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ORIGINAL ARTICLE

Effect of repetitive SCUBA diving on humoral markers of endothelial and central nervous system integrity

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Abstract During SCUBA diving decompression, there is a significant gas bubble production in systemic veins, with rather frequent bubble crossover to arterial side even in asymptomatic divers. The aim of the current study was to investigate potential changes in humoral markers of endothelial and brain damage (endothelin-1, neuron-specific enolase and S-100β) after repetitive SCUBA diving with concomitant assessment of venous gas bubble production and subsequent arterialization. Sixteen male divers performed four open-water no-decompression dives to 18 msw (meters of sea water) lasting 49 min in consecutive days during which they performed moderate-level exercise. Before and after dives 1 and 4 blood was drawn, and bubble production and potential arterialization were echocardiographically evaluated. In addition, a control dive to 5 msw was performed with same duration, water temperature and exercise load. SCUBA diving to 18 msw caused significant bubble production with arterializations in six divers after dive 1 and in four divers after dive 4. Blood levels of endothelin-1 and neuron-specific enolase did not change after diving, but levels of S-100β were significantly elevated after both dives to 18 msw and a control dive. Creatine kinase activity following a control dive was also significantly increased. Although serum S-100β levels were increased after diving, concomitant increase of creatine kinase during control, almost bubble-free, dive suggests the extracranial release of S-100β, most likely from skeletal muscles. Therefore, despite the significant bubble production and sporadic arterialization after open-water dives to 18 msw, the current study found no signs of damage to neurons or the blood–brain barrier.

Keywords Endothelin-1 · Neuron-specific enolase · S-100β · Decompression · Diving · Arterialization

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Introduction

During SCUBA diving decompression, as partial pressure of inert gas in blood and tissues exceeds ambient pressure, a significant gas bubble production often occurs. The presence of bubbles in the vessels is usually not associated with overt clinical symptoms and majority of dives result in so-called 'silent' bubbles. In most cases, the gas bubbles carried by systemic veins become trapped in the lungs which act as a filter that prevents the bubble passage into the systemic arteries (Levett and Millar 2008). However, occurrence of a large number of bubbles leaving the left heart is linked to the increased risk of decompression sickness (DCS) (Mollerlokken et al. 2012) with neurological type of DCS being its most severe presentation. The neurological DCS presumably arises due to occlusion of systemic arteries, subsequent ischemia and permanent loss of neurons induced by the bubbles that by-passed lung filtration (via patent foramen ovale or intrapulmonary shunts) (Vann et al. 2010). Another possibility for neurological decompression injuries may be endothelial

dysfunction as a consequence of the gas bubble effects on the endothelium (Brubakk and Mollerloken 2009).

Damage to the vascular endothelium by SCUBA diving has been reported in a number of studies with some recent theories speculating that a significant role in DCS is played by the endothelium, rather than strictly bubble-induced microembolizations (Brubakk and Mollerloken 2009; Madden and Laden 2009). Indeed, air diving to 18 msw (meters of sea water) impaired the endothelial integrity with increased shedding of endothelial microparticles into the circulation together with microparticles derived from leukocytes, erythrocytes and platelets (Thom et al. 2012). In addition, endothelial dysfunction was repeatedly shown following either a single or repeated dives with decreased vascular compliance and decreased response of vasculature to shear stress (flow-mediated dilation) (Brubakk et al. 2005; Marinovic et al. 2012; Obad et al. 2010). Endothelin-1, a potent vasoconstrictory endothelium-derived peptide, was shown to contribute both to arterial stiffness (decreased vascular compliance) and endothelial dysfunction (Halcox et al. 2001; Vuurmans et al. 2003) in cardiovascular disease.

We recently showed using higher resolution echocardiography that venous gas bubbles cross to systemic arteries more often than previously reported and more frequently than what can be ascribed to patent foramen ovale (PFO) (Ljubkovic et al. 2010, 2011; Vann et al. 2010), and, on many occasions, divers without PFO exhibited bubble arterialization (Ljubkovic et al. 2012). This significant number of bubble arterializations poses concern that some of these bubbles can end up in central nervous system and cause neurological injury (Ljubkovic et al. 2010, 2011, 2012). In addition, recent studies indicate that there might be subtle neurological damage as a result of cumulative effects of thousands of dives in professional and/or recreational divers showing no symptoms (Erdem et al. 2009; Gerriets et al. 2003; Tripodi et al. 2004).

There exist several serum markers of brain damage that are regularly used in clinical setting, with neuron-specific enolase (NSE) and S-100β being two of these biomarkers (Bottiger et al. 2001). Andersson et al. (2009) reported increased levels of S-100β after apnea in trained breath-hold divers indicating that prolonged voluntary apnea affects the integrity of CNS. Havnes et al. (2010) recently showed an increase in S-100β which correlated with bubble grade in rats exposed to simulated dives in hyperbaric chamber. A recent study investigated the serum levels of S-100β in five SCUBA divers following three repetitive dives using nitrox as a breathing gas (Stavrinou et al. 2011). However, the results were inconclusive due to very limited number of divers, and gas bubbling, a possible factor driving S-100β changes, was not assessed. Also, recent studies point to extracranial sources of S-100β, making interpretation of potential S-100β increases more difficult (Hasselblatt et al. 2004; Missler et al. 2002).

Therefore, the aim of the current investigation was to explore potential changes in humoral markers of endothelial and brain damage (endothelin-1, NSE and S-100β) in a group of SCUBA divers performing several consecutive dives with concomitant detailed assessment of venous gas bubble production and bubble arterialization.

Methods

Study population

This investigation was performed as part of a larger field diving study, with some of the results obtained from this study already being published elsewhere (Thom et al. 2012). Sixteen healthy male divers were included. They were 36.8 ± 1.7-year old with average height of 1.82 ± 0.01 m, weight 89.8 ± 2.5 kg and BMI 27.0 ± 0.7 kg/m^2. Also, percent body fat of the divers was determined using standardized skin-fold measurements with the methodology and detailed anthropometric data regarding this group of divers being demonstrated elsewhere (Thom et al. 2012). None of the divers had PFO [evaluated by the contrast transthoracic echocardiography described elsewhere (Ljubkovic et al. 2012)]. All divers completed the study and no one developed symptoms of DCS. All experimental procedures were conducted in accordance with the Declaration of Helsinki and were approved by the Ethics Committee of the University of Split School of Medicine and DAN institutional review board. Each method and potential risks were explained to the participants in detail and they gave their written consent before the experiment.

Dive protocol

Experimental dives included four dives in 4 consecutive days. Divers used their own equipment consisting of wet suits and SCUBA apparatus, and air was used as a breathing gas. Divers refrained from any diving and swimming activities for at least 3 days before the first dive. All dives were no-decompression to 18 msw with 47 min bottom time (and additional 2-min ascent time). A control air dive to 5 msw was performed on another occasion with 10 of the 16 divers, with diving duration, water temperature and exercise intensity being identical to the 18-msw dives. In all the dives, divers performed moderate exercise with heart rates of 53 ± 10, 53 ± 11 and 47 ± 7 % of the maximum predicted heart rate according to age (values presented for control dive, dive 1 and dive 4, respectively). Galileo dive computers (Uwatec, Johnson Outdoors Inc., Racine, WI, USA) were used to capture dive profiles and

heart rate. The water temperature for all dives was approximately 16 °C. After dives, the subjects were asked to refrain from exercise throughout the monitoring period.

Bubble grade assessment

Within 15 min postdive, the subjects were placed in the left lateral position and a phase-array ultrasonic probe (1.5–3.3 MHz) connected to a Vivid q ultrasonic scanner (GE, Milwaukee, WI, USA) was positioned precordially to obtain a clear four-chamber view of the heart. Bubble grading was assessed by two observers every 20 min for the 2-h postdive period. Gas bubbles were observed as high intensity echoes in cardiac cavities and recorded at rest and after performing three consecutive right arm flexions, and then after three consecutive right leg flexions. Movement was performed to mobilize gas bubbles generated in the venous pathway. Cardiac scanning was maintained for at least ten cardiac cycles postmovement. Bubble grading was performed according to a modified Brubakk scale (Eftedal et al. 2007; Ljubkovic et al. 2011) using the following definition: 0 no bubbles; 1 occasional bubbles; 2 at least one bubble every four cardiac cycles; 3 at least one bubble every cardiac cycle; 4 continuous bubbling with modifiers (4a at least one bubble/cm^2 in all frames, 4b at least three bubbles/cm^2 in all frames, or 4c almost complete whiteout but individual bubbles can be discerned); and 5 "white-out", individual bubbles cannot be seen.

Blood analysis

Venous blood was withdrawn 45 min before and 80 min after dive on the first day and fourth day of a single-dive daily diving series and before and after the control dives. Samples in collection tubes were centrifuged at 3,000 relative centrifugal force (RCF) for 10 min immediately after they clotted at room temperature and serum was then transferred to tubes that were held on ice. The samples were frozen at −20 °C within 60 min of serum collection and stored until analysis.

Endothelin-1 concentrations were determined by The Quantikine Human Endothelin -1 ELISA immunoassay (R&D Systems Inc, Minneapolis, USA). S-100β and NSE concentrations were measured using electrochemiluminescence 'ECLIA' Elecsys S-100 and NSE assays, respectively, using automatic analyser Cobas e 601 (Modular Analytics E170, Roche Diagnostics GmbH, Mannheim, Germany). Creatine kinase CK activities in control dive were measured with Beckman Coulter IFCC recommended method using automatic AU 2700 analyzer (Beckman coulter, Brea, CA, USA). Analyses were performed on all the samples at once.

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. Bubble 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. Linear associations between bubble grades and humoral markers, as well as body weight, BMI, fat content and humoral markers were tested by Pearson correlation analysis. The threshold for significance was set at $P < 0.05$. Analyses were done using Statistica 7.0 software (Statsoft, Inc., Tulsa, OK, USA).

Results

Humoral indicators

Serum levels of endothelin-1, NSE, S-100β and creatine kinase are shown in Table 1. Endothelin-1 and NSE were not significantly different before and after diving. S-100β, although never reaching pathological or borderline levels (above 0.5 or 0.15–0.5 μg/l, respectively) (Ingebrigtsen et al. 1995; Korfias et al. 2007), was significantly elevated after each of the tested dives, including the control dive. Creatine kinase activity following the control dive was also significantly increased, but the values remained within the normal range. Correlation analysis revealed a weak correlation between body weight, fat content and serum S100β

Table 1 Serum concentrations of endothelin-1, neuron-specific enolase (NSE), S-100β and creatine kinase before and after diving

	Predive	Postdive
Endothelin-1 (ng/l)		
Dive 1	3.31 ± 1.53	2.83 ± 1.71
Dive 4	3.31 ± 1.72	3.31 ± 1.30
NSE (μg/l)		
Dive 1	9.62 ± 1.53	10.20 ± 1.28
Dive 4	10.50 ± 1.11	10.83 ± 1.46
S-100β (μg/l)		
Dive 1	0.044 ± 0.01	0.066 ± 0.02*
Dive 4	0.045 ± 0.01	0.061 ± 0.02*
Control dive	0.049 ± 0.02	0.060 ± 0.02*
Creatine kinase (U/l)		
Control dive	131.7 ± 59.0	173.9 ± 110.3*

* $P < 0.05$ versus predive

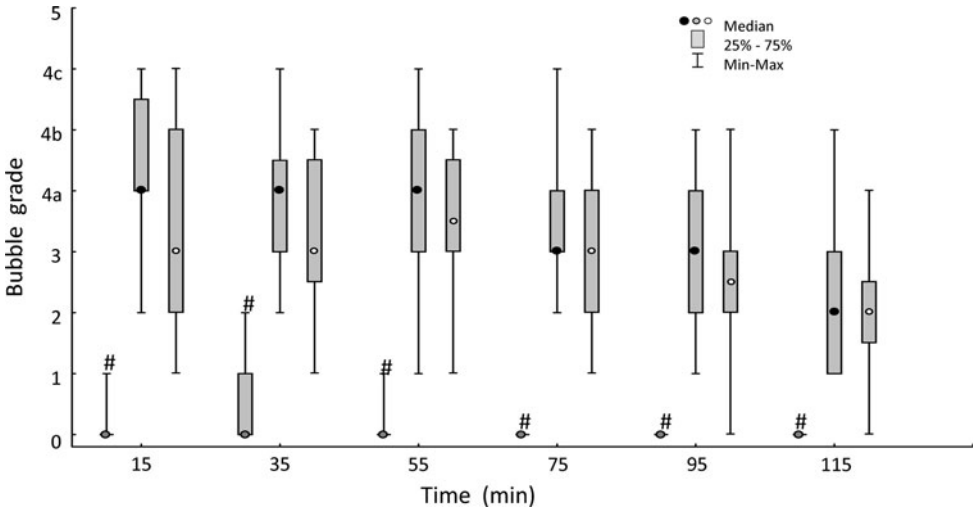

Fig. 1 Shown are medians (*circles*), 25th and 75th quartiles (*shaded squared areas*) and ranges (*error bars*) of bubble grades observed at various timepoints (15–115 min) after control dive (marked by *grey circle*), dive 1 (*black circles*) and dive 4 (*white circles*). $^{\#}P < 0.05$ versus dive 1 and dive 4

levels ($r^2 = 0.4$, $p = 0.0084$ and $r^2 = 0.6$, $p = 0.0004$, respectively). No correlations between BMI, body weight, fat content and other humoral markers (NSE, endothelin-1, creatine kinase) were found.

Bubble grade

Bubble grades at each measured timepoint after control dive to 5 msw, and first and fourth consecutive dive to 18 msw are shown in Fig. 1. At all measured timepoints, there were significantly higher bubble grades after dives to 18 msw than the control dive. There was no difference in bubble grades for any of the timepoints between dives 1 and 4. Bubble arterializations appeared in six divers after dive 1 and in four divers after dive 4. Three of the divers that arterialized after dive 1 also had arterializations after dive 4. As expected, and confirming our previous findings, all cases of arterializations were associated with high bubble grade (grade 4b and higher) in the right ventricle and were more likely to appear in the same group of divers (Ljubkovic et al. 2012). There was no correlation between the extent of bubble production and any of the tested humoral markers. Also, there was no significant difference between divers with bubble arterialization to those that did not arterialize in magnitude of changes (postdive–predive) for any of the parameters (Table 2).

Table 2 Shown are the changes in concentrations [concentration (postdive) − concentration (predive)] of endothelin-1, neuron-specific enolase (NSE), and S-100β in reference to bubble arterialization

	No arterialization	Arterialization
ΔEndothelin-1 (ng/l)		
Dive 1	−0.89 ± 1.73	0.42 ± 2.68
Dive 4	0.05 ± 1.46	0.05 ± 1.70
ΔNSE (μg/l)		
Dive 1	0.50 ± 1.07	0.76 ± 0.52
Dive 4	0.28 ± 1.26	0.38 ± 0.48
ΔS-100β (μg/l)		
Dive 1	0.02 ± 0.01	0.02 ± 0.02
Dive 4	0.01 ± 0.01	0.02 ± 0.01

The control dive to 5 msw is omitted since no arterializations were observed after this dive

Discussion

In the current study, we investigated the potential effect of SCUBA diving on humoral indicators of endothelial and neuronal damage. We used four consecutive dives to 18 msw with moderate exercise load and, despite significant production of venous gas bubbles and even sporadic arterializations, found no change in endothelin-1 or NSE, with concomitant small but significant increase in S-100β in a postdiving period. Control dive to 5 msw without bubble load also increased blood level of S-100β, as well as the serum activities of creatine kinase, suggesting that postdive increases in S-100β serum concentration were not related to intravascular bubbling.

Despite the significant venous bubble load and a dive profile that induced endothelial shedding of microparticles (Thom et al. 2012), we found no significant differences in pre and postdive blood levels of endothelin-1 in both dives 1 and 4. Since excessive production of endothelin-1 is an indicator of altered endothelial function (Iglarz and Clozel 2007), the finding of unchanged postdive endothelin-1 levels suggests that the hypothesized endothelial damage by venous gas bubbles was not sufficient to be reflected by the detectable changes in the blood. In fact, in our recent study using the same dive profile (no-deco air dive to 18 msw) (Marinovic et al. 2012), despite changes in arterial compliance, we found no significant changes in flow-

mediated dilation, indicating borderline alterations in vascular/endothelial function. Nevertheless, exposure of eight divers to hyperbaric air and oxygen at 2.5 ata for 60 min increased the plasma levels of endothelin-1 by approximately 30 % (Lund et al. 1999). The same observation was reported in another study with volunteers subjected to either hyperbaric oxygen or air at 2.8 atm, with no difference in endothelin-1 levels related to breathing gas used (Rocco et al. 2001). Despite the similar level of hyperbaria used in the aforementioned studies as compared to our study, the potential reasons for the differences in results pertaining to endothelin-1 might be due to differences in duration of hyperbaric exposure [60 min in a study by Lund et al. (1999), compared to 47 min in the current study], or longer time that passed between dive and blood withdrawal (80 min in the current study versus 20 min in others). With plasma half-life of endothelin-1 being very short (4–7 min) (Levin 1995), another possible explanation for the lack of endothelian-1 change in our study is that we could have missed detectable endothelin-1 increase in the blood, if endothelin-1 was only transiently increased by some factor acting on the endothelium *during* a dive (e.g. hyperoxia) and not *after* a dive such as venous gas bubbles. On the other hand, in a rat model of DCS, the circulating endothelin-1 levels were unchanged following decompression (Montcalm-Smith et al. 2007).

Besides endothelin-1 being an indicator of vascular function, it was also shown to be increasingly released in acute cerebral ischemia since hypoxia stimulates endothelin-1 synthesis and increases secretion of endothelin-1 from damaged endothelial and neuronal cells (Iglarz and Clozel 2007; Ziv et al. 1992). Therefore, no change in endothelin-1 also might suggest the lack of significant ischemia in the brain.

In addition to endothelin-1, measurements of NSE revealed that its blood levels are unaffected by the diving protocol used in the current study. NSE is the glycolytic dimeric enzyme predominantly found in cytoplasm of neurons and as such, it is not secreted into extracellular fluid by the intact neurons. However, following neuronal damage its concentration increases in blood (Herrmann et al. 2000). Considering that dive profile in this study produced substantial bubbling and arterialization in a significant number of divers, potential bubble-induced change in blood–brain barrier permeability and neuronal damage is not unrealistic. However, serum NSE concentrations were not increased postdive even in subjects with arterialization, indicating that neuronal damage, at least one that can be detected using this marker, did not occur. On the other hand, the peak of NSE rise is reached slowly (Schoerkhuber et al. 1999). For example, NSE levels peaked on third day after temporary global cerebral ischemia induced by cardiac arrest (Rosen et al. 2001). Considering this slow dynamics of the NSE rise, even if asymptomatic postdive neuronal damage existed in the current study, we could have not detected it on day 1 of diving, but rather on day 4 (following 4 days of consecutive diving). However, since we found no pre or postdive increase in NSE on day 4, we did not find any evidence of possible cumulative damaging effect of three previous dives on CNS integrity.

S-100β protein belongs to the family of acidic calcium-binding proteins predominantly expressed by astroglial cells, and also in adipose tissue and skeletal muscle (Hasselblatt et al. 2004), concentration of which increases after various types of brain damage (Bottiger et al. 2001). It is typically found in low concentrations in healthy individuals, with plasma levels being one-third of those found in cerebrospinal fluid. Blood–brain barrier opening would therefore be expected to significantly increase the serum S-100β concentration, making it suitable for use as the indicator of blood–brain barrier integrity and/or brain damage marker (Kapural et al. 2002). A transient small, but significant, S-100β increase was found in trained breath hold divers after apnea, which was ascribed to likely temporary opening of the blood–brain barrier (Andersson et al. 2009). In the current study, we found a small but significant increase in serum S-100β levels after both dive 1 and dive 4, without cumulative effect of consecutive dives, indicating possible blood–brain barrier damage. Although no cumulative effect of repetitive dives as in the study by Stavrinou et al. was found, we did find a significant postdive increase in S-100β. Stavrinou et al. (2011), however, found no significant elevation of S-100β after 3 days of consecutive diving using nitrox gas mixture. Since the divers in study used nitrox (oxygen-enriched mixture), we have a reason to speculate that the bubble load after these dives (although not measured) was lower than in dives performed in our study. In order to test whether the rise of S-100β after diving is related to decompression stress and bubble load, we performed a control dive to 5 msw and found the same increase in S-100β despite the negligible bubble load, indicating the more likely bubble-unrelated release of S-100β. A number of studies indicate the importance of concomitant measurement of creatine kinase together with S-100β to exclude the possibility of the extracranial release of S-100β (Hasselblatt et al. 2004; Missler et al. 2002). Serum S-100β and creatine kinase levels increased in marathon runners postrace while the glial fibrilar acidic protein, a more specific marker of brain injury, did not change, indicating extracranial origin of S-100β (Hasselblatt et al. 2004). Indeed, creatine kinase activity measurements after the control dive showed a significant increase after diving, suggesting the skeletal muscle origin of the S-100β due to swimming or immersion in cold water.

It is important to state that the current study does have certain limitations. First, it was performed in a relatively small number of subjects making it necessary to view the obtained results with caution. However, each predive and postdive measurement was performed in the same individual meaning that each diver was his own control. Also, all the divers performing the control 5-msw dive were recruited from the group that performed the four consecutive dives to 18 msw. In addition, although we did assess the bubble load and arterialization in each diver and in all the dives, we do not know how many (if any) of the bubbles indeed entered the cerebral circulation. This should be tested in a separate study where potential postdive middle cerebral artery air embolization would be correlated with indicators of neuronal damage. Last, in the current study we measured the circulating endothelin-1 concentration in serum despite endothelin-1 not being a circulating hormone, but rather a local one that is secreted preferentially to the basal side of the endothelium and acting on underlying smooth muscle cells. This makes the circulating levels of endothelin-1 a potentially imprecise indicator of its physiological activity and a chance for false-negative results realistic. Also, since we measured endothelin-1 in blood drawn at 80 min after dive, we cannot exclude that some factor acting on endothelium *during* a dive caused endothelin-1 release, which could not be detected due to endothelin's short half-life. In order to investigate the importance of endothelin-1 in diving-induced endothelial/vascular changes, a more detailed study using specific endothelin antagonists and assessing vascular function after diving would need to be performed, which was outside the scope of the current investigation.

In conclusion, we found that 4 days of consecutive no-decompression air dives to 18 msw with continuous moderate physical exercise during the bottom phase, despite the relatively high bubble production and arterialization that was observed in six divers, did not increase blood endothelin-1 and NSE levels. Although the S-100β levels were significantly increased, concomitant increase of creatine kinase during a control, almost bubble-free dive, suggests the most likely extracranial origin of S-100β. Therefore, the current study suggests that significant bubble production and arterialization in this study did not affect release of markers of endothelial, neuronal or the blood barrier damage.

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Conflict of interest The authors have no conflict of interest to report.

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