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ORIGINAL ARTICLE

Release of VCAM-1 associated endothelial microparticles following simulated SCUBA dives

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Abstract Microparticles (MP) are shed into the circulation from endothelium following activation or apoptosis. Vascular cell adhesion molecule-1 (VCAM-1) is expressed on endothelial cells following activation and here we report quantification of VCAM-1 positive microparticles (VCAM + MP) following simulated SCUBA dives, breathing either air or oxygen. VCAM + MP were quantified pre-dive (09:00 and 13:00) and post-dive (+1, +3 and +15 h) on both air and oxygen dives and compared with control samples taken from the same subjects. VCAM + MP followed a similar trend in all experiments, however both dives caused a change in endothelial state, as measured by VCAM + MP. A significant increase in VCAM + MP was observed 1 h post-air dive relative to the control ($p = 0.013$), which was not observed after the oxygen dive ($p = 0.095$). Oxidative stress (TBARS) was correlated with VCAM + MP. Data presented highlights the potential of MP as a biological marker of both endothelial state and decompression illness.

Keywords Decompression illness · SCUBA diving · Microparticles · Endothelium · VCAM-1

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Introduction

The formation of bubbles following rapid decompression after a dive has long been considered to be the major causative agent of decompression illness (DCI). Gas bubbles formed upon decompression present within the circulation are far from being inert and have been shown to interact with the blood cell population and have the potential to occlude vessels, causing ischaemic events followed by reperfusion injury in the microvasculature (Barak and Katz 2005). The detection of bubbles using 2D Doppler imaging and the association of bubble scores with DCI is not without limitations (Eckenhoff et al. 1990). The detection limit for Doppler imaging remains debatable whereby undetectable bubbles have been shown to cause DCI and conversely divers presenting with high bubble scores have remained asymptomatic (Eckenhoff et al. 1990). Therefore, novel biomarkers predictive of DCI risk potential are an attractive target in the expanding field of professional and recreational diving.

Endothelial microparticles (MP) are constantly shed into the circulation of healthy individuals (Freyssinet 2003) and have shown to be elevated in many diseased states, most notably those characterised by endothelial dysfunction (Horstman et al. 2004; Madden and Laden 2007). Bubbles formed during decompression can interact with the endothelium resulting in a loss of integrity and activation of the endothelium and result in an increased shedding of endothelial MP into the circulation (Madden and Laden 2007). Furthermore, there is evidence for bubble interaction with the endothelium causing mechanical damage, including cell-stripping and an increase in the size of the cell junctions between the endothelial cells (Barak and Katz 2005). Blood viscosity may be increased due to the presence of bubbles and complement has been shown to be activated

(DeGorordo et al. 2003). Circulating proteins can adsorb to the surface of bubbles through simple hydrophobic interactions, forming a protein layer which results in unfolding of the tertiary structure and ultimately makes possible biological interaction of the bubble with the endothelium (Eckmann and Armstead 2006). This results in a generally inflammatory pro-thrombotic state and severe DCI has common symptoms with anaphylaxis, furthermore IL-6 has been observed to increase in severe DCI (Ersson et al. 1998). It is, therefore, worthy of consideration that endothelial integrity and activation may be implicitly involved in DCI.

Previously, attempts to reduce the risk of DCI have focussed upon decompression rate and breathing of oxygen (Moon and Gorman 2003). A recent study has tested pre-breathing of oxygen prior to simulated rapid decompression in goats and found a significant reduction in bubble scores post-escape (Gennser and Blogg 2008), with no symptoms of DCI. Brubakk et al. have published numerous articles (Dujic et al. 2008; Obad et al. 2007a, b; Wisloff et al. 2004) on bubble formation and endothelial function in both human and animal models. Interestingly, they demonstrated a decrease in arterial endothelial function following a single air dive (Brubakk et al. 2005). This function has been attenuated by the administration of nitric oxide (NO) donors (Wisloff et al. 2004), antioxidants (Obad et al. 2007a, b) and by bouts of exercise (Blatteau et al. 2007; Dujic et al. 2008) before a dive, suggesting a possible role of oxidant status. The endothelium is known to be sensitive to oxidative stress and shear rate leading to vascular remodelling (Lehoux et al. 2006; Ungvari et al. 2006) and release of MP (Freyssinet 2003).

Previously, we have suggested that endothelial MP can be used as a marker of DCI stress by evaluating antigenic markers on circulating MP that not only enable specific derivation but are also reflective of the integrity of the endothelium (Madden et al. 2004). Following endothelial disruption expression of adhesion molecules are expressed following an accepted configuration. One such molecule, vascular cell adhesion molecule-1 (VCAM-1) is an attractive marker due to expression exclusively on activated endothelium as achieved following a vascular insult and is therefore is a predictive marker of a pro-inflammatory endothelium. Furthermore, blocking of the VCAM-1/very late antigen four interactions is a therapeutic target for treatment of chronic inflammatory disorders (Yusuf-Makagiansar et al. 2002).

The aim of this study was to investigate the effect of a simulated (chamber) dive breathing compressed air and oxygen on the circulating population of VCAM-1 positive MP (VCAM + MP) following decompression in healthy male subjects in comparison to their own control samples. Any changes in VCAM-1 + MP may be attributable to a change in endothelial state caused by decompression and this is discussed along with a possible role of oxidant status in release of VCAM + MP.

Materials and methods

Experimental protocol and blood sampling

Six healthy male volunteers, all with no previous dive experience, were subjected to a simulated dive of 78 min bottom time at 2.8 ATA breathing either compressed air or 100% oxygen (20 min oxygen, 5 min air cycle for 78 min bottom time). Subjects were decompressed in accordance with the USN standard air decompression tables. The dives commenced at 15:00 and each subject gave written informed consent to participate in the study. The study was approved by the local institutional ethics committee. Venous blood was collected from the antecubital vein into a tri-sodium citrate Vacuette tube (Vacuette®, Greiner Bio-one, UK) by standard venipuncture technique. Blood samples were drawn at 09:00 and 13:00 before the dive in order to assess a pre-dive baseline and subsequently 1 h post-dive (18:00), 21:00 and 09:00 the following day. All blood samples were processed immediately for assessment of MP. For comparison, control blood samples were taken from all subjects under resting conditions every 4 h from 09:00 on a single, separate day exactly a week before the first dive. The following week all subjects underwent a simulated dive in a hyperbaric chamber following the protocol above, breathing air. Exactly a week later all subjects underwent another simulated dive breathing oxygen. On all 3 days of the experimental protocol the subjects dietary intake was the same and apart from being at the hyperbaric chamber, spent the remainder of the day under resting conditions in a temperature controlled environment.

Flow cytometry analysis of MP

Blood tubes were centrifuged (160×*g*, 10 min) and platelet rich plasma aspirated and transferred to a microfuge tube and further centrifuged (1,500×*g*, 10 min) to prepare platelet poor plasma (PPP), in accordance with published methodology (Jimenez et al. 2003). Samples of PPP (25 μL) were incubated with 2 μL of either isotype matched negative control: FITC (AbD Serotec), anti-VCAM-1: FITC (AbD Serotec) in the dark for 30 min. Filtered (0.22 μm) PBS (200 μL) and counting beads (25 μL, Caltag Laboratories) were added immediately prior to analysis by flow cytometry (BDFACSCalibur). Positive MP were defined as an increase in mean fluorescence intensity over the isotype matched negative control and were quantified in relation to counting beads according to manufacturers' instructions.

Forward scatter was set to a 1.5 µm limit as defined using spherical beads (Bangs Laboratories).

Oxidative stress

Serum was analysed for lipid peroxidation, a major indicator of oxidative stress, using a commercially available thiobarbituric acid reactive substances (TBARS) kit (ZeptoMetrix, USA), according to manufacturers' instructions. Results obtained are expressed as malondialdehyde equivalents.

Peripheral blood mononuclear cell characterisation

PBMC were isolated using Histopaque™ (Sigma) according to maufacturers' instructions and stored in foetal bovine serum (Biosera) supplemented with 10% (v/v) dimethyl sulphoxide (Sigma), in liquid nitrogen until required. They were then washed in PBS and aliquoted into tubes (2×10^5 per tube) for flow cytometric evaluation. Fluorescein-tagged antibodies (CD1a, CD14, CD18, CD69, CD105 all AbD Serotec) were added (4 µL) and incubated with the PBMC for 30 min, prior to washing with PBS and analysis as above.

Statistical analysis

Statistical analyses were performed by a professional statistician using Minitab® version 14.2 (Minitab Inc., State College, PA). Analysis of covariance (ANCOVA) was used to compare post-dive using the pre-dive data as the covariate. Tukey's post hoc test was subsequently used to identify significant paired differences. The relationship between oxidant status and VCAM + MP was tested using the Pearson correlation coefficient. In relation to evidence against the null hypotheses, significance probabilities were interpreted as follows: $p \leq 0.01$, strong evidence; $0.05 \geq p > 0.01$, moderate evidence; $0.10 \geq p > 0.05$, weak evidence; $p > 0.10$, little evidence.

Results

Microparticles were assayed by flow cytometry, typically a spread is observed below 1.5 µm as seen in Fig. 1. VCAM + MP were identified as a distinct population with increased fluorescence, in comparison to MP incubated with a negative control antibody, and quantified using counting beads (Fig. 1). To gain an insight into endothelial state VCAM-1 positive MP were quantified and data are presented relative to pre-dive values at 09:00 (Fig. 2). The control samples showed a well-defined minima at 17:00, which was not observed following either simulated dive.

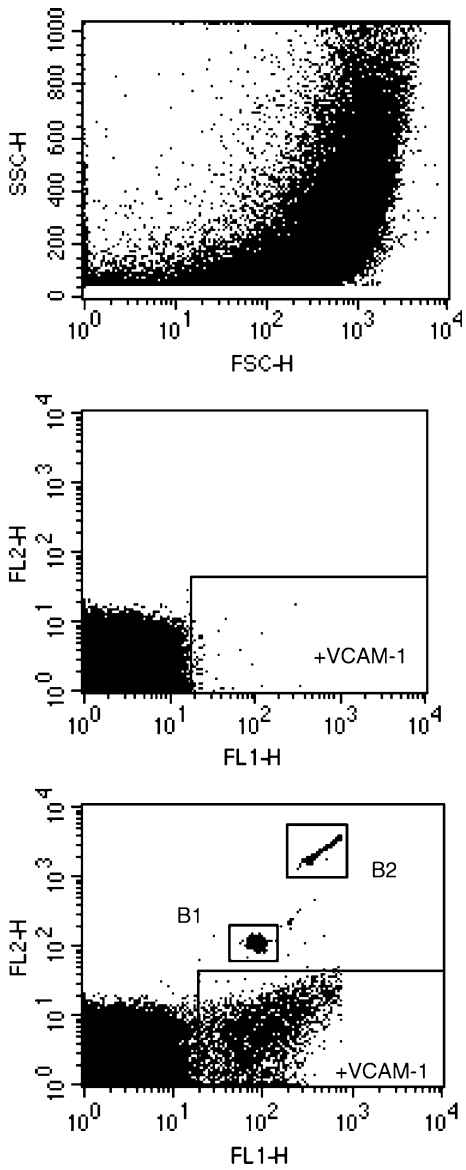

Fig. 1 Typical flow cytometry profiles of microparticles (*top*), incubated with a negative control antibody (*middle*) and VCAM-1 positive labelling (*bottom*). Counting beads (B1 and B2) in quantification of positive microparticles

No difference was observed from 09:00 to 13:00 (pre-dive) in mean VCAM + MP on any day. Following the compressed air dive VCAM + MP were over twofold higher ($p = 0.013$) when compared to control samples, whereas following oxygen breathing dive this change was not statistically significant, although weak evidence was observed ($p = 0.095$). TBARS, a measure of lipid peroxidation and therefore oxidative stress, was determined at all time points and data is shown in Fig. 3. The lowest average serum TBARS was observed after the oxygen breathing dive, a minima which was not seen in either the air dive or control samples, although no statistical significance was observed between the groups. At 09:00 the day post-dive serum

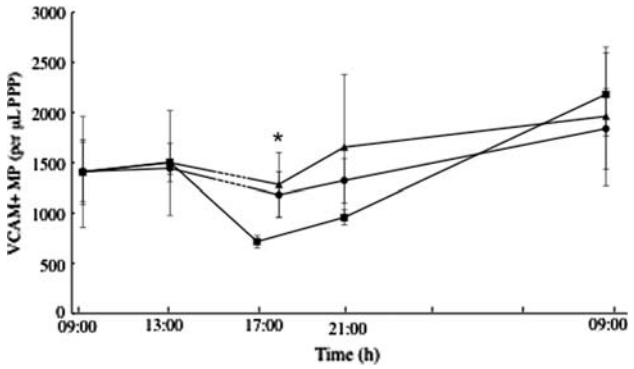

Fig. 2 Number of VCAM-1 MP during (*filled square*) control day, (*filled triangle*) compressed air and (*filled circle*) oxygen dives. Time of dive is indicated by *non-continuous line*. Data are presented as mean ($n = 6$) ± SD. *Asterisks* designates air dive was significantly different to control sample ($p = 0.013$) and oxygen dive was not ($p = 0.095$). No significance was observed at any other time

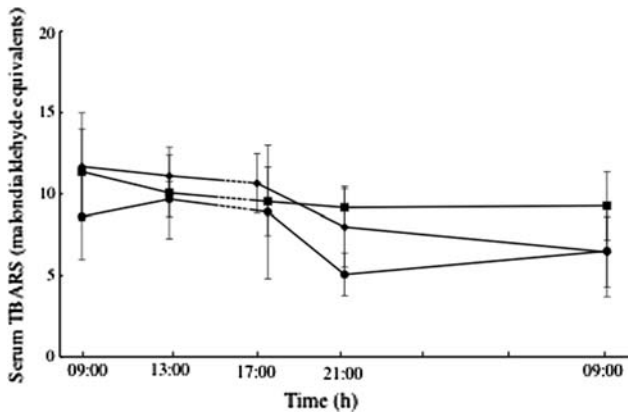

Fig. 3 Serum thiobarbituric reactive substances (TBARS) on (*filled square*) control day, (*filled triangle*) compressed air and (*filled circle*) oxygen dives. Time of dive is indicated by *non-continuous line*. Data are presented as mean ($n = 6$) ± SD. No significant differences were observed comparing dives to control samples

TBARS concentrations were similar in both oxygen and control groups, however the air dive samples were elevated in comparison. To investigate possible mechanisms behind VCAM + MP release from the endothelium, correlation of serum TBARS and VCAM + MP was assessed using Pearson's correlation coefficient (Fig. 4). A statistically significant trend for increased VCAM + MP with increased oxidant status was observed ($p = 0.001$, $R^2 = 0.26$). This correlation was further confirmed using samples obtained from a previous study (Madden et al. 2008). Correlation of TBARS and VCAM + MP on the 11 subjects from that study resulted in a similar trend ($p = 0.001$, $R^2 = 0.24$).

Upon performing flow cytometry on whole blood it was noted that subsets of both neutrophils and lymphocytes were present in four out of six subjects at 09:00 following the oxygen dive protocol. This observation was not seen with any other samples at any other times and appears quite

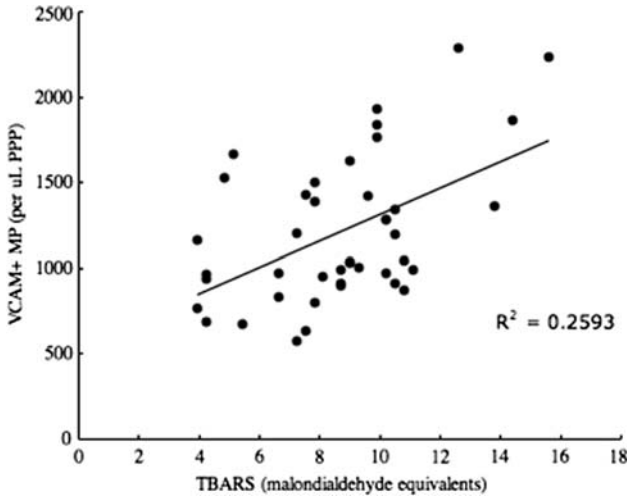

Fig. 4 Correlation of VCAM + MP (absolute count) and TBARS (malondialdehyde equivalents), $p = 0.001$

unusual. To offer an explanation for this finding, PBMCs, which were taken at the time of sampling, were tested for expression of either lymphocyte or monocyte activation markers. No changes were observed in these 09:00 samples, compared to immediate pre- or post-dive samples in either CD1a (pan-leukocyte activation marker) or CD69 (lymphocyte activation marker). Also no change in mean fluorescence was observed in the constitutive expression of CD14 (monocytes) or CD18 (leukocytes) (data not shown).

Discussion

Bubbles formed during decompression have the potential to interact and damage the endothelium and are hypothesised to be implicitly involved in the progression of DCI. Endothelial stress and damage caused by bubble contact or shear stress can result in an increased release of MP that serve as sensitive biological markers with the capacity to reflect the condition and function of the endothelium (Horstman et al. 2004). In this study, we observed an increase in the circulating population of VCAM + MP following a simulated dive breathing both compressed air, or oxygen compared to their non-diving control samples taken at the same time of day. Due to its expression exclusively on activated endothelium VCAM + MP can be employed as a very sensitive marker of endothelial function/dysfunction. We hypothesise that an increase in circulating VCAM + MP can be reflective of changes in the state of the endothelium and may have potential as a biomarker of susceptibility to DCI where vascular mechanisms are involved.

Taking into account that endothelial MP are a well-established marker of endothelial dysfunction (Horstman et al. 2004), then the lowest concentration of VCAM-1 + MP

within the circulation could speculatively be taken to be the time at which endothelial function is optimal, within the 24 h testing regime. This point occurred at 17:00 in the subjects control samples and therefore this may be taken to represent maximal (100%) endothelial function, as VCAM + MP released from endothelial cells would certainly be correlated with cell damage. Endothelial function relative to this peak for control, air and oxygen dives is shown in Fig. 5. The profiles of VCAM + MP over 24 h in the control, air and oxygen dive groups was seen to follow a similar profile, however, the dive intervention appeared to cause a reduction in endothelial function, as measured by VCAM + MP. Therefore, 1 h post-dive a significant attenuation of endothelial state was observed after breathing air, and less so breathing oxygen, however by 09:00 the following day, function had returned to levels observed 24 h previous.

The depth time profile of the dive breathing compressed air has been reported to consistently produce venous gas phase producing bubbles able to interact with the endothelium (Eckmann and Armstead 2006) resulting in the release of MP (Freyssinet 2003). Interestingly, Brubakk et al. (2005) performed the same dive on compressed air and similarly reported a 45% reduction in endothelial function 30 min post-dive, as measured by flow-mediated dilation, when breathing compressed air and a lesser reduction (27%) was observed when breathing 60% oxygen.

Previously we proposed a potential role of endothelial derived MP in DCI (Madden et al. 2004), and it was subsequently suggested that an observed decrease in arterial endothelial dysfunction may be due to release of MP on venous side due to gas bubbles and have an effect at other remote site, e.g. the arterial system (Brubakk et al. 2005). However, any arterial endothelial response to MP would depend upon the antigens carried on the MP and whether they are able to alter arterial endothelial state via antigenic interaction.

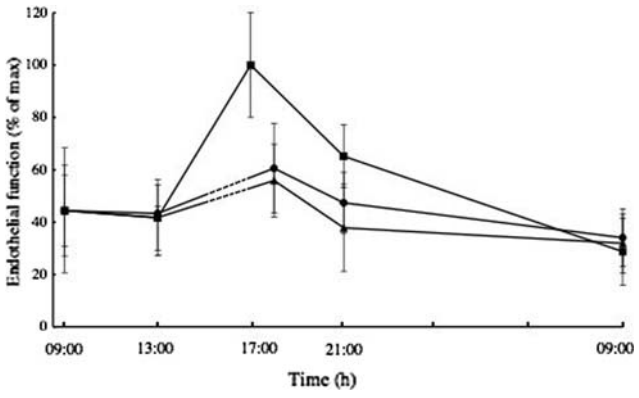

Fig. 5 VCAM-1 derived endothelial state on (*filled square*) control day, (*filled triangle*) compressed air and (*filled circle*) oxygen dives. Time of dive is indicated by *non-continuous line*. Data are presented as mean ($n = 6$) ± SD

The decrease in serum TBARS post-oxygen dive, although not significant, was unexpected as hyperbaric oxygen has previously been shown to induce oxidative stress in healthy males (Bader et al. 2007), although data presented recently did conversely show no changes in reactive oxygen metabolites after treatment for DCI using US navy treatment Table 6 (Knogoji et al. 2007). This study also demonstrated an increase in antioxidant capacity following treatment. Hyperbaric exposure has been shown to cause an increase in oxidative stress in both animal and human experiments (Bearden et al. 1999; Otler et al. 2007; Benedetti et al. 2004), however differences in subject population (healthy or diseased), methodology (specifically sample type, i.e. tissue, erythrocytes or serum/plasma) and sample timing has an influence on the outcome. The decrease in serum TBARS presented here therefore warrants further investigation; the reasons behind this finding could be numerous, for example study population, physical fitness, diet (e.g. vitamin supplements), etc.

Neutropenic rats have been shown incapable of showing symptoms of DCI (Martin and Thom 2002); leading to suggestions that endothelial expressed adhesion molecules may be implicitly involved in the onset of symptoms following decompression (Montcalm-Smith et al. 2007). Hyperbaric oxygen has been shown to inhibit neutrophil adherence (Thom et al. 1997) and platelet activation has been observed post-decompression which was found to be reduced with oxygen pre-treatment before a dive (Landofli et al. 2006).

NO maintains endothelial vasodilatory tone (McGowan et al. 1994) and has been shown to inhibit platelet adhesion and aggregation (Bult 1996) and neutrophil adherence to the endothelium (Conger and Weil 1995; Jordan et al. 1999; Ronson et al. 1999) via inhibiting expression of adhesion molecules (Decaterina et al. 1995; Ohashi et al. 1997). Induction of NO has been shown to be effective in reducing bubble formation following a dive both via administration of NO donors (Obad et al. 2007a; Wisloff et al. 2004) and possibly via induction due to exercise (Blatteau et al. 2007; Dujic et al. 2008). As mentioned earlier bubbles have the potential to occlude vessels and interact with the endothelium, causing localised activation, therefore any reduction in bubble number and size should attenuate endothelial response. However, as we have reported here, there may be an effect on the endothelium via oxidative stress and not bubble related damage, which may be linked to release of MP. The beneficial effects of NO induction may be through an antioxidant mechanism as NO has been demonstrated to possess antioxidant properties (Mohanakumar et al. 2002) and furthermore acute antioxidants have been shown to similarly attenuate decreases in endothelial function following a dive (Obad et al. 2007a). The endothelium is known to be sensitive to oxidant status

(Ungvari et al. 2006) and we have demonstrated here that release of VCAM + MP may be linked to oxidant status, thus offering a potential mechanism to attenuate endothelial response following a dive by preventing or at least reducing endothelial activation, adhesion molecule expression and MP release.

Oxidant status may be a potential factor involved in an individual's propensity to show symptoms of DCI following a dive; the research discussed has focussed upon administration of antioxidants in an attempt to reduce the effects of compression/decompression on endothelial function. Here we report for the first time quantification of VCAM-1 positive MP as a sensitive biomarker to oxidant status and their potential role as biological marker of DCI warrants further investigation.

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