The effects of acute oral antioxidants on diving-induced alterations in human cardiovascular function

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Diving-induced acute alterations in cardiovascular function such as arterial endothelial dysfunction, increased pulmonary artery pressure (PAP) and reduced heart function have been recently reported. We tested the effects of acute antioxidants on arterial endothelial function, PAP and heart function before and after a field dive. Vitamins C (2 g) and E (400 IU) were given to subjects 2 h before a second dive (protocol 1) and in a placebo-controlled crossover study design (protocol 2). Seven experienced divers performed open sea dives to 30 msw with standard decompression in a non-randomized protocol, and six of them participated in a randomized trial. Before and after the dives ventricular volumes and function and pulmonary and brachial artery function were assessed by ultrasound. The control dive resulted in a significant reduction in flow-mediated dilatation (FMD) and heart function with increased mean PAP. Twenty-four hours after the control dive FMD was still reduced 37% below baseline (8.1 *versus* 5.1%, $P = 0.005$), while right ventricle ejection fraction (RV-EF), left ventricle EF and endocardial fractional shortening were reduced much less (∼2–3%). At the same time RV end-systolic volume was increased by 9% and mean PAP by 5%. Acute antioxidants significantly attenuated only the reduction in FMD post-dive ($P < 0.001$), while changes in pulmonary artery and heart function were unaffected by antioxidant ingestion. These findings were confirmed by repeating the experiments in a randomized study design. FMD returned to baseline values 72 h after the dive with pre-dive placebo, whereas for most cardiovascular parameters this occurred earlier (24–48 h). Right ventricular dysfunction and increased PAP lasted longer. Acute antioxidants attenuated arterial endothelial dysfunction after diving, while reduction in heart and pulmonary artery function were unchanged. Cardiovascular changes after diving are not fully reversed up to 3 days after a dive, suggesting longer lasting negative effects.

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Recreational diving has become one of the most frequently performed types of outdoor sport activities, with millions of recreational divers worldwide. During dives with compressed air the divers are exposed to various environmental stresses that may affect haemodynamics and cardiovascular function, such as immersion-induced increase in preload, cold-induced increase in afterload, reduced filling of the left heart due to ventilation of high-density gas mixtures, hyperoxia, formation of intravascular nitrogen bubbles, exercise and psychological stress. We have shown that simulated chamber diving results in acute arterial endothelial dysfunction (Brubakk *et al.* 2005) and unchanged pressure in the pulmonary artery (PAP) (Valic *et al.* 2005), while field scuba diving is associated with increased PAP (Dujic *et al.* 2006*a*) and endothelial dysfunction (Obad *et al.* 2006). Four weeks of oral antioxidant supplementation with vitamins C ($1\,g\,day^{-1}$) and E ($400\,IU\,day^{-1}$) reversed acute endothelial dysfunction after diving (Obad *et al.* 2006). Reduction in endothelial function post-dive may also be present in the heart, as a reduction in endothelial function is an important factor in heart failure (Landmesser & Drexler, 2005). Immersion leads to an increase in thoracic blood volume, and 180–240 ml of blood is added to the heart volume with enlargement of all four chambers (Risch *et al.* 1978). Stroke volume is increased by about 50% after upright immersion to the neck in healthy individuals (Meyer & Bucking, 2004). This leads to an increase in right ventricular dimensions and to an increase in PAP to a degree that in some cases even can lead to pulmonary artery

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oedema (Pons *et al.* 1995). Thus, it is conceivable that immersion and hyperoxia, with the addition of gas bubble formation caused by decompression, can reduce systemic conduit as well as pulmonary artery function and cardiac endothelial–myocardial coupling, which would account for the observations.

Oxidative stress in the vessel wall and endocardium is associated with the generation of reactive oxygen and nitrogen species (ROS and RNS) by activities of several oxidases, like nitric oxide synthase (NOS), NOx oxidases, NADPH oxidases, xanthine oxidase, cytochrome-450 and cyclooxygenase, and of mitochondria (Wolin *et al.* 2005). The superoxide anion was shown to quench NO (Rubanyi & Vanhoutte, 1986), but it will also react with NO to generate peroxynitrate, which causes additional oxidative stress due to activation of oxidases and inactivation of antioxidant enzymes (Wolin *et al.* 2005). Reduction of local NO by ROS causes hyperoxic vasoconstriction and impairs NO-dependent vasodilatation (Milone *et al.* 1999; Zhilyaev *et al.* 2003). Oxidative stress has been recently implicated in different cardiovascular pathologies such as atherosclerosis (Schulz *et al.* 2004), hypertension (de Champlain *et al.* 2004), diabetes (Gonzalez-Vilchez *et al.* 2005), atrial fibrillation (Dudley *et al.* 2005) and ischaemia–reperfusion injury (Seccombe & Schaff, 1995). A variety of antioxidants, including vitamins C and E, have been shown to have a protective effect on the pulmonary endothelial function after cardiopulmonary bypass (Angdin *et al.* 2003), but also offer general endothelial protection (Pratico, 2005).

Thus, in the present study we wanted to determine whether acute oral antioxidants can attenuate the negative effects of diving on the function of the heart and the pulmonary and brachial artery in men performing standard dives.

Methods

Study design

This study is performed in two separated protocols: protocol 1, non-randomized, and protocol 2, a double blind placebo-controlled crossover study design.

Study population

Protocol 1. Seven experienced Croatian Navy male divers aged 34.1 ± 3.5 years (mean $\pm$ standard deviation, S.D.), with the average body mass index of $25.9 \pm 2.4\,\mathrm{kg\,m^{-2}}$, height 1.82 ± 0.04 m and body fat index 20.1 ± 3.4 (%, body fat/kg) took part in the study. All participants were apparently healthy non-smokers and at the time of the study had a valid medical certificate for diving. They had not used antioxidants within 4 weeks of study and were not taking other medications. All experimental procedures were conducted in accordance with the *Declaration of Helsinki*, and were approved by Ethics Committee of the University of Split School of Medicine. Each method and the potential risks were explained to the participants in detail and they gave written informed consent before the experiment.

Protocol 2. Six of the above-mentioned subjects participated in the second protocol, which was carried out 8 months after the completion of the first set of experiments.

Ergospirometry

Maximal oxygen uptake ($\dot{V}_{O_2,max}$) and heart rate (HR_{max}) were determined in all divers 2 weeks before dives using a cycle ergometer (Marquette Hellige Medical Systems 900 ERG, Milwaukee, WI, USA). Criteria for assessment of $\dot{V}_{O_2,max}$ included: (1) HR in excess of 90% of age-predicted maximum (220 – age), (2) respiratory exchange ratio (RER) ≥ 1.1, and (3) plateau (≤ 150 ml increase) in $\dot{V}_{O_2}$ with an increase in workload. If at least two of the three criteria were met, the highest $\dot{V}_{O_2}$ measured was chosen as the subject's $\dot{V}_{O_2,max}$. During the exercise protocol oxygen uptake and pulmonary ventilation were measured with a cardiopulmonary exercise testing unit (Quark b^2, Cosmed, Rome, Italy), and heart rate (HR) was registered continuously with a Polar S810i HR monitor (Polar Vantage, Finland).

Location of the study and dive protocol

Both the protocols of the study were performed at a military base over a 2 week period. The diving site was located in the vicinity of the base, to which the divers were transported by powerboat, during a 10 min drive. The site was chosen because it allowed us to perform dives of suitable depth and duration. In protocol 1, sea temperature at the bottom and at the decompression stop was 13°C for all dives, and outside temperature varied between 16 and 18°C (protocol 1). Protocol 2, the randomized study, was performed in July, with warmer sea (19°C) and outside (32–36°C) temperatures. All dives were performed by divers equipped with wet suits. The depth of the dive was set to 30 m with a descent rate of $10\,\mathrm{m\,min^{-1}}$, and each pair of divers was supplied with a diving computer (Mosquito, Suunto, Finland). The divers were told to swim at the bottom over the distance of 500 m at an exercise intensity corresponding to ~40% of their maximal heart rate; the distance covered was controlled by the personnel on the powerboat and the work load by the subjects themselves by monitoring their preselected HR during the

dive (Polar S810i HR monitor). After spending 30 min at the bottom, the ascent rate to decompression depth was 9 m min^{-1}, with a decompression stop at 3 m for 3 min. During the decompression period divers were told not to perform any exercise, as we have shown that exercise during decompression significantly reduces bubble grade (Dujic *et al.* 2005*c*). This diving protocol was used because we have shown previously that it produces a significant amount of venous bubbles even with adequate decompression (Dujic *et al.* 2005*b*). The heart rate (HR) was continuously monitored in all divers during diving with a Polar S810i HR monitor, and the data collected were downloaded to a PC and later analysed.

Timeline of measurements

Prior to experimental sessions, subjects abstained from any physical activity and diving for 48 h and fasted for 10–12 h.

Protocol 1. The divers performed two dives. The first one was a control dive without any intervention. The second one was performed 24 h later, and vitamin C and E supplementation was given 2 h before the start. Thirty minutes before and 30 min following each dive, the subjects' arterial endothelial and heart functions were investigated by high-resolution ultrasound (4 times in total).

Protocol 2. The divers performed two dives in a randomized placebo-controlled study design within 3 days of each other. Cardiovascular parameters were assessed 30 min following the dives with and without a preceding placebo or antioxidants and 24, 48 and 72 h later. One diver was not available to participate in this protocol.

Post-dive monitoring and venous bubble detection

Thirty minutes post-dive subjects were placed in the left supine position and an echocardiographic investigation with a phase array probe (1.5–3.3 MHz) of the Vivid 3 Expert and portable Logic Book XP ultrasonic scanners (GE Healthcare, Milwaukee, WI, USA) was performed by an experienced cardiologist (A.O.). High quality images were obtained in all subjects and gas bubbles were seen as high intensity echoes in the right heart and the pulmonary artery at rest and after two coughs (Valic *et al.* 2005). Images were graded as previously described (Eftedal & Brubakk, 1997) and transferred into a linear scale using the relationship described by Nishi *et al.* (2003). Detailed information about this technique is presented elsewhere (Dujic *et al.* 2004).

Endothelial function

Endothelial function was assessed according to the method of Raitakari & Celermajer (2000) in all subjects. This method determines the arterial response to reactive hyperaemia, flow-mediated dilatation (FMD) (Corretti *et al.* 2002). The subjects were placed in a quiet room with temperature about 22°C and rested for 15 min on the bench in a supine position before measurement. Participants were tested at the same time of day (from 10 to 12 a.m.) to account for diurnal variation in endothelial function and were always 4 h postprandial after the consumption of a light breakfast. All participants were asked to abstain from caffeine for at least 12 h before testing, from strenuous exercise from 48 h before, and from diving for about 1 week. Measurements were performed with a 5.7–13.3 MHz linear transducer using a Vivid 3 Expert and portable Logic Book XP ultrasonic scanner (GE Healthcare, Milwaukee, WI, USA). Brachial artery diameter was measured from longitudinal images with the lumen–intima interface visualized on both (anterior and posterior) walls. Images were acquired using ECG gating during acquisition, using the onset of the R wave to identify end-diastole. When the images were chosen for analysis, the boundaries for diameter measurement were identified manually with an electronic caliper. In parallel to the imaging of the brachial artery, mean blood velocity (MBV) was obtained using the duplex function of the linear array vascular probe. Pulsed Doppler measurements for measuring MBV were performed with the sample volume placed in mid-artery. The position of the transducer was marked to ensure the same position for all measurements, which was 3–5 cm proximal to the antecubital fossa. Once the basal measurements were obtained, arterial occlusion was created by inflating a cuff placed on the forearm to 240 mmHg for 5 min. After 5 min inflation the cuff was deflated producing a brief high-flow state resulting in artery dilatation due to increased shear stress. Flow and diameter of brachial artery were measured at time of cuff deflation and at points of every 30 s for 3 min, and at the fourth and fifth minute. Subjects were then rested in the supine position for 10 min to get back to baseline diameter. FMD was calculated as the percentage increase in brachial artery diameter from the resting state to maximal dilatation. Blood flow was calculated from the MBV measurements and the vessel diameter, assuming that the vessel was circular. All raw date were saved on the ultrasound hard disk as still and cine-loop images for later analysis. As previously described, blood velocity measurements were acquired 5 s after cuff release. Measurements of peak flow and 5 s average flow were calculated (vessel cross-sectional area × MBV) and used to quantify the hyperaemic response. Shear rate was calculated as blood flow velocity (cm s^{-1}) divided by diameter (cm) (Pyke & Tschakovsky, 2005). The accuracy

of the method was assessed in our previous study (Brubakk *et al.* 2005). Endothelial-independent dilatation was not measured with nitroglycerine in this study since it reduces venous bubble formation post-dive (Dujic *et al.* 2006*b*).

Pulmonary artery pressure

Transthoracic echocardiography (TTE) was used to estimate mean PAP before and after the dive. The transducer was pointed to the outflow tract of the right heart, with pulse wave Doppler sample volume positioned at a level of pulmonary valve annulus. The PA flow velocity was recorded during cessation of breathing on an S-VHS videotape. Doppler measurements were averaged over three consecutive cardiac cycles. AccT (the time interval between the onset and peak of pulmonary flow velocity, in milliseconds), RVET (the time interval from the onset to termination of the systolic pulmonary flow velocity, in milliseconds) and the R-R interval (milliseconds) (the time interval of the R wave of the electrocardiogram) were measured, and the AccT/RVET ratio was calculated for each cardiac cycle. We have previously used AccT/RVET as an index of the mean PAP (Valic *et al.* 2005; Dujic *et al.* 2006*a*) because others have shown a good relationship between AccT/RVET and invasively measured PAP and PVR (Kitabatake *et al.* 1983).

Heart function

Two-dimensional echocardiographic studies were performed with a phased array sector probe (1.5–3.3 MHz) of the Vivid 3 Expert ultrasonic scanner and Logic Book XP (GE Healthcare) using standard examination protocol. The apical four-chamber view was identified initially by palpation of the cardiac apex with the patient in the left lateral decubitus position. The transducer position was then adjusted as needed to obtain optimal images. The four-chamber view displays all four cardiac chambers, as well as the ventricular and atrial septa. This view was used for measurements of right ventricular end-systolic and end-diastolic volumes (RV-ESV and RV-EDV) and calculation of right ventricle ejection fraction (RVEF) using Simpson's method (Schiller *et al.* 1989). After evaluation of right heart chambers, using the same Simpson's method, quantification of left ventricular (LV) function was performed. The endocardium of the LV was traced and automatically subdivided into a series of discs for measurements of LV end-systolic and end-diastolic volume (LV-ESV and LV-EDV), and calculation of LV ejection fraction (LVEF). Because both end-diastolic and end-systolic measurements are needed for volume calculations, the ECG was continuously recorded. The ultrasound probe was positioned in the parasternal long axis to visualize structures of the left heart, primarily the inferoposterior wall and interventricular septum; two-dimensionally guided M-mode echocardiography was then performed. The cross-sectional axis of the LV at the papillary muscle tip level was measured. Measurements of LV septal, posterior wall and cavity dimensions (LV interventricular septum and posterior wall, IVS and LVPW) were measured at the end-diastolic and end-systolic period. Three consecutive cardiac cycles were measured and average values were obtained. All measurements were made according to the American Society of Echocardiography (Sahn *et al.* 1978).

The following parameters were derived from two-dimensional and M-mode measurements. LVIDd and LVIDs are left ventricular internal diameter; d in diastole and s in systole. Endocardial fractional shortening (FS) was calculated by:

$$\mathrm{FS}\,(\%) = 100 \times ((\mathrm{LVIDd} - \mathrm{LVIDs})/\mathrm{LVIDd})$$

Stroke volume (SV; ml) was derived from diastolic and systolic LV volumes with Teicholz's formula:

$$\mathrm{SV} = \mathrm{EDV} - \mathrm{ESV}$$

where EDV is end-diastolic volume and ESV is end-systolic volume (Teichholz *et al.* 1976). This value was multiplied by heart rate to obtain a value for cardiac output (CO; $\mathrm{l\,min^{-1}}$). Ejection fraction of the right ventricle (RV) and LV was calculated from the following equation:

$$\mathrm{EF}\,(\%) = (\mathrm{EDV} - \mathrm{ESV})/\mathrm{EDV}$$

EF was used as an index of myocardial contractility. The difference between thickness of LV walls before and after the dive was presented as LV IVS Δ (%) and LV posterior wall (PW) Δ (%).

Pharmacological intervention

The subjects were treated with ascorbic acid (2 g of pure powder, Ph.Eur.II, Medimon, Split, Croatia) and vitamin E (400 IU, Twinlab, Hauppauge, NY, USA) 2 h before the second dive (protocol 1). Subsequently, we performed a double-blind crossover study in which subjects were randomly assigned to either placebo or antioxidants (protocol 2). Three days were allowed between the phases of the crossover study. The randomization of the treatments was performed by the pharmacist, who was not involved in data acquisition or analysis. The investigator (A.O.) who performed the data collection and analysis was blinded to the group and treatment condition.

Statistical analysis

Data are given as the mean $\pm$ standard deviation (S.D.). Differences in arterial diameter and response to hyperaemia, heart function and pulmonary artery pressure before and after a dive were determined using Student's

Table 1. Brachial and pulmonary artery function in divers before and after control dive and dive with acute administration of antioxidants in a non-randomized protocol (protocol 1)

	Control dive		C and E dive	
	Pre-dive	Post-dive	Pre-dive	Post-dive
Bubble count (bubbles cm^{-2})				
20 min	0	1.46 ± 1.41	0	1.28 ± 1.54
20 min cough	0	2.32 ± 1.47	0	1.36 ± 1.49
Brachial artery				
Baseline diameter (mm)	4.16 ± 0.57	4.20 ± 0.62	4.24 ± 0.62	4.26 ± 0.63
FMD (%)	8.05 ± 1.75	2.13 ± 1.01*	5.09 ± 0.75†	5.42 ± 1.08
Peak flow (ml min^{-1})	773.5 ± 313.0	752.2 ± 378.8	885.7 ± 389.7	859.6 ± 324.5
Average flow in 5 s (ml min^{-1})	708.2 ± 302.3	697.0 ± 360.4	817.6 ± 372.1†	812.4 ± 320.1
Shear rate (s^{-1})	89.1 ± 30.2	83.1 ± 28.9	93.6 ± 33.7	91.4 ± 30.6
Pulmonary artery				
AccT (ms)	139.4 ± 12.4	120.0 ± 8.8*	135.2 ± 6.4	117.1 ± 5.7*
RVET (ms)	333.0 ± 15.6	356.0 ± 26.2*	336.3 ± 15.7	349.0 ± 16.9*
AccT/RVET	0.42 ± 0.04	0.34 ± 0.03 *	0.40 ± 0.03†	0.34 ± 0.01*

Values are the means ± S.D. *$P < 0.05$ comparing pre-dive to post-dive; †$P < 0.05$ comparing control pre-dive to C and E (antioxidants) pre-dive. FMD, flow-mediated dilatation; Acct, time interval between the onset and peak of pulmonary flow velocity; RVET, time interval from the onset to termination of the systolic pulmonary flow velocity.

t test for paired samples. Differences between control and experimental dives were compared using Student's *t* test for paired samples (protocol 1). To determine the effects of placebo *versus* vitamin C and E treatment in a randomized study design on all outcome variables (protocol 2), Friedman's non-parametric analysis of variance was used, while *post hoc* comparisons were done by Wilcoxon's sign rank test. Non-parametric tests were used because of the small sample size ($n = 6$). Associations between metric variables were evaluated by Pearson's coefficient of correlation. The limit of significance was set at $P = 0.05$. All analyses were done using Statistica 7.0 software (Statsoft, Inc., Tulsa, OK, USA).

Results

Venous gas bubbles

$\dot{V}_{O_2,max}$ for the group studied in protocol 1 was 41.5 ± 9.6 ml kg^{-1} min^{-1} and HR_{max} at $\dot{V}_{O_2,max}$ was 178.5 ± 9.6 beats per minute (bpm). All seven participants in protocol 1 and six in protocol 2 successfully completed the study protocols and no symptoms/signs of DCS were reported. Venous gas bubbles were seen after the dive in all subjects. Over the observation period in protocol 1 an average number of venous bubbles per square centimetre in the right heart after the first dive was 1.5 ± 1.4 and after the second dive 1.3 ± 1.5. This difference is not significant ($P = 0.18$).

In protocol 2 the number of venous bubbles per square centimetre was non-significantly reduced from 0.99 ± 1.30 without antioxidant vitamins to 0.70 ± 1.37 with the vitamins. We assume that no large patent foramen ovale was present in any subject as no bubbles were observed in the LV.

Endothelium-dependent and -independent brachial artery function

Protocol 1. Mean brachial artery diameter did not change significantly after either the control ($P = 0.2$) or the experimental dives ($P = 0.78$) (Table 1). Acute antioxidants had no significant effect on baseline diameter. Brachial artery FMD decreased from 8.1 ± 0.6 to $2.1 \pm 1.0\%$ ($P = 0.0002$) after the control dive. Twenty-four hours after the first dive FMD was $5.1 \pm 0.8\%$, which was significantly reduced below baseline ($P = 0.005$). Acute antioxidants prevented further FMD reduction after the second dive ($5.4 \pm 1.1\%$) and this response was significantly different from that following the first dive ($P < 0.0001$). Peak and 5 s post-occlusion flows were not changed after the dives. FMD reduction after the control dive correlated significantly to increases in RV-ESV ($y = -0.84x - 0.88$; $r = -0.90$, $P = 0.005$), and a similar but non-significant effect was noted for RV-EDV increase ($P = 0.067$).

Protocol 2. In protocol 2, antioxidants did not alter baseline diameters prior to the dive. Mean brachial artery diameter showed a significant increase after the dive without antioxidant vitamins ($P = 0.028$) and no change in the dive with antioxidant (Table 2). Brachial artery FMD decreased from 8.1 ± 1.5 to 2.5 ± 0.7 ($P = 0.028$) after the dive without antioxidant vitamins. It remained at reduced values for the next two days, only to return to baseline

Table 2. Brachial and pulmonary artery function in divers before and after control dive and dive with acute administration of antioxidants in a randomized protocol (protocol 2)

	Control dive		C and E dive	
	Pre-dive	Post-dive	Pre-dive	Post-dive
Bubble count (bubbles cm^{-2})				
20 min	0	0.83 ± 1.35	0	0.23 ± 0.38
20 min cough	0	0.99 ± 1.30	0	0.70 ± 1.37
Brachial artery diam (mm)				
Baseline	4.50 ± 0.34	4.62 ± 0.37*	4.50 ± 0.34	4.52 ± 0.37
24 h	—	4.53 ± 0.37	—	4.50 ± 0.34
48 h	—	4.50 ± 0.34	—	4.50 ± 0.34
72 h	—	4.50 ± 0.34	—	4.50 ± 0.34
Brachial artery FMD (%)				
Baseline	8.11 ± 1.51	2.49 ± 0.67*	7.72 ± 0.94	5.62 ± 0.92*†
24 h	—	5.57 ± 1.12*	—	7.55 ± 0.73
48 h	—	6.49 ± 1.95*	—	7.70 ± 0.91
72 h	—	7.57 ± 0.98	—	7.74 ± 0.94
Pulmonary artery AccT/RVET				
Baseline	0.44 ± 0.03	0.35 ± 0.02*	0.44 ± 0.02	0.36 ± 0.01*†
24 h	—	0.43 ± 0.03*	—	0.43 ± 0.01
48 h	—	0.42 ± 0.02*	—	0.43 ± 0.02
72 h	—	0.43 ± 0.02	—	0.43 ± 0.02

Values are means ± S.D. *$P < 0.05$ comparing pre-dive to post-dive; †$P < 0.05$ comparing control pre-dive to C and E (antioxidants) pre-dive. diam, diameter; FMD, flow-mediated dilatation; Acct, time interval between the onset and peak of pulmonary flow velocity; RVET, time interval from the onset to termination of the systolic pulmonary flow velocity.

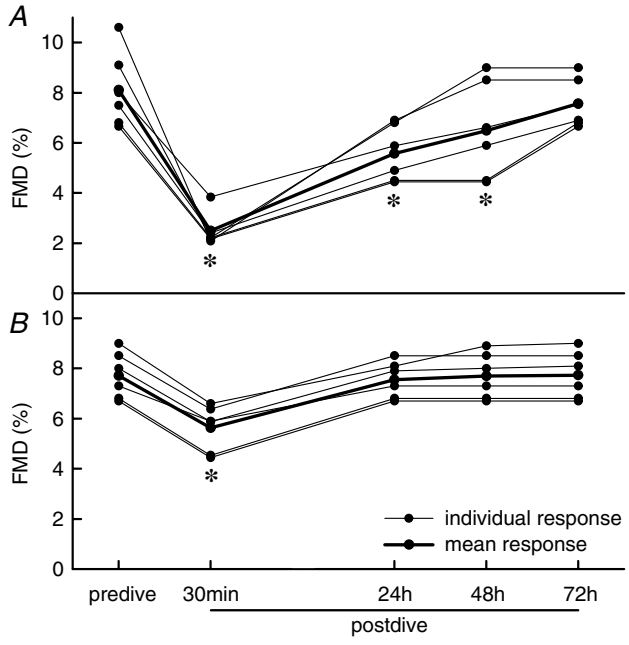

Figure 1. Flow-mediated dilatation (FMD) at pre-dive and post-dive (30 min, 24 h, 48 h and 72 h) periods for control dive (*A*) and dive after application of antioxidants (*B*) in protocol 2
Values are represented as individual responses (thin lines) and mean response (thick line) for both dives; *significant difference ($P < 0.05$) between pre-dive and post-dive values; †significant difference ($P < 0.05$) between dives with and without antioxidant treatment.

on the third day. When diving with vitamins C and E, FMD was significantly reduced from 7.7 ± 0.9 to 5.6 ± 0.9 ($P = 0.028$). This reduction was less than the decrease in the dive without antioxidant vitamins ($P = 0.0039$); the recovery time for FMD was within 24 h (Fig. 1). Values for corrected FMD (taking into account level of sheer stress) yielded similar results (data not shown).

Pulmonary artery pressure

Protocol 1. In protocol 1 pulmonary artery acceleration time (AccT) and right ventricle ejection time (RVET) ratio decreased significantly from 0.42 ± 0.04 to 0.34 ± 0.03 ($P = 0.003$) after the control dive (Table 1). Twenty-four hours after the dive the Acct/RVET ratio was still significantly lower than at baseline (0.4 ± 0.03, $P = 0.018$). The second dive caused a significant AccT/RVET reduction from 0.4 ± 0.03 to 0.34 ± 0.01 ($P = 0.0001$). This response was not different from that following the first dive, suggesting no effect of antioxidants ($P = 0.43$). For both dives the reduction in AccT/RVET was due to a similar decrease in AccT and an increase in RVET (Table 1). The reduction in AccT/RVET after the control dive correlated with the reduction in RV ejection fraction (RV-EF) ($y = -1.9x + 63.4$; $r = 0.85$; $P = 0.003$). The bubble numbers after the control dive did not correlate

Table 3. Cardiac volumes and function in divers before and after control dive and dive with acute administration of antioxidants in non randomized protocol (protocol 1)

	Control dive		C and E dive	
	Pre-dive	Post-dive	Pre-dive	Post-dive
Left heart				
Cardiac output (l min^{-1})	5.58 ± 1.06	4.25 ± 0.64*	5.61 ± 1.02	4.39 ± 0.71*
Heart rate (bpm)	59.7 ± 4.1	54.1 ± 5.0*	61.0 ± 3.1	54.6 ± 3.1*
Stroke volume (ml)	93.2 ± 14.4	78.5 ± 8.7*	91.6 ± 13.5	80.2 ± 10.3*
LV-EDV (ml)	145.6 ± 19.3	140.8 ± 18.6	148.0 ± 19.3	147.9 ± 19.2
LV-ESV (ml)	52.5 ± 6.5	66.6 ± 14.4*	54.0 ± 7.1	65.2 ± 8.3*
LV-EF (%)	63.8 ± 2.8	55.9 ± 2.9*	62.8 ± 3.1†	55.2 ± 1.6*
LV-IVS delta (%)	32.3 ± 12.7	13.9 ± 6.4*	28.9 ± 5.4	15.6 ± 10.0*
LV-PW delta (%)	49.5 ± 10.4	35.0 ± 10.4*	43.7 ± 11.3	34.9 ± 10.5*
EndoFS (%)	35.1 ± 2.2	29.5 ± 1.6*	34.4 ± 2.3†	29.0 ± 1.1*
Right heart				
RV-EDV (ml)	58.5 ± 17.7	64.0 ± 17.5*	61.8 ± 17.2	66.8 ± 17.7*
RV-ESV (ml)	21.8 ± 7.4	25.3 ± 7.0*	23.8 ± 6.5†	27.1 ± 6.9*
RV-EF (%)	63.3 ± 3.8	60.2 ± 3.2*	61.2 ± 3.3†	59.1 ± 3.6*

Values are means ± S.D. *$P < 0.05$ comparing pre-dive to post-dive; †$P < 0.05$ comparing control pre-dive to C and E (antioxidants) pre-dive. LV, left ventricle; RV, right ventricle; EDV, end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction; IVS, interventricular septum; PW, posterior wall; EndoFS, endocardial fractional shortening.

with AccT/RVET decrease ($P = 0.077$), indicating no relationship to the increase in PAP.

Protocol 2. Responses in protocol 2 followed in great similarity results from protocol 1 (data given in Table 2). Since the follow up period in protocol 2 lasted for 3 days we detected statistical difference in time needed for recovery between dives without and with vitamins C and E. While it took 3 days for the AccT/RVET ratio to return to baseline when no antioxidant therapy was administered, its prescription reduced it to only 1 day (Fig. 2).

Cardiac volumes and function

Protocol 1. Cardiac output (CO) was similarly reduced after both dives due to a concomitant decrease in stroke volume (SV) and heart rate (HR) (Table 3). Antioxidants did not cause any change in this response and all three parameters (CO, SV and HR) returned to the baseline values before the second dive.

All divers showed a significant increase in RV-EDV and RV-ESV after both dives, suggesting no effect of antioxidants. Twenty-four hours after the control dive, RV-ESV was significantly higher than the baseline value ($P = 0.001$), while a non-significantly similar change in RV-EDV was found ($P = 0.06$). RV-EF decreased from 63.3 ± 3.8 to 60.2 ± 3.2% after the first dive ($P = 0.03$) and from 61.2 ± 3.3% to 59.1 ± 3.6% after the second dive ($P < 0.0001$). The observed change in RV-EF was not different in the two dives ($P = 0.39$). RV-EF after the first dive was still 3% below baseline 24 h later ($P = 0.03$) (Table 3).

LV-ESV was significantly increased after both control and experimental dives ($P = 0.02$ and 0.0007), while

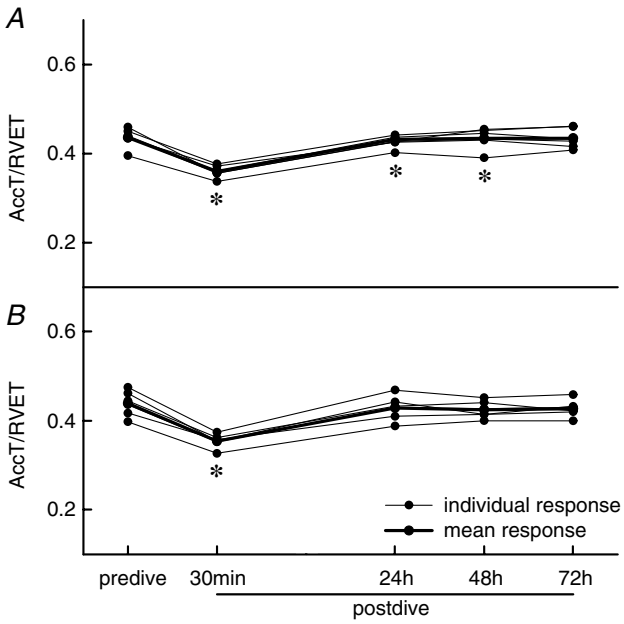

Figure 2. Changes in a Acct/RVET ratio (for estimation of mean PAP change) at pre-dive and post-dive (30 min, 24 h, 48 h and 72 h) period for control dive (*A*) and dive after application of antioxidants (*B*) in protocol 2
Values are represented as individual responses (thin lines) and mean response (thick line) for both dives; *significant difference ($P < 0.05$) between pre-dive and post-dive values.

Table 4. Cardiac volumes and function in divers before and after control dive and dive with acute administration of antioxidants in randomized protocol (protocol 2)

	Control dive		C and E dive	
	Pre-dive	Post-dive	Pre-dive	Post-dive
Left heart				
Cardiac output (l min^{-1})				
Baseline	5.44 ± 0.79	4.54 ± 0.58*	5.53 ± 0.68	4.59 ± 0.53*
24 h	—	5.28 ± 0.78	—	5.41 ± 0.82
48 h	—	5.42 ± 0.64	—	5.31 ± 0.73
72 h	—	5.38 ± 0.63	—	5.44 ± 0.70
Stroke volume (ml)				
Baseline	85.94 ± 12.96	81.08 ± 10.50*	85.65 ± 11.34	81.30 ± 10.23*
24 h	—	84.50 ± 12.81	—	84.86 ± 13.04
48 h	—	84.14 ± 11.52	—	84.18 ± 11.81
72 h	—	83.98 ± 11.14	—	85.37 ± 12.13
LV-EDV (ml)				
Baseline	134.09 ± 19.27	138.49 ± 19.29*	135.18 ± 19.11	139.18 ± 19.02*
24 h	—	134.69 ± 18.43	—	135.10 ± 20.70
48 h	—	134.07 ± 19.42	—	134.96 ± 18.85
72 h	—	134.37 ± 19.30	—	134.30 ± 19.78
LV-ESV (ml)				
Baseline	48.15 ± 7.94	57.41 ± 10.49*	49.54 ± 9.63	57.88 ± 10.31*
24 h	—	50.20 ± 8.02	—	49.93 ± 8.52
48 h	—	49.93 ± 9.43	—	50.78 ± 8.53
72 h	—	50.40 ± 9.25	—	48.93 ± 9.27
EndoFS (%)				
Baseline	35.08 ± 2.65	31.26 ± 2.41*	34.42 ± 2.95	31.07 ± 2.19*
24 h	—	34.17 ± 2.82	—	34.32 ± 2.22
48 h	—	34.14 ± 2.63*	—	34.04 ± 2.21
72 h	—	34.03 ± 2.18	—	34.67 ± 2.81
Right heart				
RV-EF (%)				
Baseline	61.65 ± 3.07	55.69 ± 2.60*	61.43 ± 2.80	55.82 ± 2.08*
24 h	—	60.58 ± 3.79*	—	59.98 ± 2.94*
48 h	—	61.03 ± 3.28	—	61.37 ± 2.72
72 h	—	61.33 ± 2.61	—	61.88 ± 2.93

Values are means ± S.D. *$P < 0.05$ comparing pre-dive to post-dive; †$P < 0.05$ comparing control pre-dive to C and E (antioxidants) pre-dive. Post-dive period contains data on values obtained immediately post-dive, 1, 2 and 3 days after the dive. LV, left ventricle; RV, right ventricle; EDV, end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction; IVS, interventricular septum; PW, posterior wall; EndoFS, endocardial fractional shortening.

LV-EDV was unchanged after both dives (Table 3). LV-EF decreased significantly from 63.8 ± 2.8 to 55.9 ± 2.9% after the control dive ($P = 0.0007$) and from 62.8 ± 3.1 to 55.2 ± 1.6% after the dive with antioxidants ($P = 0.0003$). Twenty-four hours after the control dive LV-EF was still significantly reduced below baseline ($P = 0.04$), while LV-ESV was non-significantly increased ($P = 0.08$). Regional (interventricular septum end-systolic *versus* end-diastolic difference, IVS Δ and LV posterior wall end-systolic *versus* end-diastolic difference, LV-PW Δ) and global (endocardial fractional shortening, endoFS) LV contractility indices were significantly decreased after both dives, except for a non-significant decrease in LV-PW Δ after the second dive ($P = 0.09$). Twenty-four hours after the first dive, endoFS was still significantly reduced below baseline ($P = 0.04$), while a non-significant change in LV-PW Δ was found ($P = 0.06$). Acute administration of antioxidants did not influence the observed responses in LV volumes and functional parameters. High LV mass index was found in all seven divers at baseline with an average of around 260 g, indicating athlete's heart-like adaptation due to regular sports activity in these professional military divers.

Protocol 2. Responses in CO, SV and HR were similar between protocols 1 and 2 (data in Table 4). RV-EF decreased in both dives (without and with antioxidant treatment). However, 3 days of monitoring of the divers

showed decreased right ventricular systolic function not improved by vitamin C and E ingestion that persisted until the second day (Table 4).

LV-ESV and LV-EDV were increased in both groups after dives and returned to baseline values before the next day (Table 4). Markers of left ventricular systolic function, namely LV-EF and endo FS, decreased in dives without and with antioxidant treatment (Table 4 and Fig. 3). In dives without vitamin C and E administration, left ventricular systolic function showed a statistical difference from baseline during all three days of observation. Acute administration of antioxidants improved recovery of the left ventricular systolic function (Table 4 and Fig. 3).

Discussion

Three important messages emerge from this study. First, a single field dive with compressed air is associated with reduced function of the heart and the brachial artery endothelium together with an increase in pulmonary artery pressure in man, similar to what was seen in a previous study (Dujic *et al.* 2006*a*). Second, several cardiovascular functional parameters were not reversed to baseline 48–72 h after the dive with preceding placebo administration, indicating a longer lasting deleterious effect of a single dive. Third, acute antioxidant treatment with vitamins C and E reverses brachial endothelial dysfunction, while the reduction in the post-dive heart and pulmonary artery function is unaffected.

Endothelial function of the brachial artery

Recently, we reported that a simulated dive, producing low bubble loads, caused resting brachial vasodilatation and a reduction in arterial endothelial function and attributed these changes to hyperoxia and bubble formation (Brubakk *et al.* 2005). In addition, divers breathing 60 kPa oxygen for 80 min had a significant increase in arterial diameter with a non-significant reduction in flow-mediated dilatation (FMD). Others have supported our findings of unchanged vascular responses with hyperoxia in healthy adults (Waring *et al.* 2003). In the present study, a larger reduction in post-dive FMD (74%) was noted than in our previous simulated dives (42%). FMD is most probably mediated by nitric oxide (NO) produced by the endothelial cells, as the response can be nearly completely abolished by N^{ω}-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor (Mullen *et al.* 2001). Since divers are exposed to hyperoxia during diving (80 kPa oxygen for 30 min in the current diving profile), it seems reasonable to use antioxidants in order to improve diving-induced endothelial dysfunction. We have recently shown that 4 weeks of oral vitamin C (1 g day^{-1}) and E (400 IU day^{-1}) supplementation prevented the increase in resting vessel diameter and significantly reduced post-dive decrease in FMD (Obad *et al.* 2006). The findings in this study, by using a single oral antioxidant combination composed of 2 g of vitamin C and 400 IU vitamin E, confirms this observation, showing that even a single dose of antioxidants reversed diving-induced changes in endothelial function. This finding was later confirmed with randomized placebo-controlled study design. Most investigations have tested the beneficial effect of acute vitamin C, applied either intra-arterially or orally, in similar dosage used in this study. Our data support the hypothesis that oxidative mechanisms play a major role in the changes observed after a single air dive.

While hyperoxia seems to play a major role in reducing FMD after a dive, it is probably not the only effect, as the antioxidants were able only partly to eliminate this effect. Venous bubble formation may conceivably have an effect on arterial endothelial function. We have previously shown in the rabbit that low bubble load will lead to endothelial dysfunction in the pulmonary artery between 1 and 6 h after exposure to infused bubbles (Nossum *et al.* 2002). Activation of endothelium in the venous circulation produces endothelial microparticles that can initiate endothelial dysfunction at remote sites (Brodsky *et al.* 2004). These microparticles have a size of a few micrometres and could possibly pass through the lung filter and

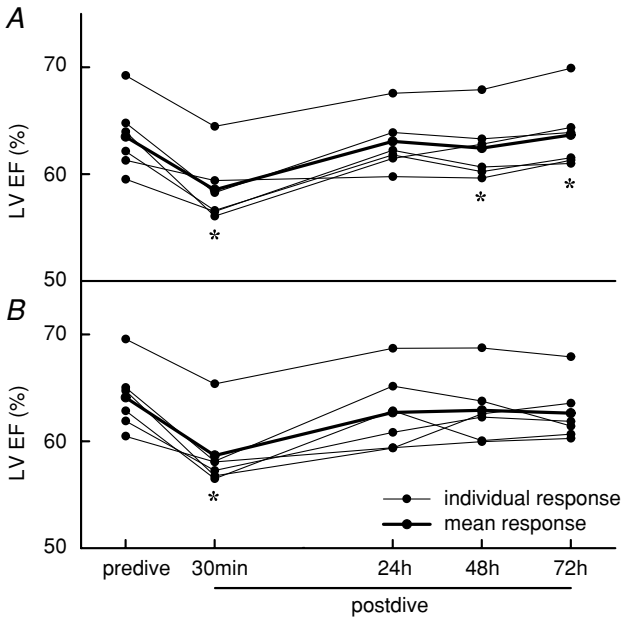

Figure 3. Change of left ventricle (LV-EF) systolic function at pre-dive and post-dive (30 min, 24 h, 48 h and 72 h) period for control dive (*A*) and dive after application of antioxidants (*B*) in protocol 2

Values are represented as individual responses (thin lines) and mean response (thick line) for both dives; *significant difference ($P < 0.05$) between pre-dive and post-dive values.

enter the arterial system. Therefore, changes in arterial endothelial function can occur without direct contact with the bubbles. It is worth mentioning that a positive, but non-significant, relationship between the reduction in FMD and bubble formation was seen in our previous study (Brubakk *et al.* 2005). As these relationships are based on few data, the observations must be viewed with considerable caution.

FMD did not return to baseline values 24 h after the first dive in the current study. Before the second dive FMD was 5.1%, which was still 37% below the response seen before the first dive. In the subsequent experimental design, post-dive FMD was investigated up to 3 days after the first dive. FMD recovered 3 days after the dive without antioxidants, whereas in the dive with antioxidants this occurred much faster (1 day). Since most recreational dives are conducted as a multi-day diving series (e.g. 4–5 dives in a week), it would be interesting to investigate in future studies the cumulative negative effects of diving on arterial endothelial function. The increased oxidative stress is counteracted by efficient antioxidant defence mechanisms. Acute negative effects of single dives on endothelial function may be offset by up-regulation of endogenous antioxidant mechanisms, so that the acute increase in oxidative stress and inflammation may not persist. At present this is unknown.

Pulmonary artery pressure

This study confirms our previous findings of increased PAP after a single open sea air dive to 30 m of sea water in experienced divers (Dujic *et al.* 2006*a*). These observations are different from what was seen in previous studies using a simulated hyperbaric (Valic *et al.* 2005) or hypobaric protocol (Diesel *et al.* 2002), where no PAP increase could be seen. The PAP increase was accompanied by an increased right ventricular end-diastolic and end-systolic volume and a reduced ejection fraction and cardiac output, as was reported previously (Dujic *et al.* 2006*a*). At present the mechanism causing increases in PAP and pulmonary vascular resistance (PVR) after only a single field dive is unknown. Cold sea water and immersion potentiate greater central pooling of blood in the thoracic area than in dry dives. This leads to an increase in right ventricular dimensions and to an increase in PAP and PVR to a degree that in some cases even can lead to pulmonary oedema (Pons *et al.* 1995). Gas bubble formation seems to play a minor role, as bubble formation in this study was moderate and significant pressure increases were seen in divers with no detectable bubbles. It must, however, be borne in mind that the detection threshold for vascular gas bubbles probably is in the range of 20–30 μm, so that smaller bubbles may still be present. Venous bubbles lodging in the pulmonary circulation may cause mechanical, humoral and biochemical effects. Humoral factors, like tromboxane A2, histamine, endothelin and serotonin, have been implicated in pulmonary microembolization by bubbles (Malik, 1983). In addition, a significant reduction in arterial P_{O_2}, on average 20 mmHg, was found after dives with air decompression, together with a decrease in diffusing lung capacity for CO (Dujic *et al.* 1993). Alveolar hypoxia is associated with pulmonary vasoconstriction due to increased calcium entry through receptor- and store-operated calcium channels located in endothelial caveolae (Murray *et al.* 2006).

Endothelial cells of the pulmonary vasculature are one of the primary targets of the hyperoxic insult resulting in extensive vascular leakiness, further augmenting oxidative injury of the lung tissue (Parinandi *et al.* 2003). As mentioned previously, divers are exposed to hyperoxia during diving. We found that an increase in PAP was the same in both dives with and without antioxidants, indicating that oxidative stress is not an important factor in diving-induced changes in the pulmonary circulation. This finding was confirmed later with a follow-up study. However, we found that PAP did not return to the pre-dive values up to 2 days after the dive, again suggesting longer lasting effects. Furthermore, this effect is independent of venous gas bubbles since the similar bubble grade was noted after both dives. Therefore, other causes of pulmonary vasoconstriction are probably involved (humoral factors, hypoxia and immersion).

Heart function

We have shown that a single open sea air dive is associated with acute, post-dive depressions in lung respiratory function and cardiac output (Dujic *et al.* 2005*a*). Reduction in cardiac output was due to a decrease in stroke volume and heart rate, in the presence of increased systemic vascular resistance and unchanged arterial blood pressure (Dujic *et al.* 2005*a*). A reduction in AccT/RVET ratio after control dives in the current study (indicating an increase in mean PAP) correlated negatively with right ventricle ejection fraction, suggesting increased acute right ventricular afterload. Marabotti *et al.* (1999) have also found that a recreational scuba dive is associated with a right ventricular overload and an impairment of both RV and LV diastolic performance 2 h after a dive in subjects who produced venous gas bubbles. In addition, a reduction in LV function was also seen in divers without detectable bubbles. Pro-brain natriuretic peptide was increased up to 4 h after 1 h scuba dives at 10 msw depth (Gempp *et al.* 2005), suggesting that diving involves a mechanical strain on the heart, since this peptide is used as an index of ventricular function (Sullivan *et al.* 2005). In animal models, the decrease in cardiac output after dives was related to reduced venous return and increased afterload

of the right and/or left ventricle (due to increased PAP and systemic vascular resistance, respectively) (Bove *et al.* 1974; Vik *et al.* 1993; Butler *et al.* 1989). In the present study we found additionally evidence of LV systolic myocardial dysfunction (reduction in ejection fraction and increase in end-systolic volume) and reductions in global and regional contractility indices. This may be due to reduced contractility and increased afterload, which was found in our previous report (Dujic *et al.* 2006*a*). The observation that the reduced post-dive heart function did not return to baseline values 24 h later indicates longer lasting effects. This was later expanded with a follow-up study in which we investigated cardiovascular function up to 3 days after a dive with and without preceding antioxidants. Most cardiovascular parameters returned to the baseline value within 24 h, but for some (e.g. FMD, AccT/RVET, LV-EF, endoFS and RV-EF) it took another 24 h. This would suggest that even a single dive could have negative effects on cardiovascular function lasting several days.

The endothelial dysfunction observed post-dive in our previous studies (Brubakk *et al.* 2005; Obad *et al.* 2006) and in this report is probably also present in the heart, as a reduction in endothelial function is an important factor in heart failure (Landmesser & Drexler, 2005). In this study FMD reduction after the control dive correlated significantly with right ventricle end-systolic volume, suggesting similar mechanisms. However, acute negative effects of diving on heart function were also present with acute antioxidants whereas this was not the case with arterial endothelial function, suggesting other underlying mechanisms for the transient negative effect of diving upon heart function. This should be explored in future studies.

In summary, acute antioxidants have a beneficial effect on the brachial artery endothelial function after diving. However, this treatment does not attenuate negative changes on heart and pulmonary artery function. Functional alterations in the cardiovascular system after diving are not fully reversed within 24–72 h after a single dive, suggesting longer lasting negative effects.

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