Effects of scuba diving on vascular repair mechanisms

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ABSTRACT

A single air dive causes transient endothelial dysfunction. Endothelial progenitor cells (EPCs) and circulating angiogenic cells (CAC) contribute synergistically to endothelial repair. In this study (1) the acute effects of diving on EPC numbers and CAC migration and (2) the influence of the gas mixture (air/nitrox-36) was investigated. Ten divers performed two dives to 18 meters on Day (D) 1 and D3, using air. After 15 days, dives were repeated with nitrox-36. Blood sampling took place before and immediately after diving. Circulating EPCs were quantified by flow cytometry, CAC migration of culture was assessed on D7. When diving on air, a trend for reduced EPC numbers is observed post-dive, which is persistent on D1 and D3. CAC migration tends to improve acutely following diving. These effects are more pronounced with nitrox-36 dives. Diving acutely affects EPC numbers and CAC function, and to a larger extent when diving with nitrox-36. The diving-induced oxidative stress may influence recruitment or survival of EPC. The functional improvement of CAC could be a compensatory mechanism to maintain endothelial homeostasis.

INTRODUCTION

Healthy endothelial cells fine-tune vascular homeostasis by regulating vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation [1]. Prolonged and/or repeated damage will ultimately exhaust protective mechanisms, resulting in loss of integrity and endothelial dysfunction [2].

Circulating endothelial progenitor cells (EPCs) are recruited from the bone marrow in response to endothelial damage and differentiate into mature cells with endothelial characteristics. There is substantial evidence that EPCs mediate vascular repair and prevent the progression of atherosclerosis [3]. Circulating angiogenic cells (CACs) are derived from the monocyte-macrophage lineage and are involved in endothelial repair [4]. Although CACs have low proliferative capacity and fail to form blood vessels *in vivo*, they contribute to vascular homeostasis in a paracrine fashion by producing angiogenic cytokines [5]. Patients at risk for [6] or with established coronary artery disease [7] have lower numbers of EPCs. In addition to this quantitative deficit, dysfunction of CACs correlates with impaired endothelial function [8]. Interventions that improve endothelial function, such as physical exercise and medical therapy with statins, are considered to have a potent EPC-mobilizing effect and to increase the angiogenic capacity of CACs [9-11].

Decompression sickness (DCS) is a potential problem for a growing number of professional and recreational divers. During diving, compressed air is taken up at high pressure and saturates tissues. When divers ascend to the surface, especially after a brisk rise, this gas is partly released into the circulation and forms bubbles. The latter are commonly considered the main cause of DCS [12]. However, divers with no bubbles or a low bubble score, assessed with ultrasonic scanning, have been shown to develop DCS, whereas – conversely – divers with high bubble scores may have no symptoms.

Madden LA, *et al.* hypothesized that these gas bubbles are not the causal mechanism of DCS, but should rather be regarded as an exacerbating factor [13]. Indeed, a single scuba dive (self-contained underwater breathing apparatus) with compressed air acutely induces vascular oxidative stress, leading to transient endothelial dysfunction [14]. Breathing oxygen at higher-than-normal pressure increases the oxidant status in the circulation [15-17]. Superimposed on oxidative stress, bubbles may aggravate endothelial dysfunction by damaging the vascular endothelium through ischemia/reperfusion through physical contact with the endothelial cell layer or by an increase in shear stress. During recovery after hyperoxia, an increase of hypoxia-induced factor expression occurs in human umbilical vein endothelial cells, associated with an increase of matrix metalloproteinases activity, suggesting that endothelial cells "interpret" the return to normoxia after hyperoxia as a hypoxic stimulus [18].

The nitrogen concentration in compressed air (78%) limits bottom time, requires a longer surface interval between dives and subsequently reduces the number of dives that can be performed in a day. Other gas mixtures, such as enriched air, also called nitrox, contain higher-than-normal oxygen levels and, consequently, less nitrogen. Diving with nitrox reduces nitrogen uptake in tissues and thereby extends dive time and reduces bubble formation during decompression. On the other hand, stringent depth limits need to be respected to avoid oxygen toxicity while diving with nitrox.

In this study, the aim was to determine the acute effects of consecutive deep dives on endothelial repair mechanisms (EPC numbers and CAC migratory capacity). Secondly, the effects of air and nitrox dives by using similar no-decompression diving profiles in the same cohort of divers were compared.

METHODS

Study population

Ten male non-smoking military scuba divers participated in this study, which is part of a larger project and described in detail elsewhere [19]. All subjects were highly experienced divers with more than 1,000 hours of diving with both air and technical gases (nitrox or trimix). Each method and potential risks were explained to the participants in detail, and all subjects gave their written informed consent. The experimental procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Split School of Medicine.

Dive protocol and timeline of measurements

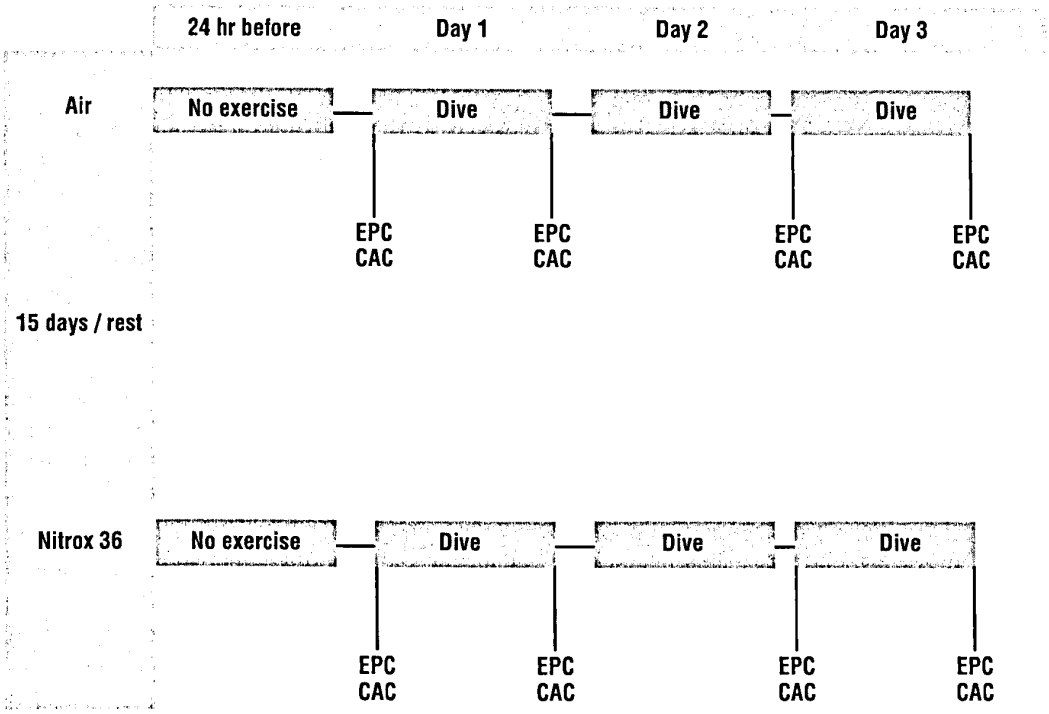
Divers performed three consecutive dives with air and three consecutive dives with nitrox-36 (36% oxygen and 64% nitrogen) as breathing gases, with two weeks' break between the air and nitrox-36 dives (Figure 1). All dives were no-decompression to 18 meters of sea water, with 47 minutes of bottom time. Water temperature was 20 ± 3°C at the surface and 16 ± 1°C at the bottom. The divers refrained from exercise 24 hours before diving since pre-dive exercise was shown to influence physiological parameters post-dive [20].

In vitro assessment of endothelial function was performed on the first and third dive of each diving series. Venous blood was sampled pre- and post-dive at an onshore diving facility. Blood samples (20 ml) were collected in acid citrate dextrose tubes. The first 3 ml of blood were discarded in order to prevent contamination with circulating endothelial cells due to vascular trauma [21]. Samples were processed immediately or stored at 4°C and analyzed within two hours.

Quantification of CD34+/KDR+/CD45- progenitor cells

The number of circulating EPCs (defined as CD34+/KDR+/CD45- cells) was determined by flow cytometry in whole blood samples. Briefly, 200 µl of whole blood was pre-treated with an FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated with anti-KDR-PE (R&D Systems, Minneapolis, USA), anti-CD34-PC5 (Beckman Coulter, Marseille, France) and anti-CD45-ECD (Beckman Coulter, Marseille, France) antibodies for 30 minutes at 4°C. A dump channel was created on CD45+ cells to exclude leukocytes. SYTO 13 (Molecular Probes, Invitrogen, Eugene, USA) was used to eliminate non-nucleated cells. After red blood cell lysis with ammonium chloride (Stem Cell Technologies, Vancouver, Canada), 106 events were recorded on a Coulter Epics XL flow cytometer (Beckman Coulter Corporation, Miami, USA). Fluorochrome and isotype-matched controls as well as unstained cell samples were measured and processed as negative controls. The numbers of CD34+/KDR+/CD45- cells were analyzed in the lymphocyte region using FACS Diva software and expressed as positive events/10^6 total events.

FIGURE 1: Study design



Divers performed three consecutive dives with air and three consecutive dives with nitrox-36. In between these diving series, they refrained from diving for 15 days. The divers avoided strenuous exercise 24 hours before diving. Numbers of circulating endothelial progenitor cells (EPC) and function of circulating angiogenic cells (CAC) were evaluated pre and post-diving, on the first and third dive of each diving series.

CAC culture and characterization

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation with Histopaque (Sigma-Aldrich, Saint Louis, Mo. USA) from 20 ml of peripheral blood. PBMC were cultured in Endothelial Growth Medium-2-MV (EGM-2-MV, Clonetics, Lonza, Walkersville, Md. USA), supplemented with 20% fetal bovine serum (FBS, Invitrogen, Eugene, Ore. USA). EGM-2-MV is composed of Endothelial Basal Medium (EBM)-2, supplemented with hydrocortisone, hEGF, VEGF, hFGF, R3-IGF-1, ascorbic acid and gentamicin-amphotericin B. Cells were seeded on 24-well dishes (BD, Franklin Lakes, N.J. USA), manually coated with human fibronectine (Invitrogen, Eugene, Ore. USA), at density of $1x106$/well. After 48 hours in culture, non-adherent cells were removed by thorough washing with phosphate buffer saline (PBS), and the adherent cells were maintained for five additional days. On Day 7, CAC migratory activity was evaluated.

CAC migratory capacity

At Day 7 of culture in a 24-well dish, growth medium was changed to EBM-2 supplemented with 0.5% bovine serum albumin (BSA, Invitrogen, Eugene, Ore. USA) in order to starve CACs. After three hours, CACs were harvested using PBS/ EDTA by repeated pipetting. In the upper chamber of a transwell with a pore size of 5 μm (Corning Costar, New York, N.Y. USA) $2x105$ cells were placed. In the lower chamber, vascular endothelial growth factor (VEGF, 50 ng/ml, R&D systems, Minneapolis, Minn. USA) and stromal cell-derived factor (SDF)-1α (100 ng/ml, R&D systems, Minneapolis, Minn. USA) were added to EBM-2/0.5% BSA. After four hours, viable CACs that had migrated to the lower chamber were counted manually using a hemacytometer. CACs counted in the negative control transwell (no addition of chemo-attractants) were subtracted from this number. Migrated CACs were expressed as percentage of total cell number added to the transwell (positive control).

Table 1: Subject characteristics

	n = 10
Male (%)	100
Age (yrs)	36.6 ± 2.3
Height (cm)	181 ± 1.7
Weight (kg)	94.4 ± 2.4
Heart rate (bpm)	61.6 ± 3.5
Systolic blood pressure (mmHg)	119 ± 2
Diastolic blood pressure (mmHg)	77 ± 2

Data are mean ± SEM

Statistical analysis

Continuous data are expressed as mean (± standard error of the mean, SEM). Normality of data was assessed using the one-sided Kolmogorov-Smirnov test. Due to non-normal distribution, EPC measures were normalized by natural logarithmic transformation prior to analysis. Pre- and post-exercise data were compared using paired sampled Student t-test. All tests were two-sided and a p-value < 0.05 was considered statistically significant. All analyses were performed using PASW Statistics 18.0 (SPSS Inc., Chicago, Ill., USA).

RESULTS

Characteristics of subjects and dives

Ten male non-smoking military divers with a mean age of 36.6 years were enrolled (Table 1). During participation in this study, none of the divers developed DCS.

Effect on numbers of circulating endothelial progenitor cells (EPCs)

Figure 2A shows the evolution of CD34+/KDR+ EPCs for each gas mixture separately. Diving with air led to a decrease in EPC numbers on Day 1, but this difference did not reach statistical significance (- 36%; p=0.39). The number of EPCs was not affected after diving with air on Day 3. Although diving with nitrox-36 had no effect on Day 1, EPCs clearly decreased following the dive on Day 3 (- 56%; p=0.019).

Effect on function of circulating angiogenic cells (CACs)

In Figure 2B, diving-induced changes in migratory capacity of CACs are depicted for air and nitrox-36 separately. Diving with air did not affect the chemotactic response of CACs towards VEGF and SDF-1α, neither on Day 1 nor on Day 3. A significant increase of 36% (p=0.036) could be shown when divers used nitrox-36 on Day 1. Repetitive diving with nitrox-36 did not lead to a further improvement.

DISCUSSION

The present study is the first to describe the acute effects of scuba diving on the numbers of circulating CD34+/KDR+ EPCs and on the functional capacity of CACs. Immediately after diving, numbers of EPCs were decreased, while the migratory capacity of CACs improved acutely. Both these effects were more pronounced when a nitrox-36 mixture was used compared to compressed air.

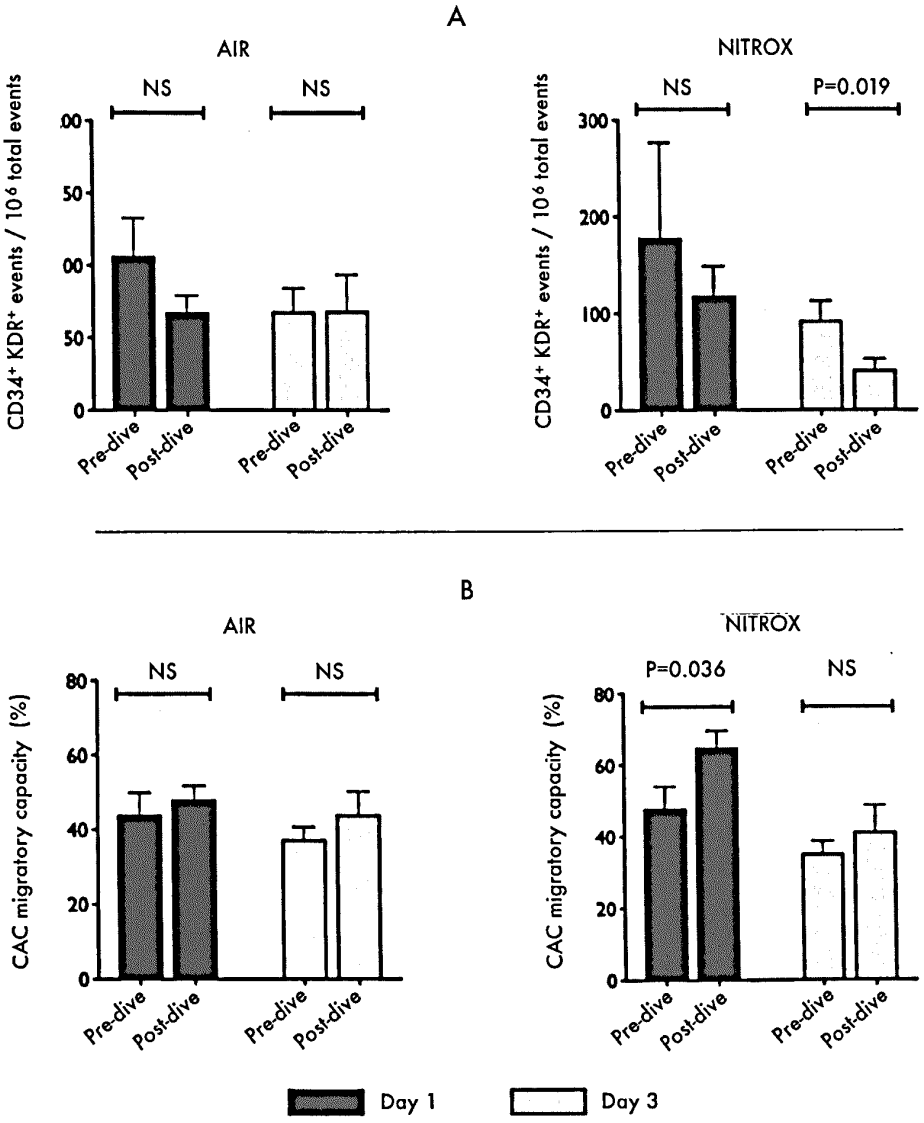
Effects of diving with nitrox on endothelial function

Impaired endothelium-dependent vasodilatation following a single scuba dive [14], does not fully recover after successive dives, indicating that repetitive diving may induce long-term alterations in vascular function [22]. Whereas the exact mechanisms that lead to endothelial dysfunction are still incompletely understood, hyperoxia-induced production of reactive oxygen species (ROS), reduction in the bioavailability of nitric oxide (NO) and direct mechanical damage to the endothelium during decompression, are considered to play an important role [23].

In order to increase dive time and to reduce bubble formation during decompression, a trend toward the preferential use of technical gases, such as nitrox-36, has developed during recent years. Diving with nitrox-36, however, increases the risk of oxygen toxicity, as the oxygen fraction in this mixture is higher compared to air. Breathing higher oxygen concentrations in hyperbaric conditions as well as breath-hold diving enhances the generation of reactive oxygen species [24-26].

These data were collected as a part of a larger study in which the effect of nitrox-36 and air dives on vascular function was assessed [19]. In that study, Marinovic, *et al.* reported that the diving-induced reduction in endothelial function was more prominent following nitrox-36-dives compared to air-dives, despite significantly lower venous gas bubble loads with nitrox-36 [19]. Although speculative, the higher production of reactive oxygen species (ROS) during nitrox dives and the subsequent scavenging of NO could have amplified this effect. However, neither administration of tetrahydrobiopterin (BH4), nor antioxidants, such as vitamin C, decreased pulmonary artery pressure after diving [27].

FIGURE 2: Acute effect of diving on circulating cells with vasculogenic potential



(A) Numbers of circulating EPCs are shown separately for air and nitrox-36 dives. Diving with air led to a decrease in EPC numbers on Day 1 but this difference did not reach statistical significance (- 36%; $p=0.39$). Diving with nitrox-36 had no effect on Day 1, but EPCs were reduced by 56% following the dive on Day 3.

(B) Migratory capacity of CACs was not altered when diving with air. A significant increase of 36% was observed with nitrox-36 on Day 1. Repetitive diving with nitrox-36 did not lead to a further improvement.

Data are mean ± SEM.

Effect of diving on endothelial progenitor cells

Apart from the delicate balance between NO and ROS production, endothelial homeostasis depends on efficient endothelial repair, a process in which bone marrow-derived cells with high vasculogenic potential are involved. Upon stimulation, EPCs are mobilized from the bone marrow into the circulation and are presumed to integrate into dysfunctional/damaged endothelial cell-layer of peripheral tissues.

Our group, as well as others, has shown that a single maximal exercise bout elicits an increase in the numbers of circulating EPCs in both healthy subjects and in cardiovascular patients [28,29]. Guerrero, *et al.* showed beneficial effect of exercise training on endo-thelial function in established hypertension in rats [30]. In the study by Fismen, *et al.*, heat stress, hyperoxia and dive affected several biochemical parameters that may play important roles in the mechanisms protecting against the adverse effects of saturation diving [31]. The high vascular oxidative load of a maximal exercise bout causes a temporary decrease in endothelium-dependent vasodilatation, which is followed by a substantial improvement 12 to 24 hours later [32]. Such an acute period of vascular stress appears to stimulate repair mechanisms, including the mobilization of CD34+/KDR+ EPCs, which could be considered as an adequate physiological response.

In contrast, the present study showed a clear decrease in EPCs immediately after a dive, an effect that was more pronounced when diving with nitrox-36. It is speculative that impaired NO bioavailability, resulting from the abundant generation of superoxide (O_2-) anions following nitrox diving and their reaction with NO to form peroxynitrite ($ONOO^-$), limit the process of EPC recruitment. Indeed, Laufs, *et al.* showed that EPC recruitment was blunted in eNOS knockout mice, confirming the central role of NO [33].

ROS-induced apoptosis of EPCs is another plausible mechanism for the observed decrease in EPCs. Tie, *et al.* demonstrated that addition of oxidized low-density lipoprotein to EPC cultures generates O_2- and H_2O_2, leading to apoptosis by inactivating the phosphoinositide 3-kinase/Akt pathway [34].

In addition, the release of pro-inflammatory cytokines in response to diving, may account for apoptosis in circulating EPCs [35]. Although data on the acute effects of diving on inflammatory cytokines are lacking in humans, it has been reported in rats that a decompression trauma acutely increased levels of interleukin-6 [36].

Effect of diving on circulating angiogenic cells

Several investigators have elucidated the role of CACs in re-endothelialization and neovascularization [5,37]. In the present study, a significant improvement in the functional properties of CACs after a single dive with nitrox-36 was observed, whereas diving with compressed air induced no changes. This could be regarded as a protective effect in an attempt to restore the injury at the level of the endothelium, elicited by a single dive.

CACs have been described to be extremely resistant to vascular oxidative stress. This particular feature may explain why, in contrast to the detrimental effect of ROS on EPC mobilization, the functional properties of CACs are not affected [38]. It has been shown for various cell types that migratory capacity towards SDF-1α is enhanced after priming with TNF-α [39]. This could be due to an increased CAC sensitivity to SDF-1α, by modulating CXCR4 signal transduction without affecting receptor expression [40]. Whether diving with nitrox-36 is associated with a larger release of pro-inflammatory cytokines compared to compressed air will be investigated in future experiments.

LIMITATIONS

The present study is limited by the small number of subjects included, which advocates cautious interpretation of the data. In this particular study design, however, each subject acts as its own control in pre- and post-dive effects and in evaluating the effects of the different gas mixtures, which strengthens the results. Moreover, the effect of diving with nitrox-36 was remarkably consistent in evaluation of endothelial repair mechanisms. Therefore, we think that the effects of diving on endothelial repair merits further study in a larger study cohort.

CONCLUSION

In the current study it is shown that diving reduced EPC numbers immediately after diving. In contrast, CAC migration tends to improve acutely following diving. Recruitment of EPCs may be hampered by reduced NO bioavailability or increased apoptosis. Nevertheless, the immediate functional improvement of CACs could contribute to maintaining endothelial homeostasis. Remarkably, these responses are more pronounced when diving with a nitrox-36 gas mixture, suggesting that such a mixture elicits more endothelial dysfunction.

PERSPECTIVES

Endothelial dysfunction is a risk factor for cardiovascular diseases and an independent prognostic marker of cardiovascular events [41]. Endothelial function diminishes with aging, even in healthy subjects. Since this study included young men, future studies should point out whether there is a differential effect of diving on endothelial repair mechanisms in older subjects.

According to some reports, more than two million people practice scuba diving worldwide. Although manifest cardiovascular disease precludes diving, cardiovascular risk factors (*e.g.*, hypertension, smoking, overweight and diabetes), may be commonly encountered in recreational divers. Numbers of EPCs are already decreased, and function of CACs is impaired in individuals at cardiovascular risk, which contributes to the development of cardiovascular disease [42]. As more patients with cardiovascular risk factors undertake diving, investigation of the implications of diving on endothelial repair in this specific population is of major interest.

The authors report that no conflict of interest exists with this submission. ■

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