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Ascorbic acid supplementation diminishes microparticle elevations and neutrophil activation following SCUBA diving

Ming Yang,[1] Otto F. Barak,[2] Zeljko Dujic,[2] Dennis Madden,[2] Veena M. Bhopale,[1] Jasjeet Bhullar,[1] and Stephen R. Thom[1]

[1]*Department of Emergency Medicine, University of Maryland School of Medicine, Baltimore, Maryland; and* [2]*Department of Physiology, University of Split School of Medicine, Split, Croatia*

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Yang M, Barak OF, Dujic Z, Madden D, Bhopale VM, Bhullar J, Thom SR. Ascorbic acid supplementation diminishes microparticle elevations and neutrophil activation following SCUBA diving. *Am J Physiol Regul Integr Comp Physiol* 309: R338–R344, 2015. First published July 1, 2015; doi:10.1152/ajpregu.00155.2015.—Predicated on evidence that diving-related microparticle generation is an oxidative stress response, this study investigated the role that oxygen plays in augmenting production of annexin V-positive microparticles associated with open-water SCUBA diving and whether elevations can be abrogated by ascorbic acid. Following a cross-over study design, 14 male subjects ingested placebo and 2–3 wk later ascorbic acid (2 g) daily for 6 days prior to performing either a 47-min dive to 18 m of sea water while breathing air (~222 kPa N_2/59 kPa O_2) or breathing a mixture of 60% O_2/balance N_2 from a tight-fitting face mask at atmospheric pressure for 47 min (~40 kPa N_2/59 kPa O_2). Within 30 min after the 18-m dive in the placebo group, neutrophil activation, and platelet-neutrophil interactions occurred, and the total number of microparticles, as well as subgroups bearing CD66b, CD41, CD31, CD142 proteins or nitrotyrosine, increased approximately twofold. No significant elevations occurred among divers after ingesting ascorbic acid, nor were elevations identified in either group after breathing 60% O_2. Ascorbic acid had no significant effect on post-dive intravascular bubble production quantified by transthoracic echocardiography. We conclude that high-pressure nitrogen plays a key role in neutrophil and microparticle-associated changes with diving and that responses can be abrogated by dietary ascorbic acid supplementation.

decompression; microparticles; neutrophil activation; oxidative stress

THE FOCUS OF THIS WORK IS to improve understanding of the pathophysiology for decompression sickness (DCS). DCS is a systemic pathophysiological process that occurs after tissues become supersaturated with nitrogen or some alternative gas used to dilute O_2 in breathing mixtures during activities, such as deep sea diving, high-altitude aviation, and space exploration. A series of studies has demonstrated that circulating microparticles (MPs), membrane-encapsulated cell fragments with diameters of 0.1 to 1 μm, are elevated in animals and humans after simulated or bona fide underwater diving (14–16, 20, 25, 26, 29). In a murine model, MPs have been shown to initiate a systemic inflammatory process postdecompression that is related to neutrophil activation (27, 28, 31, 32). Injuries identified in decompressed animals can be recapitulated by injecting decompression-induced MPs into naïve mice (28, 31, 32).

SCUBA diving causes upregulation of antioxidant genes and elevations of plasma and intracellular antioxidant enzyme levels (3, 5, 17, 21–23). These changes are assumed to occur due to elevations of O_2 partial pressure associated with breathing air at depth. Findings from murine studies and also several trials involving human divers have led us to hypothesize that MP production may actually occur because of high-pressure inert or ballast gas (such as nitrogen [N_2]) exposures, rather than being a consequence of elevations in O_2 partial pressure per se or due to decompression (20, 26). Neutrophils generate MPs when exposed to elevated partial pressures of helium (He), N_2, or argon (Ar), even when there is no elevation of O_2 partial pressure beyond that associated with ambient air (24). This occurs due to oxidative stress initiated by singlet O_2, which is generated by collision complexes between O_2 and inert gases. Singlet O_2 triggers a cascade of cytoskeletal modifications that generate MPs. These events are inhibited by ascorbic acid, which is an efficient singlet O_2 scavenger, but a relatively poor scavenger for more highly reactive oxygen species, nitric oxide (NO), and related agents, such as peroxynitrite (2, 8). MP production, neutrophil activation, and associated vascular injuries in mice exposed to high-pressure air can be inhibited by ascorbic acid (30).

Dietary supplementation with ascorbic acid and vitamin E attenuates endothelial dysfunction in human SCUBA divers (18, 19). Therefore, we chose to evaluate whether ingestion of ascorbic acid prior to diving may diminish MPs production in human divers. As a component to this study, MPs were probed for nitrotyrosine. Nitration of protein tyrosine residues to form 3-nitrotyrosine by highly reactive species, such as peroxynitrite, occurs even in the presence of reduced thiols and other antioxidant scavengers (1, 7). Our rationale for probing nitrotyrosine comes from more basic studies showing that intrinsic characteristics of MPs from decompressed mice, rather than merely the elevation in their number, cause vascular and neurological injuries (27, 28, 31, 32). One component to this process pertains to augmented activity of nitric oxide synthase-2 (NOS-2 or inflammatory/inducible NOS) in cells and within MPs (24, 28). A subset of MPs in mice subjected to high pressure have been shown to contain nitrogen dioxide, which could react with proteins to generate nitrotyrosine (28). Nitrotyrosine is elevated in neutrophils after SCUBA diving and in

Address for reprint requests and other correspondence: S. R. Thom, Dept. of Emergency Medicine, Univ. of Maryland, 655 W. Baltimore St., Bressler Research Bldg., Rm. 4-013, Baltimore, MD 21201 (e-mail: sthom@smail.umaryland.edu).

isolated neutrophils exposed to elevated partial pressures of He, N_2, or Ar (21, 24).

The central place of bubbles as an inciting factor for DCS is widely accepted, yet most decompression procedures generate asymptomatic blood-borne bubbles (4, 12, 13). Inert gases inhaled while breathing are taken up by tissues in proportion to the ambient pressure, and when pressure is reduced, some of the gas released from tissues forms bubbles due to the presence of gas cavitation nuclei (6, 33, 34). Therefore, for the current study, we also measured intravascular gas bubbles after diving. Whereas ascorbic acid had no impact on intravascular bubbles, dietary supplementation abrogated neutrophil and MP changes triggered by diving. Moreover, exposure to O_2 at a partial pressure experienced by the divers did not cause these changes. We conclude that elevated partial pressures of ballast gases, such as N_2, are required for blood-borne changes, consistent with recent ex vivo studies (24).

METHODS

Subjects. Fourteen male divers were recruited for this investigation. All performed the SCUBA dives, and 10 were available to return for the studies involving 60% O_2 breathing. The experimental procedures in the study were all performed in accordance with the Declaration of Helsinki and were approved by Institutional Ethical Committees; all participants provided fully informed, written consent.

Study protocol. Prior to SCUBA dives or exposures to 60% O_2 at ambient pressure, half of the subjects ingested placebo tablets, while the other half ingested ascorbic acid (1 g) twice daily for 6 days and again 2 h before diving or O_2 exposures. Following a cross-over study design, after a 2- to 3-wk study interruption, subjects switched groups by ingesting the opposite type of tablet before undertaking the dive or

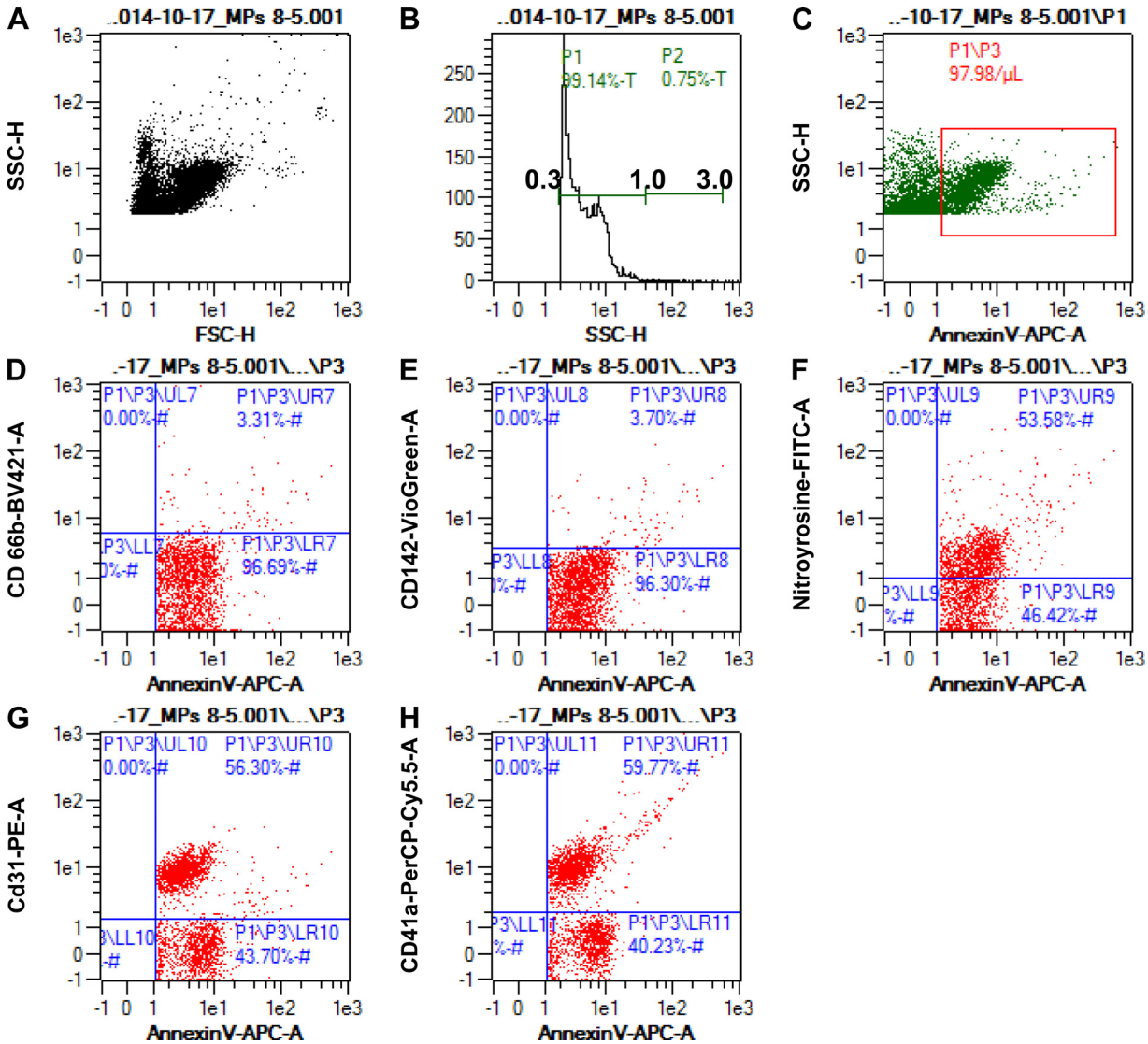

Fig. 1. Gating strategy for microparticle (MP) evaluations. After forward scatter (FSC-H) and side scatter (SSC-H) interrogation (*A*), the particle histogram is bracketed using the locations of microbeads with 0.3 µm, 1.0 µm, and 3.0 µm diameters (*B*). Particles between 0.3 and 1 µm in diameter are analyzed, and positive surface staining for annexin V is defined on the basis of the Fluorescence-Minus-One Control Test (FMO), which is established daily for each analysis and shown in *C* as a red box. Subsets of MPs expressing surface markers for CD66b (*D*), CD142 (*E*), nitrotyrosine (*F*), CD31 (*G*), and CD41 (*H*) are also identified on the basis of grids established according to FMO. Thus, particles 0.3 to 1 µm in diameter staining positive for annexin V and also positive for one of the additional surface proteins are quantified as those particles found in the upper right quadrant of *D* through *H*.

60% O_2 exposure. Inhalation of 60% O_2 was chosen as a stressor because this achieves the same partial pressure of O_2 that occurs while breathing air at 18 msw.

All procedures/protocols involved with SCUBA diving have been described in a previous publication (25). In brief, subjects were certified divers with diving experience ranging from 4 to 25 yr, who provided their own diving equipment. The dives were performed in November 2014 at the same location near Split, Croatia, as described previously (25). A 2- to 3-min surface swim through calm water was required for divers to reach the descent point and again to return to the exit point. The water temperature for the dives was ~17°C with minimal current. Divers had refrained from any diving and swimming activities for at least 7 days before the study. The diving profile, monitored electronically (Uwatec Galileo Sol, Johnson Outdoors, Racine, WI), involved a direct descent to 18 m while breathing compressed air (hence, 281 kPa O_2) for an actual bottom time of 47 min, then a 2-min ascent to the surface. Subjects swam continuously while at depth at a pace intended to represent a sustained moderate to moderately heavy work rate. Vascular gas emboli (bubbles) were monitored by transthoracic echocardiography (TTE) with a phase array probe (1.5–3.3 MHz) using a Vivid q scanner (General Electric, Waukesha, WI) on each subject every 30 min for 1.5 h post-dive in a beachside room ~50 steps from the dive site. These studies were conducted at rest and after arm or leg exercise to elicit bubble release from vascular margins, exactly as described in our previous report (25). Bubble grading employed a modified Brubakk scale that has been used in several studies (12). The grading system is as follows: 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 [(a = at least one bubble per cm^2 in all frames), (b = at least three bubbles per cm^2 in all frames), or (c = almost complete "whiteout" but individual bubbles can still be discerned)] and 5, whiteout where individual bubbles cannot be discerned. Venous blood was collected by a trained phlebotomist 30 min prior to and at 30 and 120 min after the dives. Blood was drawn into Cyto-Chex BCT test tubes that contain a proprietary preservative (Streck, Medimark Europe, Grenoble, France). The volume drawn per sample (two tubes) was ~5 ml.

Ambient pressure exposures were conducted for 47 min with subjects breathing 60% O_2 (balance of gas was pure N_2) from tight-fitting face masks equipped with an O_2 reservoir bag and ball valve to prevent rebreathing exhaled gas. Subjects were seated during these exposures and blood obtained in Cyto-Chex BCT tubes before and at 2 h after the exposure.

Materials. Unless otherwise noted, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Annexin binding buffer and the following antibodies purchased from BD Pharmingen (San Jose, CA): FITC-conjugated Annexin V, APC-conjugated Annexin V, FITC-conjugated anti-human myeloperoxidase (MPO), BV421-conjugated anti-human CD66b, P-phycoerythrin (PE)-conjugated anti-human CD31, R-PE-conjugated anti-human CD18, and PerCP/Cy5.5-conjugated anti-human CD41. VioGreen conjugated anti-human CD142 was purchased from Miltenyi Biotec Corp (Auburn, CA), Alexa Fluor 488 conjugated anti-nitrotyrosine was purchased from Abcam (Cambridge, MA).

Standard laboratory procedures. Blood samples were sent by express mail to the University of Maryland, where all analyses were performed within 48 h after arrival, ~4 to 7 days from time of collection. As described previously, MPs and neutrophil characteristics remain unchanged when samples stored at either 4°C or at room temperature are processed in a time span of 3 wk from time of collection (25). Briefly, a small sample of the whole, fixed blood was set aside for evaluation of neutrophils and plasma separated for syndecan-1 analysis using a commercial kit (R&D Systems, Minneapolis, MN). The remaining blood was centrifuged for 5 min at 1,500 *g*, the supernatant was made with 12.5 mM EDTA and then centrifuged at 15,000 *g* for 30 min. Aliquots of the 15,000 *g* supernatant were stained with antibodies for MPs analysis by flow cytometry.

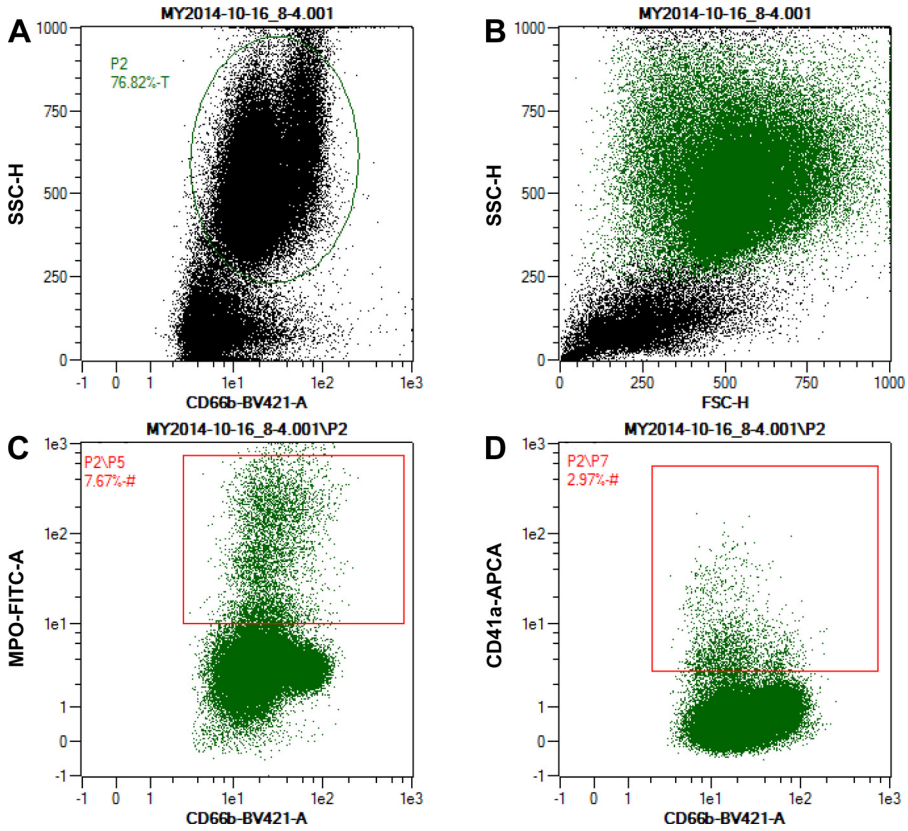

Fig. 2. Gating strategy for neutrophil evaluations. Antibody to CD66b is added to whole blood and positive events (defined based on daily FMO procedures) are plotted against SSC-H. A gate is generated based on size (SSC-H) (*A*), and back-gating (*B*) shows the population (green area) defined as neutrophils. The fraction of neutrophils exhibiting positive staining for MPO (*C*) and CD41 (*D*) is established based on FMO and shown in red boxes.

Table 1. *Intravascular bubble scores*

	30 min		60 min		90 min	
	Placebo	Ascorbate	Placebo	Ascorbate	Placebo	Ascorbate
Max	4C	4C	4B	4C	4B	4A
75th	4B	4B	4B	4B	4A	4A
Med	4A	4A	4A	4A	3	3
25th	3	4A	3	4A	2	3
Min	0	3	3	3	0	2

Measurements were carried out 30, 60, and 90 min after SCUBA diving, while subjects were at rest and after arm or leg movement. For most divers, the highest bubble scores were found after leg exercises, so these data are listed in the table. Maximum (Max), median (Med), minimum (Min), as well as 75th and 25th percentile scores are shown. There were no significant differences in scores between placebo and ascorbate groups.

Flow cytometry. Flow cytometry analysis of MPs and neutrophils was performed with an eight-color, triple-laser MACSQuant (Miltenyi Biotec, Auburn, CA) using the manufacturer's acquisition software. All reagents and solutions used for MPs analysis were sterile and filtered (0.2 μm filter). MPs were stained with annexin V and analyzed as previously described, including micro-beads with diameters of 0.3 μm (Sigma), 1.0 μm, and 3.0 μm (Spherotech, Lake Forest, IL) to assess the size of particles (25, 26). Surface markers were evaluated with use of the "Fluorescence-Minus-One Control Test". We define MPs as annexin V-positive particles with diameters up to 1 μm. Neutrophils in whole blood were identified by CD66b staining and surface expression of MPO and CD41 assayed, as previously described (25, 26). Gating strategies for MPs and neutrophil analyses are outlined in Figs. 1 and 2.

Statistical analysis. Parametric data are expressed as means ± SE, and nonparametric data are expressed as median, 25th, and 75th percentile values. We used SigmaStat software (Systat, Point Richmond, CA) for the statistical analysis. MP numbers, MP subtypes, neutrophil activation, and all parametric data were analyzed by two-way repeated-measures ANOVA followed by the Holm-Sidak test to evaluate the impact of time from decompression and use of ascorbic acid. Bubble scores (nonparametric data) were analyzed using repeated-measures ANOVA on ranks. Significance was accepted at $P < 0.05$ for a two-tailed *t*-test.

RESULTS

Diving and intravascular bubbles. Divers reported no adverse effects from any of the SCUBA activities. Post-dive circulating bubbles quantified by transthoracic echocardiography were assessed while subjects were at rest and after arm or leg movement. There were no significant differences in scores between placebo and ascorbate groups at rest or after limb movements. The distribution of highest bubble scores are shown in Table 1.

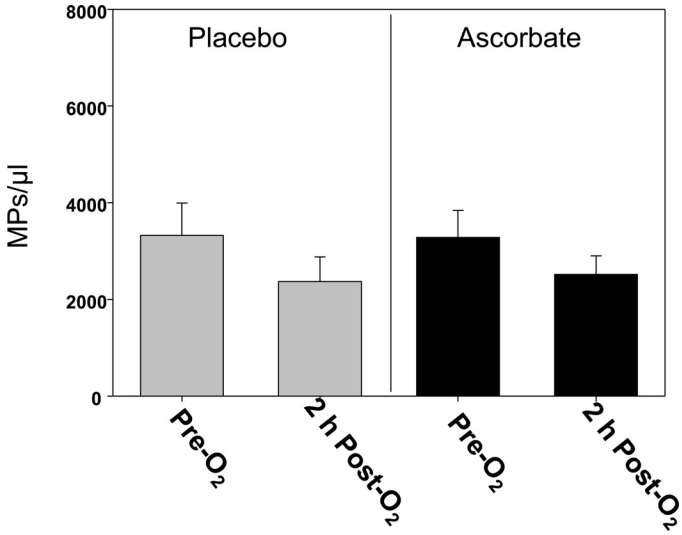

Fig. 4. MPs in blood following 60% O_2 breathing. Placebo and ascorbic acid ingestion was as described in the caption for Fig. 1. MPs values were not statistically significantly different.

MP elevations. Circulating MP counts related to diving are shown in Fig. 3. Significant elevations were found at both 30 min and 2 h post-dive in the placebo group, whereas no elevations occurred in divers after ingesting ascorbic acid for 1 wk. When subjects breathed 60% O_2 for 47 min, no elevations of MPs were identified (Fig. 4).

MPs surface protein expression patterns. MP subtypes were characterized by surface markers for vascular cell proteins and nitrotyrosine. Fig. 5 shows the number of annexin V-positive MPs coexpressing each surface protein marker. Significant differences vs. pre-dive values were found for all MP subtypes in the placebo group, but no elevations occurred in the ascorbate group. No subtype differences were observed after exposure to 60% O_2 in either the placebo or ascorbate groups (data not shown).

Neutrophil activation. Figure 6 shows measurements of neutrophils obtained before and after diving. The neutrophil population was first identified by surface expression of CD66b, and then expression of myeloperoxidase (MPO) was assessed as an index of neutrophil activation. Surface expression of the platelet-specific protein CD41 was also assessed as a measure

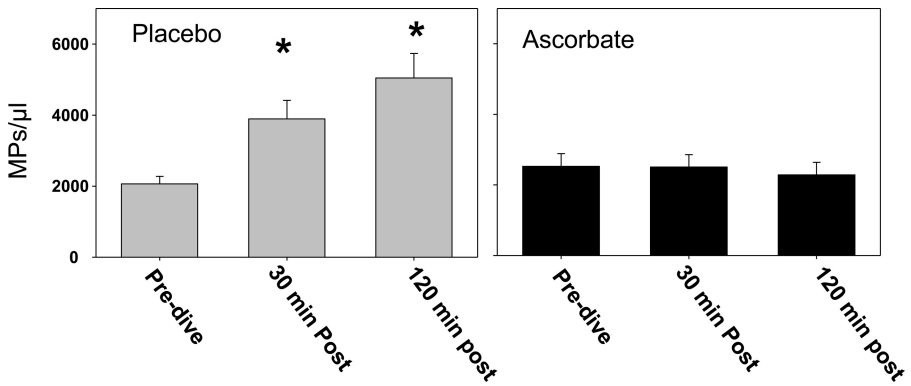

Fig. 3. MPs in blood after diving. Total MPs in blood of subjects before dive and 15 and 120 min after dive are shown. As indicated, subjects ingested placebo or ascorbic acid (1 g) twice daily for 6 days, as well as on the morning prior to diving. Values are expressed as means ± SE. Post-dive values that are statistically significantly different from the pre-dive values are shown by an asterisk (*).

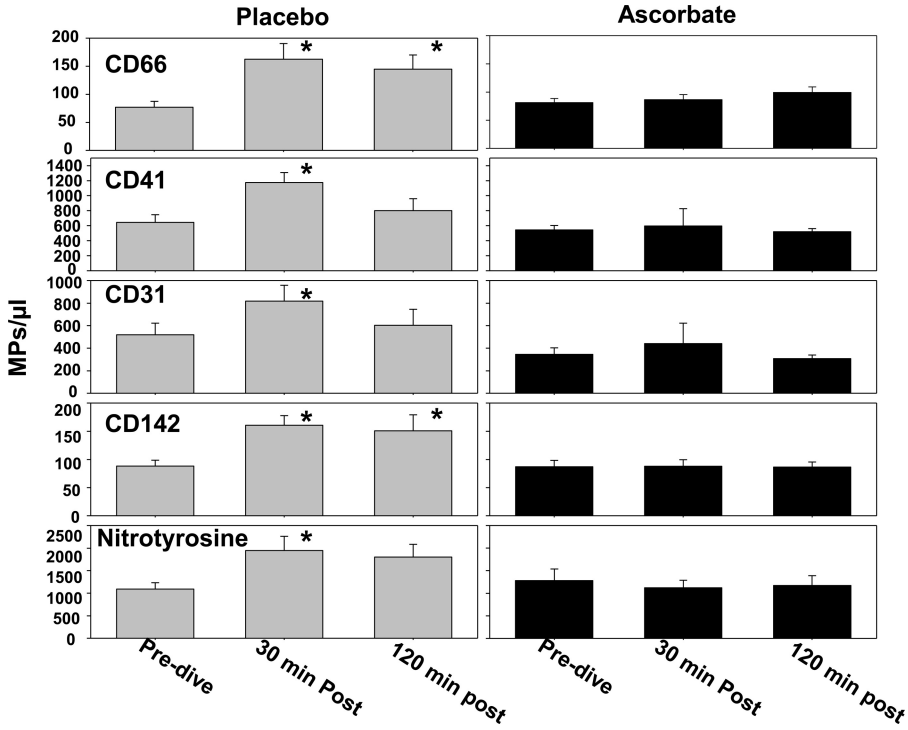

Fig. 5. Pre-diving and post-diving MPs expressing surface markers for various antigens. MPs expressing the neutrophil protein CD66b are shown in the first panel, while those bearing the platelet-specific CD41 in the second, CD31 (PECAM) in the third, CD142 or tissue factor in the fourth, and nitrotyrosine in the fifth. Values are expressed as means ± SE. Post-dive values that are statistically significantly different from the pre-dive values are shown by an asterisk (*).

of neutrophil-platelet interactions. Significant elevations were found at both 30 min and 2 h post-dive in the placebo group, whereas no elevations occurred in divers after ingesting ascorbic acid for 1 wk. No elevations in neutrophil activation or platelet-neutrophil interactions were identified after breathing 60% O_2. Fold changes after 60% O_2 compared with the preexposure values for MPO and CD41 in the placebo group were 1.16 ± 0.23 and 0.99 ± 0.07, respectively (not significantly different vs. preexposure, NS), and for the ascorbic acid group 1.06 ± 0.20 and 1.14 ± 0.08 (NS).

Syndecan-1 levels. Syndecan-1 is a heparin sulfate proteoglycan bound to the endothelium that can be shed in response to oxidative stress (9, 10). There were no statistically significant changes in blood-borne syndecan-1 levels related to diving. Prior to diving, the placebo group level was 37.6 ± 2.9 pg/ml plasma and after ingesting ascorbic acid 34.8 ± 6.8 (NS). At 30 min post-dive, the values in the placebo and ascorbic acid groups were 30.9 ± 4.1 and 38.1 ± 4.7, pg/ml plasma, respectively (NS) and at 2 h 33.0 ± 3.0 and 30.7 ± 3.4 pg/ml plasma, respectively (NS).

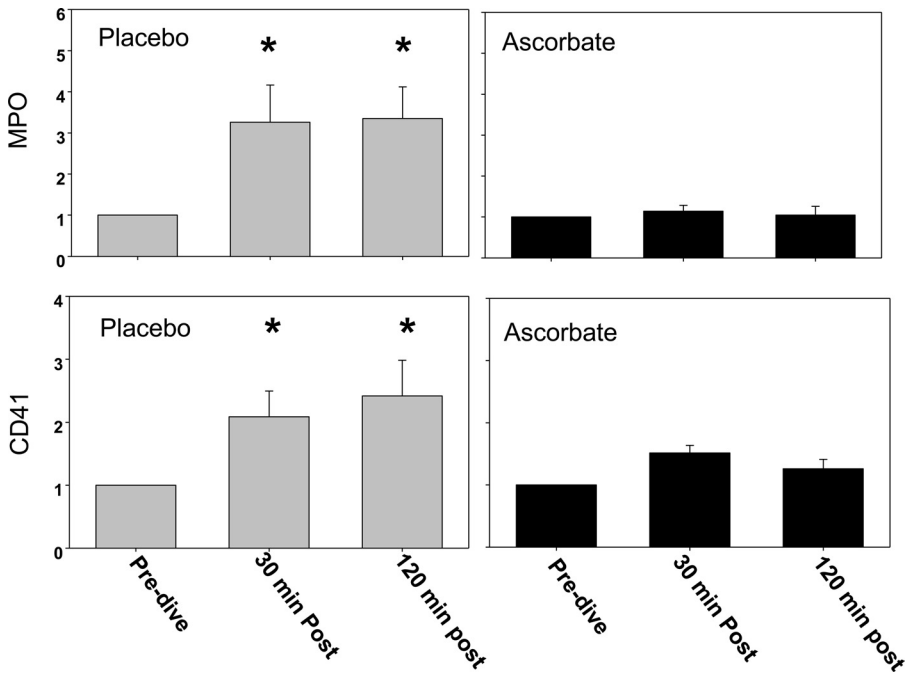

Fig. 6. Neutrophil-associated changes after diving. Neutrophils were identified by CD66b staining and the percent of cells exhibiting co-surface expression of myeloperoxidase (MPO) and platelet-specific CD41 were assessed by flow cytometry. Data are normalized and expressed as fold-change from pre-dive values to ease comparisons. There were no significant differences in pre-dive values between placebo and ascorbic acid groups. Values are expressed as means ± SE. Post-dive values that are statistically significantly different from the pre-dive values are shown by an asterisk (*).

DISCUSSION

There is substantial evidence that oxidative stress occurs with SCUBA diving (3, 5, 17, 21–23). We found that prophylactic ingestion of ascorbic acid was effective for inhibiting MP elevations and neutrophil activation due to diving. This finding has several interesting implications. First, it underscores the notion that MP generation from high-pressure exposures may be an oxidative stress response. This view is also supported by several other studies (24, 28, 30–32). Further, because ascorbic acid is not a particularly potent inhibitor of highly reactive agents such as peroxynitrite and nitrogen dioxide, our results offer insight into the mechanism of MP production due to diving. This is the first report of MPs expressing nitrotyrosine, a manifestation of protein nitration likely resulting from production of peroxynitrite or some similarly reactive nitrogen species. Increased presence of nitrotyrosine on MPs is consistent with results in a mouse model and also a report of nitrotyrosine elevations in neutrophils obtained from SCUBA divers (21, 24). Because dietary ascorbic acid supplementation abrogates elevations of nitrotyrosine-bearing MPs, the results suggest that ascorbic acid acts at sites proximal to pressure-related production of the reactive species capable of nitration reactions. In neutrophils, ascorbic acid inhibits MPs generation because it limits singlet oxygen production, which is generated because of successive events linked to cytoskeletal modifications that activate NADPH oxidase and NOS-2 (24). This same pathway could be occurring in circulating cells in vivo, leading to nitrotyrosine-bearing MPs.

Exposure to an elevated partial pressure of O_2 without high-pressure N_2 did not cause MP elevations or changes on neutrophils. With regard to comparisons between open-water diving and the 60% O_2 inhalation study, there are clearly differences between these stresses other than ballast gas (primarily N_2) partial pressure. We have previously carried out a series of open-water control dives at just 5 msw depth, which demonstrated that stresses due to swimming the same underwater course as used in the current study in sea water at the same temperature while breathing air from SCUBA apparatus generated no significant changes in annexin V-positive particle number or various subgroups (25). Therefore, we conclude that high-pressure nitrogen is necessary for the elevations in MPs and neutrophil activation associated with diving.

The sites where diving-related oxidative stress arise are not fully elucidated. Clearly, neutrophils can be a source (24). Post-dive MPs bear proteins derived from neutrophils (CD66b) and platelets (CD41), CD142, which may come from endothelial cells, monocytes, macrophages, and platelets, as well as CD31, which is highly expressed at endothelial cell intercellular junctions but is also found on the surface of platelets, monocytes, neutrophils, and some types of T-cells. Because MPs interact and share surface proteins, finding various proteins on particles does not mean that MPs bearing a particular marker for, say, neutrophils (CD66b) were all derived from neutrophils (25, 26, 31, 32). We investigated plasma syndecan-1 as a possible alternative approach to surveying oxidative stress at the endothelial lining. While the null result is interesting, the physiological relevance is unclear. When elevations are found, this can arise due to proteolysis, but we do not know whether oxidation-induced syndecan-1 liberation is a generalized phenomenon (11).

There is now considerable precedence for MP elevations associated with SCUBA diving (14–16, 20, 25, 26, 29). Murine studies suggest that MPs play a role in decompression pathophysiology and possibly with gas bubble nucleation (27, 28, 31, 32). Maneuvers that decrease the incidence of DCS also diminish MP production (14, 15). A causal relationship between MPs and DCS in humans is, however, still lacking. Our observation that MP elevations can be abrogated by dietary ascorbic acid supplementation offers a novel approach for further new studies of, perhaps, more provocative dive profiles.

Perspectives and Significance

Neutrophil activation and elevations of MPs, especially those expressing nitrotyrosine, appear to be oxidative stress responses to diving. Ascorbic acid abrogates these changes, which suggests that the same mechanistic pathways are occurring in human divers as have been described in murine studies and ex vivo isolated cell experiments. Given that these blood-borne events trigger vascular and neurological injuries in the mouse decompression stress model, alternative approaches in addition to traditional decompression tables may improve diver safety.

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Present address: Otto F. Barak, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.Y., O.F.B., D.M., V.M.B., J.B., and S.R.T. performed experiments; M.Y. and S.R.T. analyzed data; M.Y., O.F.B., D.M., J.B., and S.R.T. interpreted results of experiments; M.Y., O.F.B., Z.D., D.M., V.M.B., J.B., and S.R.T. edited and revised manuscript; M.Y., O.F.B., Z.D., D.M., V.M.B., J.B., and S.R.T. approved final version of manuscript; O.F.B., Z.D., and S.R.T. conception and design of research; S.R.T. prepared figures; S.R.T. drafted manuscript.

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