconstriction can be observed during longer hyperoxia [17].

MATERIALS AND METHODS

Study population

After written informed consent and Ethics Committee approval, nine male experienced scuba divers (minimum certification “Autonomous Divers” according to ISO 24801-2 with at least 50 logged dives) and five breath-hold divers (at least four years of experience) volunteered for this pilot study. They were selected from a large sports divers population in order to obtain a consistent group for age, body composition and health status: non-smokers with regular but not excessive physical activity (aerobic exercise one to three times a week). Prior to entering the study, they were assessed on fitness to dive. None of the subjects had a history of previous cardiac abnormalities, and none of them were under any cardio-active medication.

Dive profile and timeline of measurements

All measurements were made in a pool environment (Nemo33, Brussels, Belgium) with a water temperature of 33°C, thus needing no thermal protection suit. All participants were asked to refrain from strenuous exercise for 48 hours before testing. The diet of all subjects was controlled, avoiding nitrate-rich foods [18]. All tests were done in the morning, and the last meal was about 10 hours prior to testing.

Each scuba diver performed a dive to a depth of 25 meters for 25 minutes. This depth-time profile falls within accepted “no-decompression limits” [19]. Descent speed was at 15 meters per minute; ascent speed was at 10 meters per minute to the surface, with no safety stop (none required according to the U.S. Navy dive table used).

Each breath-hold diver performed successive dives to a depth of 20 meters for a total immersion time of 25 minutes. The breath-hold divers performed their dives in pairs so that each diver also served as safety buddy for the other.

Measurements

Endothelial function

Arterial endothelial function was assessed before and after each scuba dive or series of breath-hold dives by measuring the flow-mediated dilation (FMD) of the brachial artery [20]. Since it is the relative FMD variation which is of interest here, the FMD measurement was assessed according to the methodology used by Brubbakk et al. [5] for the same kind of in-field analysis following a standardized protocol and guidelines [21]. The FMD was measured with a 5-10 MHz transducer (Mindray DP 6600, Mindray, China). The brachial artery diameter was measured from longitudinal images with the lumen–intima interface visualized on both (anterior and posterior) walls. Boundaries for diameter measurement were identified automatically by means of a boundary tracking software (FMD-I software, FLOMEDI, Belgium) and optically controlled by a researcher.

Once the basal measurements were obtained, the sphygmonanometric cuff, placed above the ultrasound probe, was inflated and held at 50 mmHg above systolic pressure for five minutes. Occlusion up to five minutes produces a transient artery dilation attributable to NO synthesis [22].

After ischemia the cuff was rapidly deflated, and the brachial artery was monitored for an additional four minutes automatically. All measurements were taken by the same experienced operator to increase consistency of measurements. The FMD was computed as the percentage change in brachial artery diameter measured at peak dilation.

Post-occlusion reactive hyperemia (PORH)

The relative dilation of small arteries was measured by post-occlusion reactive hyperemia (PORH). This is a technique which was recently demonstrated useful in measuring peripheral vascular function [23]. A plethysmographic probe (Cardiovarisc, FLOMEDI, Belgium) was placed on the index finger of both hands during the entire FMD procedure. During FMD, the
amplitude tracing of the two fingers was recorded. In the arm undergoing hyperemia, baseline amplitude was recorded. During cuff inflation, flow is occluded and restored after cuff release (hyperemic period). In the contralateral control finger, flow continues throughout and pulse amplitude undergoes minimal changes. In this test, the response of the pulse wave amplitude to hyperemia was calculated from the hyperemic fingertip as the ratio of the post-deflation pulse amplitude to the baseline amplitude.

To obtain the pulse amplitude ratio, we divided previous ratio by the corresponding ratio at the control hand as described in Kuznetsova et al. [23]. Photoplethysmography works by having an infrared light at a wavelength of 940 nm illuminate the skin and then measuring the amount of light reflected back to a photodiode, which converts it into an electrical current. The changes in light absorption measured reflect the path length that the light has to travel in the bloodstream and therefore the degree of dilation of the artery. The pulse trace was displayed and recorded.

The pulse amplitude ratio was calculated as the waveform amplitude just before occlusion, divided by the waveform amplitude at baseline. The percentage of pre-occlusion values, normalized to the magnitude of variation of the other arm, was automatically measured to avoid any environmental interference.

**Circulating nitric oxide**

Venous blood samples were collected in an EDTA tube before and after either the scuba or breath-hold dives and then immediately centrifuged at 1400g (1400 x 9.81m/s²) for 10 minutes at 4°C. Plasma samples were stored at -80°C and analyzed within the following six months. Plasma levels of nitrite and nitrate, NO metabolites, were determined by a colorimetric method (Fluka, Industriestrasse 25CH-9471 Buchs, Switzerland). The NO2/NO3 Assay Kit contains dyes, nitrate reductase, enzyme co-factor, buffer solution and NO2, NO3 solutions as standards. Total NO metabolites are thus detectable. The suitable NO2 detection range is from 10 to 100 µM. At the time of the nitrite/nitrate (NOx) assay, plasma samples were ultra-filtered through 10 kDa molecular weight cut-off filters and centrifuged at 4000g for 60 minutes at 20°C in order to remove hemoglobin, which is known to interfere with subsequent spectrophotometric measurements. NOx concentration in different dilutions of plasma ultrafiltrate was determined by colorimetry based on the Griess reaction, which consist of three main steps: 1) enzymatic conversion of nitrate into nitrite using nitrate reductase in the presence of NADPH; 2) incubation with Griess reagent to convert nitrite into a chromophore compound; and 3) quantitative estimation of nitrite concentration by spectrophotometric measurement of the absorbance at 550 nm. Standards for calibration curves were prepared with sodium nitrite and taken through the full assay procedure.

**Statistical analysis**

Statistical analyses were conducted using GraphPad Prism 5 (La Jolla, Calif., USA) on the computer. Data are given 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 non-parametric Wilcoxon Rank Sum test was used. Statistical significance level was set at p<0.05.

**RESULTS**

When comparing scuba and breath-hold divers, as far as age (40.1 ± 5.8 years vs. 34.4 ± 9.9 years), height (180.2 ± 5.2 cm vs. 182.6 ± 3.4 cm), weight (82.9 ± 10.3 kg vs. 78.5 ± 9.8 kg) are concerned; both groups are comparable. There was no significant difference in anthropometric data between groups (p>0.05).

Because of the design of the study, the structure of the immersion was different between groups. Indeed, breath-hold divers were asked to perform a series of successive dives (up to 25 minutes of total immersion time). The average number of repetition was 10 ± 0.84 dives, with an average immersion time of 25.6 minutes ± 58 seconds. The mean recovery period between successive breath-hold dives was 4.91 ± 1.31 minutes. The breath-hold divers performed their dive in pairs, allowing each diver to serve as safety buddy for the other. The variation in recovery time is therefore understandable. All divers completed the study, and no one developed symptoms of decompression sickness.

**Basal diameter of brachial artery and FMD**

In the scuba group, the mean of basal diameters (pre-occlusion diameters) of the brachial artery was significantly reduced after compared to before scuba diving (85.76 ± 14.60 % of pre dive values, which corresponds to a reduction of 14.24%; p=0.019). Similarly, a reduction of the FMD was observed.
after a scuba dive (94.26 ± 7.33 % of pre dive values which corresponds to a reduction of 5.74 %; p = 0.047). In breath-hold divers, a difference in basal diameters was observed, however non-significant, between pre and post dive (86.56 ± 24.19% of pre dive values which corresponds to a reduction of 13.44 % ; p > 0.05). Curiously, a FMd reduction was also observed after breath-hold diving (95.43 ± 3.51% of predive values which corresponds to a reduction of 4.57 % ; p=0.043). There was no difference between the scuba and the breath-hold group. Results of FMd assessment are shown in Figure 1.

**Post-occlusion reactive hyperemia (PORH)**

A reduction of PORH was observed after scuba diving (73.38 ± 26.33 % of pre-dive values, which corresponds to a reduction of 26.62 %; p=0.024), while an increase of PORH was observed after breath-hold diving (149.5 ± 28.37 % of pre-dive values which corresponds to an increase of 49.5%; p=0.017). A significant difference was observed (Figure 1) for PORH between breath-hold and scuba divers (p=0.0004).

**Circulating nitric oxide**

No significant difference in NO concentration was found before and after a single scuba dive of 25m/25 minutes (100.5 ± 35.33 % of pre-dive values, which corresponds to an increase of 0.5 %; p>0.05) but circulating NO significantly increased after a series of successive breath-hold dives (154.4 ± 21.9 % of pre-dive values which corresponds to an increase of 54.4 %; p=0.005). Unlike the FMd, which did not show any differences between the two groups (p>0.05), in the breath-hold group the percentage of circulating NO after diving compared to pre-dive was significantly higher (Figure 2) than in the scuba group (p=0.008).

**DISCUSSION**

In the current study, signs of endothelial dysfunction, indicated by a reduction of FMD, are found in both groups, and no significant difference was observed between the two groups. In scuba divers, the reduced FMD confirms the findings of other authors. (4,5,15,24,25). However in breath-hold diving the reduction of FMd is associated with an increase in circulating NO, whereas in scuba diving it is not. The mechanisms that cause endothelial dysfunction after scuba diving are not clear yet. Hyperoxia-induced oxidative stress has been evoked, since it is known that hyperoxia leads to vasoconstriction [26,27]. The generally accepted hypothesis is that the increase in oxygen partial pressure promotes oxidative stress, which is at the origin of endothelial dysfunction [26]. This is because it has been shown that hyperoxia enhances the production rate of anion superoxide [28], a powerful reactive oxygen species (ROS) which interacts with NO, leading to its destruction and to the production of...
peroxinitrite (ONOO-) [29]. Indeed, the FMD reduction after scuba diving is partly prevented by administration of vitamins C and E for four weeks before the dive [4,15, 24]. ROS not only scavenge NO and decrease NO bioavailability, but also oxidize tetrabiopterin (BH4), a major co-factor of endothelial nitric oxide synthase (eNOS) into dihydrobiopterin (BH2), which induces a reduced liberation of NO by uncoupling of eNOS [30]. An impaired BH4 bioactivity is involved in hypertension-induced endothelial dysfunction [31,32]. So, if a co-factor of eNOS is impaired, an FMD reduction will also be observed. Furthermore, oxidative stress causes a down-regulation of eNOS, which leads to a decrease of NO-production [33]. This in turn leads to reduced NO-mediated vasodilation, as assessed by decreased basal diameter (pre-occlusion) of the brachial artery and flow-mediated vasodilation.

As shown in Figure 3, flow-mediated dilation (FMD) and nitric oxide (NO) were measured before and after the dive. No variation in NO is observed after the dive. Our hypothesis is that diving at 25m for 25 minutes leads to hyperoxia, increasing the amount of superoxide anion (O2·-). O2·- reacts with NO to form ONOO-. NO is thus less available to participate in the FMD. Therefore, a reduced FMD is observed after the scuba dive.

Our data show that endothelial function is impaired in the microcirculation after a scuba dive, as indicated by a reduced PORH. However the lack of difference in nitrite/nitrate levels between pre- and post-dive values supports the assumption that the FMD reduction after scuba diving may not be due to changes in NO synthesis/release, but rather to a decreased availability of NO. This can be due to a bigger oxidative stress inactivating and/or sympathetic and parasympathetic activities as a consequence of hyperoxia. The latter leads to alterations in cardiovascular function and autonomic control during the exposure and even after coming to normoxic
breathing [16]. In addition, a decrease in volemia and cardiac preload have been commonly reported after water immersion [34] or diving [35]. Furthermore, one can hypothesize that the increased PO2 during scuba diving will moderately increase vascular resistance as shown while breathing hyperoxic mixtures [16,36,37]. A reduction of cardiac stroke volume and cardiac output as well as a moderate reduction of ejection fraction have been reported during scuba diving (38,39); all are concomitant with the reduced FMD observed after a scuba dive.

In breath-hold diving an FMD reduction was also observed after a series of successive breath-hold dives. To our knowledge, these are the first results to be reported on breath-hold divers. Decreased FMD seems to indicate that breath-hold diving acts on endothelial function. Surprisingly, opposite effects on large arteries and peripheral circulation are observed, and the FMD reduction is accompanied by an increase in circulating NO. As peripheral vessels are smaller, shear stress is more important in these vessels than in the brachial artery. Moreover, it is known that there is an inverse relationship between artery size and the magnitude of endothelium-dilation [40]. Bigger arteries like the brachial artery are less sensitive to shear stress. It is not unusual to find a different reaction between endothelial function in large arteries and post-occlusive reactive hyperaemia in the finger [41].

It is accepted that FMD is modulated by the NO production by endothelial cells [42,43]. An explanation for the increased circulating NO after breath-hold dives could be that it requires more physical effort than scuba diving. Indeed at the surface, breath-hold divers have positive buoyancy. They have to make efforts to go down during the first meters. When they come back to the surface, breath-hold divers have to fin from the bottom until they recover their neutral buoyancy. On the other hand, scuba divers are heavier and go down easily without doing exercise. To come back to the surface, scuba divers usually use their buoyancy control device and no important physical exercise.

It is known that physical exercise increases NO production [44], and under normal conditions exercise training improves endothelial function, which is directly related to an increase in NO bioavailability in the smooth muscle [45]. In this breath-hold diving experiment we could not find this effect. It is hypothesized that despite the increased NO production, exercise produces oxidative stress [46,47]. Exercise and/or (at least in part) the intermittent hypoxia and the subsequent hypoxia [48] during the dive, which both lead to a reduced availability of NO can explain this endothelial reduced response. Indeed, greater oxidative stress increases the level of superoxide anion (O2-) which interacts readily with NO to form peroxynitrite, thus reducing the availability of NO in the vascular smooth muscle (See Figure 4). The increased PO2 during breath-hold immersion is so short, that it is difficult to consider it significant; nevertheless it has been shown that for breath-hold divers even short hyperoxic [49] or hypoxic situations [50] act as a powerful trigger for physiological responses with successive breath-hold dives.

It seems thus that NO is inactivated during diving, even if hypoxia is intermittent. We hypothesize that there is oxidative stress during breath-hold diving leading to endothelial dysfunction. In a following study, oxidative stress markers (as peroxinitrites) should be measured. It is of interest to estimate the amount of ROS produced during such activities. Of course, other concomitant factors can be present, as gas bubbles may lead to endothelial damage [6,7]. But this hypothesis is less supported because breath-hold divers dive on a single inhalation [14] of atmospheric air not really prone to provoke nitrogen supersaturation. Therefore, although physical exercise may well be responsible for an increase in NO production, the concomitant hypoxia seems to have neutralized the NO, resulting in more circulating metabolites, while reducing the bioavailability of NO to participate in vasodilation.

Contrary to scuba diving, the reduction of the basal diameter of the brachial artery between pre- and post-breath-hold dives was not significant. Since PORH amplitude increases after breath-hold diving, this suggests a reduced peripheral vascular resistance, contrary to the FMD, which decreases. As circulating NO level increases after breath-hold diving, we can consider that the responsiveness to circulating NO is greater in small arteries, such as digital arteries [51], than in large arteries, chosen for FMD measurements. Indeed, Noon et al. [52] have suggested that endogenous nitric oxide production might be more important in regulating microvascular blood flow in regions like the fingertips. Furthermore, these authors administered an inhibitor of nitric oxide synthase, and nevertheless proved a reaction via laser-doppler flowmetry, showing that circulating NO might be of primary interest.
Nitric oxide (NO) and flow-mediated dilation (FMD) were measured before and after the dive. The divers had to perform successive breath-hold dives adding up to 25 minutes. An increased NO accompanied by a reduced FMD is observed after the dive. It is hypothesized that there is an increase of oxidative stress due to exercise or (at least in part) to the transient increase in PO2 during the dive and/or an inhibition of the eNOS by the subsequent hypoxia, which both lead to a reduced availability of NO. Therefore, a reduced FMD is observed after the series of breath-hold dives.

CONCLUSION
After both scuba and breath-hold diving, a decreased FMD was observed. Simultaneously, an increase in circulating NO was observed in the breath-hold diving group, whereas no such variation was observed in scuba divers. As demonstrated in several studies, scuba diving induces an increase in oxidative stress, which induces a reduced reactive vasodilation. This is not linked to NO release because no variation in NO level is observed after a scuba dive. It can thus be due to an inactivation of NO, probably through oxidative stress.

During breath-hold diving a similar reduction of FMD can be shown despite increased plasmatic NO levels. Since the surrounding conditions were comparable between the scuba and the breath-hold group, we can consider that the reduced FMD in both groups is due to a reduction of available NO at the level of vascular smooth muscle, and that the increased level of circulating NO in the breath-hold group was mainly due to the increased physical exercise compared to the scuba group. The response to exercise in the small arteries near the muscle is bigger than the effect of hyperoxia, leading to opposite effects on large arteries and peripheral circulation.

The reduction of bioavailable NO seems to be due either to the transient hyperoxia and/or to the subsequent moderate hypoxia in the breath-hold diving group. We conclude that both breath-hold and scuba diving reduce FMD but the implicated NO-dependent mechanisms are different. Nevertheless this is a study on a few breath-
hold divers, although statistical tests take into account this limited number of measurements, the results have to be viewed with caution. It would then be interesting to make this experiment again with more breath-hold subjects.

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REFERENCES


