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Successive deep dives impair endothelial function and enhance oxidative stress in man

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Summary

The aim of this study was to assess the effects of successive deep dives on endothelial function of large conduit arteries and plasma pro-oxidant and antioxidant activity. Seven experienced divers performed six dives in six consecutive days using a compressed mixture of oxygen, helium and nitrogen (trimix) with diving depths ranging from 55 to 80 m. Before and after first, third and sixth dive, venous gas emboli formation and brachial artery function (flow-mediated dilation, FMD) was assessed by ultrasound. In addition, plasma antioxidant capacity (AOC) was measured by ferric reducing antioxidant power, and the level of oxidative stress was assessed by thiobarbituric acid-reactive substances (TBARS) method. Although the FMD was reduced to a similar extent after each dive, the comparison of predive FMD showed a reduction from 8·6% recorded before the first dive to 6·3% before the third ($P = 0·03$) and 5·7% before the sixth dive ($P = 0·003$). A gradual shift in baseline was also detected with TBARS assay, with malondialdehyde values increasing from $0·10 \pm 0·02$ μmol l^{-1} before the first dive to $0·16 \pm 0·03$ before the sixth ($P = 0·005$). Predive plasma AOC values also showed a decreasing trend from $0·67 \pm 0·20$ mmol l^{-1} trolox equivalents (first day) to $0·56 \pm 0·12$ (sixth day), although statistical significance was not reached ($P = 0·08$). This is the first documentation of acute endothelial dysfunction in the large conduit arteries occurring after successive deep trimix dives. Both endothelial function and plasma pro-oxidant and antioxidant activity did not return to baseline during the course of repetitive dives, indicating possible cumulative and longer lasting detrimental effects.

Introduction

During a self contained underwater breathing apparatus (SCUBA) dive, inert gas (usually nitrogen) is taken up by the tissues proportionally to the diving depth and exponentially to the time spent under increased ambient pressure. During ascent and after surfacing, the accumulated gas must be eliminated and some of it is released in the form of emboli. It is generally considered that these venous gas emboli (VGE) are the major contributors for triggering the decompression sickness (DCS), which can be presented with symptoms ranging from skin rash and joint pain to severe neurological dysfunction and death. However, in most cases, VGE are 'silent', occurring without any acute clinical signs and are assumed to have no negative effects. Nevertheless, it was shown that injection of VGE in the absence of a dive can cause a reduction in pulmonary artery endothelial function in pigs and rabbits (Nossum *et al.*, 1999, 2002). Similarly, diving-induced VGE were related to an asymptomatic reduction of the endothelial function in humans as assessed by flow-mediated dilatation (FMD) of the brachial artery (Brubakk *et al.*, 2005). This effect lasted for 3 days after a single air dive and was partially reversed by the acute and a long-term predive supplementation of antioxidants (Obad *et al.*, 2007a,b), implicating oxidative stress as an important contributor to the postdive endothelial dysfunction.

With the use of various breathing gas mixtures such as trimix (composed of oxygen, helium and nitrogen), recreational and professional divers are enabled to dive deeper than with compressed air and, despite using breathing mixtures with lower percentage of oxygen, they are frequently exposed to even higher absolute levels of oxygen. Hyperoxia increases the formation of reactive oxygen species (ROS) and can reduce the bioavailability of nitric oxide (NO), either by rapidly reacting with it (Rubanyi & Vanhoutte, 1986) or by impairing the function of NO synthase through the interaction with its cofactor tetrahydrobiopterine (Mayer & Andrew, 1998). Con-

sidering the physiological actions of NO in the vasculature, both effects can compromise the vascular function. Furthermore, since most of the recreational dives are conducted as a multi-day diving series (e.g. 4–5 dives per week), the relevant question about the possibility of the accumulated negative effects of repeated diving on arterial endothelial function emerges. Thus, the aim of the present study was to investigate the endothelial function, the level of oxidative stress and plasma antioxidative responses before and after repetitive deep trimix dives in the sea water.

Materials and methods

Findings presented in the manuscript represent an independent subset of data that was collected as a part of a larger study.

Study population

Seven experienced male divers aged 38·4 ± 7·4 years, with average body mass index of 25·8 ± 2·3 kg m^{-2} and height 1·8 ± 0·1 m took part in the study. Forced vital capacity was 105·6 ± 10·8%, and forced expiratory flow in the first second was 104·8 ± 7·0% of predicted values. All participants were apparently healthy non-smokers with normal blood chemistry, and at the time of the study had a valid medical certificate for diving. 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 potential risks were explained to the participants in detail, and they gave written informed consent before the experiment.

Location of the study and a dive protocol

The diving site was located in the relative vicinity of the field laboratory, and divers were transported to the site by a powerboat during a 20–30 -min ride. The site was chosen because it allowed us to perform dives of the suitable depth and duration. Sea temperature at bottom and at the decompression stop was 17°C for all dives, and outside temperature was 15-18°C. Divers were equipped with dry suits and regularly serviced open-circuit breathing equipment. The participating divers were active members of the Croatian Search and Rescue Unit, and the study was performed during their scheduled exercise in technical diving with nitrox and trimix. Each diver performed six dives in six consecutive days (one dive per day) with the diving depth ranging from 55 to 80 metres of sea water (msw). The diving profiles and the breathing gas mixtures were determined by the group leader according to the diving exercise requirements. The bottom times varied between 7 and 13 min, and total dive time including decompression varied between 65 and 75 min. Trimix 14/45 (14% O_2, 45% He and 41% $N_{2)}$ was used for the descent and ascent up to 21 msw in the first three and up to 39 msw in the last three dives. Trimix 30/20 (30% O_2, 20% He and 50% N_2) was used during decompression from 39 to 21 msw in the last three dives. Nitrox 50 (50% O_2 and 50% N_2) was used during decompression from 21 msw until resurfacing in all the dives. A descent rate of 10 m min^{-1} and an ascent rate of 9 m min^{-1} to a decompression stop were monitored by a Galileo Sol diving computer (Uwatec, Johnson Outdoors Inc., Racine, WI, USA). During the decompression period, divers were told not to perform any exercise, as we have shown that exercise during decompression significantly reduces the emboli grade (Dujic *et al.*, 2005). The heart rate was continuously monitored in all divers during the dives with Polar belts (Polar Electro, Kempele, Finland). Decompression profiles were determined using V-planner software according to Varying Permeability 1 Model (VPM-B) (Yount & Hoffman, 1986).

Timeline of measurements

Echocardiographic parameters (emboli grade, FMD) and plasma antioxidant and pro-oxidant responses were assessed in all divers on days 1, 3 and 6. All measurements (except the emboli grade that was assessed only postdive) were performed before and after each dive at approximately same time of day (morning) and within 60 min upon surfacing.

Detection of venous gas embolism

Sixty minutes postdive, the 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 ultrasonic scanner (GE, Milwaukee, WI, USA). High quality images were obtained in all subjects, and gas emboli 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). 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 (Raitakari & Celermajer, 2000). This method measures the FMD and determines the arterial response to reactive hyperemia (Corretti *et al.*, 2002). The subjects were placed in a quiet room with temperature about 22°C and were resting for 15 min in a supine position before the measurements. Participants were tested at the same time of the day to account for diurnal variation in endothelial function. All participants were asked to refrain from caffeine and strenuous exercise for at least 12 and 48 h before testing, respectively. Measurements were performed with 5·7–13·3 MHz linear transducer using a Vivid 3 Expert as described previously (Obad *et al.*, 2007a,b). Briefly, 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. Once the basal measurements were obtained, arterial occlusion was created by inflating a cuff placed on the upper arm to 240 mmHg for 5 min. After 5 -min inflation, the cuff was deflated producing a brief high-flow state resulting in artery dilatation because of increased shear stress. FMD is most probably mediated by NO produced by the endothelial cells, as the response can be nearly completely abolished by l-NMMA (Mullen *et al.*, 2001). 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. FMD was calculated as the percentage increase in brachial artery diameter from the resting state to maximal dilatation.

Chemicals

Trolox, 2,4,6–tri(2-pyridil)–s–triazine (TPTZ), ferric chloride hexahydrate, hydrochloric acid, 1,1,3,3-tetrametoxypropane, sodium acetate trihydrate and glacial acetic acid were purchased from Sigma-Aldrich Chemie (Steinheim,Germany), thiobarbituric acid (TBA) from Fluka Chemie GmbH (Buchs, Switzerland), tricloracetic acid from Panreac Quimica S.A.U (Barcelona, Spain). Deionized (Milli Q) water was used for preparation of all solutions and reagents.

Plasma antioxidant capacity

The antioxidant capacity (AOC) of plasma was measured by ferric reducing antioxidant power (FRAP) assay. In this assay, antioxidants are evaluated as reductants of Fe^{3+} to Fe^{2+}, which is chelated by TPTZ to form a Fe^{2+}–TPTZ complex absorbing at 593nm (Benzie & Strain, 1996). Absorbance was monitored by UV–VIS spectrophotometer (Specord 200, Analytik Jena Inc., Jena, Germany), equipped with a six-cell holder and a thermostatically controlled bath. All measurements were carried out in triplicate. Results were compared with a standard curve prepared daily with different concentrations of trolox, a water-soluble analogue of vitamin E, and expressed as mmol/L trolox equivalents.

Thiobarbituric acid-reactive substances (TBARS)

TBARS assay is based on reaction of malondialdehyde (MDA), one of end products of lipid peroxidation, with TBA (Esterbauer & Cheeseman, 1990). To correct for background absorption, the absorbance values at 572 nm were subtracted from those at 532 nm, which represent the absorption maximum of the TBA:MDA adduct (Lapenna *et al.*, 2001). Absorbance was monitored by the above-mentioned UV-spectrophotometer. All measurements were carried out in triplicate. Results were compared with a standard curve prepared daily with different concentrations of MDA and expressed as μmol/L MDA.

Statistical analysis

Data are given as mean ± standard deviation (SD). Normality of the distribution was confirmed for all parameters using Kolmogorov-Smirnov test. All the comparisons of parameters measured for a single dive (predive and postdive values) were performed using Student's t test for paired samples. Emboli grades are presented as median (25–75% quartile range) and were compared using non-parametric Friedman analysis of variance. In case of a significant difference, the Wilcoxon sign rank test was applied for the particular comparison. To examine whether the parameters changed over the course of consecutive dives (potential cumulative effects), the ANOVA for repeated measures with Bonferroni *post hoc* analysis was used. The limit of significance was set at $P<0{\cdot}05$. Analyses were carried out using Statistica 7.0 software (Statsoft, Inc., Tulsa, OK, USA).

Results

All seven divers successfully completed the study, and no symptoms/signs of DCS were reported. Venous gas embolism was found after each dive, with the highest bubbling in the third dive compared to the first and sixth dive 2 (1·0–2·5), 3 (2·5 – 3·5), 1(1·0 – 1·8), respectively ($P = 0{\cdot}043$). Mean brachial artery diameter showed a slight increase after the assessed dives that was significant after the sixth dive (3·84 ± 0·5 mm versus 3·89 ± 0·6 mm ($P = 0{\cdot}29$); 3·86 ± 0·5 mm versus 3·91 ± 0·5 mm ($P = 0{\cdot}10$); and 3·77 ± 0·4 mm versus 3·83 ± 0·4 mm ($P = 0{\cdot}025$); predive versus postdive values in the first, third and the sixth dive, respectively). No change in baseline predive brachial artery diameter over these multiple dives was detected. Brachial artery FMD decreased from 8·6 ± 1·3% to 3·0 ± 0·8% ($P<0{\cdot}001$) after the first dive. Similar response was noted after the third dive (from 6·3 ± 0·8% to 2·9 ± 0·9%, $P<0{\cdot}001$) and the sixth dive (from 5·7 ± 0·6% to 2·6 ± 0·2%, $P<0{\cdot}001$) (Fig. 1). In comparison with the baseline FMD

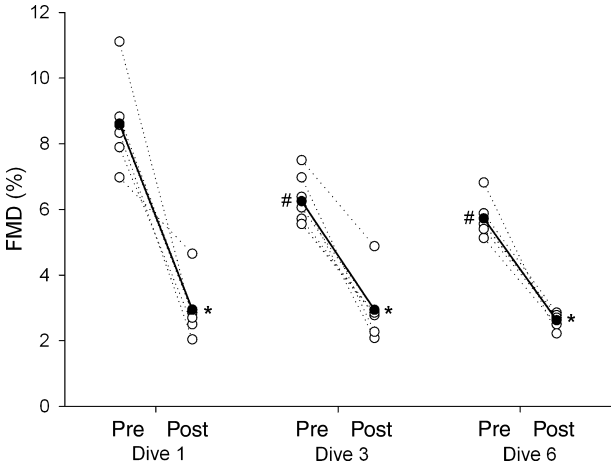

Figure 1 Change in flow-mediated dilation (FMD) before (PRE) and after (POST) each dive. Dotted lines represent individual responses, thick solid lines denote average response for an individual dive. *$P<0{\cdot}05$ versus predive value on the same day; #$P<0{\cdot}05$ versus predive on day 1.

(assessed before the first dive), predive FMD was significantly decreased before the third and the sixth dives. Values for normalized FMD (taking into account the level of shear stress) yielded similar results (data not shown).

TBARS assay, an indicator of the oxidative stress level, revealed a significant increase in lipid peroxidation only after the first dive (Fig. 2). Moreover, baseline values increased from 0·10 ± 0·02 μmol l^{-1} MDA before the first dive to 0·16 ± 0·03 μmol l^{-1} MDA before the sixth dive ($P = 0·004$).

Plasma AOC, as assessed by the FRAP assay, was unchanged after the dives (Fig. 3). However, a declining trend in AOC was observed between the values detected before the first dive and the predive values on the last day of the series (0·67 ± 0·20 versus 0·56 ± 0·12 mmol l^{-1} Trolox equivalents; respectively ($P = 0·08$)).

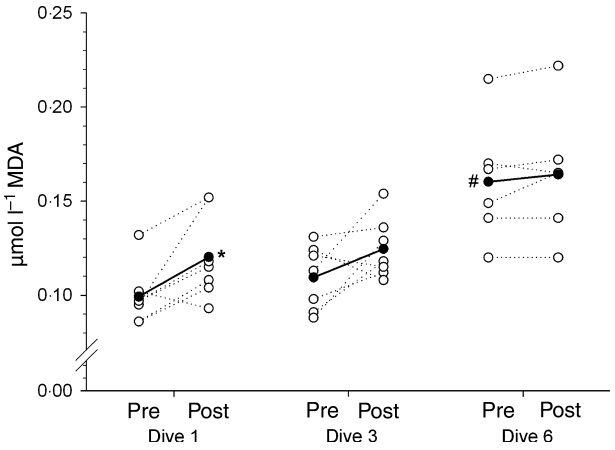

Figure 2 Change in lipid peroxidation, assessed by thiobarbituric acid-reactive substances assay, indicating the amount of oxidative stress before (PRE) and after (POST) each dive. Dotted lines represent individual responses, thick solid lines denote average response for an individual dive. MDA, malondialdehyde. *$P<0·05$ versus predive value on the same day; #$P<0·05$ versus predive on day 1.

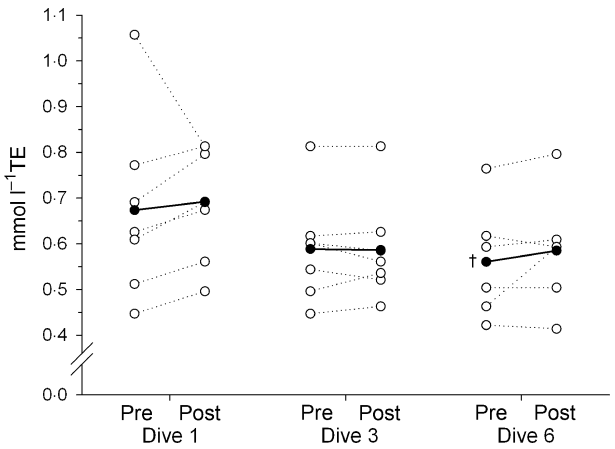

Figure 3 Change in plasma antioxidant capacity, as assessed by the ferric reducing antioxidant power assay, before (PRE) and after (POST) each dive. Dotted lines represent individual responses, thick solid lines denote average response for an individual dive. TE, trolox equivalents. †$P = 0·08$ versus predive on day 1.

Discussion

This study is the first to report acute endothelial dysfunction after successive trimix dives in sea water. Furthermore, the predive endothelial function decreased for about 35% from the first to the sixth consecutive dive, suggesting cumulative negative effects. Concomitant cumulative effect in the level of oxidative stress was also present after all investigated dives.

Endothelial dysfunction represents one of the earliest events in the pathogenesis of cardiovascular disease and is observed in several clinical conditions. Having in mind that SCUBA diving is performed regularly by millions of recreational and professional divers worldwide, as well as that the diving population is ageing and may include unfit individuals or even those with chronic diseases such as hypertension, the potential acute or long-term negative cardiovascular effects on human health require investigation. In addition to endothelial dysfunction, it has been well documented that a single SCUBA dive is associated with other asymptomatic changes of cardiovascular and lung function such as an increase in pulmonary artery pressure (Dujic *et al.*, 2006a), right ventricular overload (Marabotti *et al.*, 1999) and a reduction in left ventricular contractile function (Obad *et al.*, 2007a,b).

The mechanisms that cause endothelial dysfunction in the peripheral arteries after air and trimix diving are presently unknown. Most likely candidates are hyperoxia-induced oxidative stress and peripheral vasoconstriction, endothelial activation with induced adhesion molecules expression and vascular gas emboli, which may further exacerbate the impaired endothelial homeostasis by ischaemia/reperfusion, physical contact or by an increased shear stress. Indeed, hyperoxia was shown to play a major role in reducing the FMD after an air dive, because antioxidant agents were able to attenuate this effect (Obad *et al.*, 2007a,b). Breathing compressed air, trimix or nitrox gas mixtures with even higher absolute levels of oxygen that can even come close to the toxicity cut-off, enhances the ROS generation and augments the oxidative stress (Bearden *et al.*, 1999; Ferrer *et al.*, 2007). In the current study, the average oxygen partial pressure that the divers were exposed to during the underwater sojourn was 0·83 ± 0·25 atm, 0·85 ± 026 atm and 0·99 ± 0·25 atm for the first, third and the sixth dive, respectively, with the peak oxygen partial pressure sometimes reaching 1·5 atm. However, despite differences in oxygen tension between trimix dives from the current study and air dives from our previous studies, we found no significant difference in the extent of FMD reduction between these different dives (Obad *et al.*, 2007a,b). Possible explanation for this finding is that the maximum effect was already reached by the oxidative stress the divers were exposed during an air dive to 30 m (from our previous study) and that further increase in oxidative stress cannot additionally decrease the endothelia function. However, the role of hyperbaric oxygen in development, endothelial dysfunction is not yet clear cut. In a recent study comparing endothelial function following chamber dives with air and oxygen, a reduction in endothelial function was

found only after air, but not after oxygen dive (Madden *et al.*, 2010). According to that study, it appears that hyperbaric oxygen alone is not sufficient stress to cause endothelial dysfunction and that reduction in endothelial function requires presence of nitrogen emboli (although VGE were not assessed in that study). In another study by the same group (Vince *et al.*, 2009), no increase in lipid peroxidation as assessed by TBARS measurement was seen after both air and oxygen chamber dives. A possible explanation for the discrepancy between our results and results from these studies is that our dives were performed in the field with all other influences that a field dive brings (effect of immersion, exercise, temperature, resistance to breathing, psychological stress), or other factors (study population, timing of TBARS measurement, diet, etc.).

Biological effects of the highly reactive ROS depend on the fine balance between their production and elimination. ROS are known to react with NO, reducing its bioavailability (Rubanyi & Vanhoutte, 1986). This is partly counterbalanced by the increased inducible NO synthase expression and nitrite levels (Ferrer *et al.*, 2007), thus buffering the hyperoxic peripheral vasoconstriction. The hyperoxia associated with SCUBA diving and resulting oxidative stress was also shown to be ameliorated by increased expression of antioxidant enzymes (catalase, glutathione peroxidase) (Ferrer *et al.*, 2007; Sureda *et al.*, 2009). In this study, we assessed the antioxidative capacity in plasma using FRAP assay and found no significant change between predive and postdive values (Fig. 3). However, there was a tendency for cumulative AOC reduction, suggesting a potential depletion of antioxidants during the course of repetitive dives. This is in accordance with a previous study by Benedetti *et al.*, where a similar reduction in antioxidant status was found in patients undergoing hyperbaric oxygen therapy.(Benedetti *et al.*, 2004) This effect, together with the observed gradual increase in lipid peroxidation (as assessed by TBARS assay, Fig. 2), might be an important player in the cumulative FMD decrease during the course of repetitive dives.

Another factor that may contribute to the endothelial dysfunction is endothelial damage elicited by the venous gas emboli. Previous studies showed that infusion of gas emboli impaired the endothelial function in the pulmonary artery in rabbits (Nossum *et al.*, 2002). As venous emboli are trapped in the pulmonary circulation, they were presumed to have no further effects on the arterial side. Furthermore, it is assumed that, for the emboli to affect the systemic arteries, a right-to-left shunt, such as in the persistent foramen ovale, was necessary. In the current study, arterialization of VGE was detected only once; therefore, we believe that direct interaction of VGE with endothelium on systemic arteries in general did not occur. However, it is possible that changes in endothelial function occur without the arteries being in direct contact with the emboli. Studies have shown that activated endothelium releases endothelial microparticles and they can initiate endothelial dysfunction at remote sites (Brodsky *et al.*, 2002). Endothelial microparticles are size of a few microns and could pass the lung filter and enter the arterial system. Recently, an increased release of vascular cell adhesion molecule-1 (VCAM-1) positive endothelial microparticles following simulated chamber air dive has been reported (Vince *et al.*, 2009). VCAM-1 is expressed on endothelium that is activated by an insult to the vasculature and is considered to be a marker of pro-inflammatory endothelium. In the same study by Vince *et al.*, increase in microparticles was not observed when hyperbaric oxygen was used instead of air, suggesting that release of microparticles is indeed emboli related. In another study, increase in endoglin-positive microparticles was also seen after air dive, but not hyperbaric oxygen dive (Madden *et al.*, 2010). Although it is generally accepted that VGE are the major causative factor in development of a DCS by crossing to systemic arterial side, occluding vessels and causing multiple ischaemia/reperfusion injuries, the relationship between the DCS and the VGE is still ambiguous. VGE can be detected after majority of recreational and professional dives. However, they are a poor predictor of the likelihood of the DCS symptoms (Eckenhoff *et al.*, 1990). Moreover, DCS can occur without detected VGE (grade 0) or, surprisingly, it may not be present even with VGE grade 5 ('white-out' of the right heart) (Bakovic *et al.*, 2008). Thus, although the absence of VGE is a good indicator of decompression safety, the sole presence of VGE is not enough to cause DCS. Furthermore, our newest unpublished experimental data indicate that many field dives that result in arterialization of VGE remain completely asymptomatic. One of the recently emerged hypotheses is that DCS is the disease of vascular endothelium and that VGE are only the exacerbating factor (Madden & Laden, 2009). It may well be possible that DCS (in cases where it occurs without the apparent violation of diving protocol) is an idiosyncratic reaction, where under certain conditions and in susceptible individuals, the interaction between VGE and endothelium starts the vicious cycle resulting in the occurrence of the DCS symptoms. A line of evidence supporting such hypothesis is in the study by Ward *et al.* (Ward *et al.*, 1987) where susceptibility to DCS was correlated with sensitivity to complement activation by air emboli.

In the current study, we observed a reduction in the number of VGE occurring postdive, as the diving series progressed in consecutive days. Also, as indicated before FMD was continuously reduced postdive, meaning that the bioavailability of NO (that is supposed to be the mediator of the FMD) is reduced. Our previous studies have shown that administration of NO reduces the number of VGE postdive (Dujic *et al.*, 2006a,b). The logical question that follows is, if the NO bioavailability is reduced (as indicated by the FMD results), what causes the diving acclimatization. In a recent study by Pontier *et al.*, similar observations were made (Pontier *et al.*, 2009). There, they measured VGE production before and after 3 months of intense dive training and found a significant reduction in the emboli production. At the same time, endothelial function was monitored and was found unchanged after this dive training period. Therefore, they also conclude that the reduction in VGE production cannot be attributed to changes in endothelial function and consequently NO production, but is the result of

other biochemical mechanisms. Some of the potential candidates for diving acclimatization are heat shock proteins (HSP). HSP were shown to attenuate the air emboli-induced acute lung injury (Huang *et al.*, 2003). Moreover, a DCS-inducing rapid decompression 24 h prior to another dive, increased expression of HSP70 and attenuated the severity of neurological DCS caused by the subsequent exposure to high pressure and rapid decompression in rabbits (Su *et al.*, 2004).

If our results are looked from another perspective, whereby VGE are thought to be responsible for the endothelial dysfunction, the question is how the reduced number of emboli results in the same level of endothelial dysfunction. A possible explanation is that endothelial dysfunction lasts for at least 24 h after a single dive (Obad *et al.*, 2007a,b). Therefore, even a smaller emboli load in the consecutive dive could cause a smaller per cent change in FMD postdive, which adds up to the already reduced baseline endothelium function from the previous dive/day.

In conclusion, the present study demonstrated that diving activities associated with the use of increasingly popular gas mixtures (trimix) acutely impair the endothelial function of the large conduit arteries and augment the generation of the ROS. These effects become even more accentuated during the course of multiple repetitive dives and, although still asymptomatic, pose a potential risk for development of acute cardiovascular incidents. Therefore, a caution must be made when planning and performing multi-day diving schemes, especially in individuals with additional factors indicating a compromised cardiovascular system homeostasis. Furthermore, recent hypotheses based on endothelium dysfunction mechanism for development of DCS suggest that evaluation and calculation of endothelial damage might become necessary when assessing the risk and planning the diving activities. (Madden & Laden, 2009)

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