Original articles

Acute antioxidant pre-treatment attenuates endothelial microparticle release after decompression

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Key words
Decompression, endothelium, antioxidants, treatment, diving research

Abstract
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Purpose: The hyperbaric and hyperoxic effects of a dive have been demonstrated to elicit changes in oxidative stress, endothelial function and microparticle (MP) release. Endothelial MP, which are small membrane vesicles shed from the endothelium, have been suggested as a valid *in vivo* marker of endothelial function. Furthermore, recent research has shown an increase in CD105 MP post-dive to be associated with a decline in endothelial function. The aim of this study was to ascertain whether antioxidant (AOX) pre-treatment can attenuate increased CD105 MP release post-dive.
Methods: Five healthy, male, pressure-naive subjects completed two simulated dives (control and intervention) breathing compressed air to a depth of 18 metres' sea water for 80 min. For the intervention dive, all subjects received a commercially available AOX pill containing vitamins C and E, selenium and beta-carotene 2 h pre-dive. CD105 MP, total antioxidant capacity (TAC) and thiobarbituric reactive substances assay (TBARS) were determined pre-dive, at depth, immediately and 4 h post-dive.
Results: In the control dive, there was a significant increase in CD105 MP immediately post-dive when compared with at depth ($P < 0.001$) and pre-dive ($P = 0.039$) values. Antioxidant pre-treatment significantly attenuated this release of CD105 MP post-decompression ($P = 0.002$). There were no significant changes in TBARS or TAC.
Conclusion: These results may provide evidence of the potential use of AOX pre-treatment as an effective endothelial pre-conditioner for divers.

Introduction

Decompression illness (DCI) is an inherent risk with scuba diving. While diving with compressed air, nitrogen is taken up by the tissues in proportion to the depth and time spent underwater. During ascent to the surface some of this gas may be released from tissues in the form of bubbles.[1–2] The formation of these bubbles has been previously acknowledged within the literature as the major factor relating to the development of DCI.[3] However, the occurrence of bubbles may be a poor predictor of DCI as divers with no/low bubble scores have developed DCI, whilst divers with high bubble scores have remained asymptomatic.[4] Consequently, recent research has investigated other potential markers of DCI including, but not limited to endothelial function, oxidative stress and increased endothelial microparticle (MP) release.[5–9]

Elevations in reactive oxygen and/or nitrogen species production are thought to reduce nitric oxide bioavailability causing vasoconstriction and subsequently may result in the initiation and progression to endothelial dysfunction.[6] The endothelium is particularly sensitive to changes in oxidative stress.[10] Circulating endothelial MP, shed from the endothelium, have been postulated as a useful *in vivo* measure of endothelial state and a potential marker for DCI.[8,9] Any mechanisms by which the transition to a more oxidising environment can be attenuated may be beneficial in preventing endothelial dysfunction. To date, pre-dive pre-conditioning methods have included the use of hyperbaric oxygen breathing, physical activity, heat exposure and antioxidants (AOX).[11–16]

Endogenous AOX defences may be insufficient to cope during a scuba dive and may lack efficacy in preventing transition to a more oxidative state.[15] Therefore, exogenous AOX may be beneficial in supplementing endogenous AOX.[17] Vitamins C and E have been successfully implemented to maintain endothelial function within the clinical setting following cardiovascular surgery, as well as being used more generally to offer endothelial protection.[18,19] Moreover, exogenous AOX supplementation has been successfully implemented to reduce oxidative stress following simulated dives in rats.[20,21] The use of AOX as a pre-dive pre-conditioning strategy in reducing oxidative stress and/or endothelial dysfunction in humans has yielded contradictory results; however, markers of oxidative stress are often not reported.[15,22] Consequently the usefulness of pre-dive AOX treatment as a potential

endothelial pre-conditioner remains to be elucidated. Therefore, the aim of this study was to ascertain whether AOX pre-treatment can attenuate the increased CD105 MP expression observed post decompression, and the increased oxidative stress that may be associated with diving. CD105 MP was selected as a constitutively expressed endothelial membrane protein (endoglin) and as such is present on MP released from the endothelium.[8]

Methods

SUBJECTS

Five subjects with no prior diving experience (mean age 23 ± 6 years, height 183 ± 8 cm, weight 78 ± 12 kg), volunteered to take part in this study. All subjects were healthy, male non-smokers and recreationally active. They provided written informed consent, and completed a pre-exercise medical questionnaire. Subjects were requested to abstain from alcohol, high-fat food, caffeine, and unaccustomed, vigorous physical activity for 24 hours prior to study commencement, and compliance was monitored via a pre-study questionnaire. Ethical approval for the study was obtained in accordance with departmental and university ethical procedures and followed the principles outlined in the Declaration of Helsinki.

EXPERIMENTAL PROTOCOL

All subjects participated in a control dive (AIR) and an intervention dive (AOX) separated by two weeks. The two dives were identical except that the subjects took an AOX pill prior to one of the dives. Subjects reported to the laboratory at 0930 h and were transported to a hyperbaric chamber via minibus at 1030 h. The simulated dry dives, conducted in a 6-person multiplace chamber and commenced at 1200 h, were to a maximum depth equivalent to 18 metres' sea water (msw, 284 kPa). Compression time was five minutes, time at depth 60 min and ascent time 15 minutes, including two 5-minute stops at 6 msw and 3 msw.

BLOOD SAMPLING

Venous blood samples were obtained pre-dive, at depth, immediately post-dive and 4 h post-dive from an antecubital vein using a standard venipuncture technique with a 21-gauge needle. Preceding each blood collection (not including the 'at depth' sample), subjects rested supine for 10 minutes. Blood was collected into commercially available sodium citrate vacuette tubes (Vacuette®, Greiner, UK) and the first draw was discarded due to the possibility of endothelial damage from the venipuncture.

ANTIOXIDANT PRE-TREATMENT

Subjects consumed a standardised breakfast consisting of shredded wheat (30 g) and semi-skimmed milk (250 ml) and ingested an AOX pill 2 h before the dive (at 1000 h). The commercially available AOX pill (Super antioxidant formula capsules, Holland and Barrett, UK) contained vitamin C 500 mg, vitamin E 268 mg, selenium 50 µg and beta-carotene 15 mg.

MICROPARTICLE QUANTIFICATION

MP were quantified using a BDFACS Calibur flow cytometer (BDBiosciences, UK) and the gating strategy defined in accordance with a previous study.[8] Sodium citrate blood tubes were centrifuged (200 *g*, 10 min) and platelet-rich plasma aspirated and transferred to a microfuge tube and further centrifuged at 1500 *g* for 3 min to prepare platelet-poor plasma (PPP). Anti-CD105:FITC (4 µL, AbD Serotec), and negative control: FITC (4 AbD Serotec) were incubated in the dark with PPP (25 µL) for 30 min prior to addition of Caltag counting beads (25 µL) (Caltag Medsystems, UK) and 200 µL of filtered (0.22 µm) phosphate buffering solution. Samples were subsequently analysed using CellQuest software (BDBiosciences, UK). Forward scatter was set as a trigger determined by the scatter properties of megamix beads (Biocytex, France). MP were quantified as an absolute count per µL PPP in relation to counting beads according to the manufacturer's instructions.

MEASUREMENT OF LIPID PEROXIDATION

Venous blood was alloquated into potassium EDTA tubes (Vacuette®, Greiner, UK) mixed and then spun at 1500 *g* for 10 min. The resulting EDTA plasma was then removed and transferred into 1.5 ml polypropylene tubes and stored at -80^{O}C. Lipid peroxidation was analysed utilising a commercially available thiobarbituric acid reactive substances (TBARS) kit (TBARS Assay Kit, Zeptometrix, USA) according to the manufacturer's instructions. Absorbance was read at 530 nm. Results are expressed as MDA equivalents.

MEASUREMENT OF TOTAL ANTIOXIDANT CAPACITY

Total antioxidant capacity (TAC) was measured using a commercially available kit (TAC Assay Kit, BioVision, CA, USA) according to the manufacturer's instructions. The protein mask included in the kit was not utilised; therefore, measurements include enzyme activity as well as small AOX molecules. Results are expressed as trolox equivalents.

STATISTICAL ANALYSIS

Statistical analysis was completed using IBM SPSS Statistics 18 (SPSS Inc., Chicago, IL). No power analysis was performed. The changes in CD105 MP, fluorescence (FL), TBARS, and TAC across condition (control and AOX) and time (pre-dive, at depth, immediately post-dive and 4 h post-dive) were analysed using linear mixed models for repeated

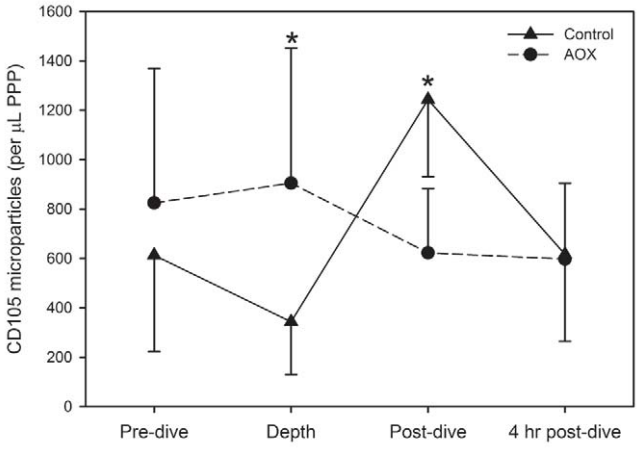
measures. Different covariance structures were assumed and the one that minimised the Hurvich and Tsai's criterion was chosen for the final model. Quantile-quantile plots showed the models for CD105 MP and TBARS exhibited right-skewed distributed residuals, which were corrected using natural log transformations of the observed data. Where a significant F ratio was observed, *post hoc* comparisons with Sidak-adjusted *P* values were used to identify which pairs of means were significantly different. Two-tailed statistical significance was accepted as $P < 0.05$.

Results

The simulated dives were both well tolerated, with no subjects reporting symptoms of DCI. The mean CD105 MP are shown in Figure 1. There were no significant main effects for condition (AIR or AOX, F = 0.2, $P = 0.66$) or time (F = 3.0, $P = 0.051$) for CD105 MP. However, there was a significant condition and time interaction (F = 6.8, $P = 0.002$). In the AIR (control) condition there was a significant increase in CD105 MP from pre-dive ($P = 0.039$) and at depth ($P < 0.001$) to immediately post-dive. The decrease in CD105 MP from immediately post-dive to 4 h post-dive did not reach statistical significance ($P = 0.058$). No significant changes across time were observed in the AOX condition ($P \geq 0.82$).

The mean FL, TBARS, and TAC for each time point in the two experimental conditions are shown in Table 1. No significant main effects for condition (F = 0.08, $P = 0.78$) or time (F = 2.1, $P = 0.13$), or an interaction effect (F = 0.4, $P = 0.77$), were observed for FL. Similarly, no significant main effects for condition (F = 2.5, $P = 0.16$) or time (F = 0.4, $P = 0.68$), or an interaction effect (F = 0.7, $P = 0.53$), were observed for TBARS. A main effect for condition for TAC was observed, where, on average, the TAC was higher in the control condition than in the AOX condition (F = 8.4, $P = 0.013$). A significant main effect also was observed for time (F = 4.6, $P = 0.039$), where TAC decreased from pre-dive to depth ($P = 0.036$), but no significant change was observed thereafter ($P = 0.44$). The interaction between condition and time was not statistically significant (F = 0.7, $P = 0.53$).

Figure 1
Mean (SEM) CD105 microparticle concentrations observed before, during and after simulated dives with antioxidant (AOX) and without (Control) pre-treatment; *n* = 5; * *P* < 0.05



Discussion

The techniques used in this study are robust. Flow cytometry is currently the only viable method for measuring microparticles, supported by a recent study.[23] The TAC and TBARS kits are commercially available kits that are certified and validated for use with human serum/plasma samples.

The main finding in the present study was that CD105 MP within the circulation were significantly lower post-dive ($P = 0.002$) in the AOX group compared to the control dive. A return to CD105 MP levels observed pre-dive was seen after 4 h, showing that the MP generated are removed within this time frame and, therefore, a return to a homeostatic condition was observed. Increases in both CD105 MP and CD106 MP have previously been observed following a dry chamber dive breathing air.[8,9] TAC was shown to be significantly decreased at depth when compared to both pre- and post-dive. This suggests AOX present within the circulation may have been utilised to deal with increased reactive oxygen species (ROS) at depth. The temporary reduction of TAC observed at depth

Table 1
Mean fluorescence intensity (MFI) of CD105 MP, thiobarbituric acid reactive substances (TBARS) and total antioxidant capacity (TAC) before, during and after a simulated dive in two groups (*n* = 5) with (AOX) or without (Control) antioxidant pre-treatment (see text for statistical analysis)

		Pre-dive		At depth		Immediately post-dive	
CD105 MP MFI	Control	33.2	(9.0)	31.7	(6.9)	36.3	(9.8)
(arbitrary units)	AOX	36.0	(4.3)	28.5	(11.0)	33.6	(4.7)
TBARS (MDA equivalents)	Control	17.3	(10.6)	25.0	(11.4)	31.4	(15.7)
(nmol mL^{-1})	AOX	25.6	(22.2)	17.5	(6.1)	18.8	(10.0)
Total antioxidant capacity	Control	30.1	(2.0)	28.1	(1.9)	30.7	(4.0)
(mmol mL^{-1})	AOX	27.7	(2.8)	25.4	(2.4)	27.0	(5.2)

may have a role within our previous hypothesis, in which it was suggested that there may be temporary endothelial dysfunction.[23] Although TBARS appears to increase at depth and post-dive, an effect that may be countered by exogenous AOX, these changes were not statistically significant, most likely due to the small number of subjects and low power of the study.

Previous, yet similar, work found that the increase in forearm vascular resistance (28 ± 10%) usually associated with hyperoxia was significantly attenuated following vitamin C administration.[24] Additionally acute endothelial dysfunction can be attenuated following both acute and chronic AOX pre-treatment, suggesting that AOX may have a prophylactic effect on endothelial dysfunction after diving.[15,16] Notably during the chronic protocol, the last dose of AOX was administered 3–4 h prior to the dive; therefore, the observed effect may have been an acute effect of the AOX and not a result of chronic treatment. Acute pre-treatment with AOX has been suggested to be more beneficial than chronic treatment.[25,26] In light of these findings, AOX pre-treatment was administered 2 h pre-dive in the present study and significantly attenuated subsequent CD105 MP release compared to the control dive. Furthermore, recent work within our laboratory has demonstrated a significant increase in CD105 MP post-dive, which was associated with a decline in endothelial function.[8] Hyperoxia-induced ROS production rapidly inactivates nitric oxide production, potentially causing vasoconstriction and ultimately contributing to the initiation and progression to endothelial dysfunction.[17]

The present study failed to demonstrate any significant changes in oxidative stress. Furthermore, chronic (four weeks) AOX pre-treatment with vitamins C and E has also failed to prevent hyperoxia-induced oxidative stress following a 2 h hyperbaric, hyperoxic exposure.[22] These results may suggest that hyperbaric hyperoxia is insufficient to cause any significant changes in oxidative stress within the circulation. However, it is more likely that other mechanisms, such as gas-bubble formation, are responsible for endothelial dysfunction and increased MP release following a dive.[9] Although gas bubbles *per se* are perhaps not the sole mechanism responsible for the development of DCI, *de novo* formation of bubbles may cause vascular endothelial cell stripping through mechanical interaction with the endothelium, and consequently cause an increase in MP release.[8,23,27]

In conclusion, a single dose of AOX 2 h prior to a dry chamber dive attenuated the release of CD105 MP from the endothelium. The mechanism(s) responsible for this endothelial protection remain to be elucidated and further research is required in order to ascertain whether AOX pre-treatment is an effective endothelial pre-conditioner for divers.

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