The impact of different gas mixtures on inflammatory responses in advanced recreational divers

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Key words
Air; Decompression sickness; Deep diving; Diving research; Inflammation; Trimix

Abstract
(Rocco M, Maggi L, Loffredo C, Pelli M, Di Benedetto P, Fiorelli S, Simmaco M, De Blasi RA. The impact of different gas mixtures on inflammatory responses in advanced recreational divers. Diving and Hyperbaric Medicine. 2021 June 30;51(2):140–146. doi: 10.28920/dhm51.2.140-146. PMID: 34157728.)
Introduction: Decompression sickness (DCS) is considered a 'bubble disease'. Intravascular bubbles activate inflammatory responses associated with endothelial dysfunction. Breathing gas has been proposed as a potential risk factor but this is inadequately studied. Different gases are used in scuba diving. Helium-containing 'trimix' could theoretically mitigate inflammation and therefore reduce DCS risk. This study determined the effect of air and trimix on the inflammatory response following dives to 50 metres of sea water, and evaluated the differences between them in advanced recreational divers.
Methods: Thirty-three divers were enrolled in this observational study and were divided in two groups: 17 subjects were included in the air group, and 16 different subjects were included in the trimix (21% oxygen, 35% helium, 44% nitrogen) group. Each subject conducted a single dive, and both groups used a similar diving profile of identical duration. A venous blood sample was taken 30 min before diving and 2 h after surfacing to evaluate changes in interleukins (IL) IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, tumour necrosis factor α (TNFα), vascular endothelial growth factor (VEGF), Interferon γ (IFN-γ), monocyte chemoattractant protein 1 (MCP-1) and epithelial growth factor (EGF) after diving.
Results: No differences were observed between groups in demographic data or diving experience. Following the dive, IL-6 values showed a slight increase, while IL-8 and EGF decreased in both groups, without significant variation between the groups.
Conclusions: In physically fit divers, trimix and air gas mixture during deep diving did not cause relevant changes in the inflammatory markers tested.

Introduction

Decompression illness is a condition that includes decompression sickness (DCS) and arterial gas embolism (AGE). DCS is a 'bubble disease', where bubbles form due to gas supersaturation in tissues during ascent.[1] A correlation between the degree of bubbling and the risk of DCS has been identified, even if the presence of circulating bubbles cannot be used to predict DCS in individuals.[2]

Intravascular bubbles may trigger an inflammatory response associated with endothelial dysfunction.[3] Using a rat model of DCS some authors have suggested that bubbles may be the cause of decompression-induced endothelial damage, which results in the release of various inflammatory mediators.[4] Moreover, intravascular inert gas bubbles have been linked to the elevation of circulating microparticles observed both in humans and in animal models of diving and these microparticles are associated with inflammation and neutrophil activation.[5] In addition, acute changes of inflammatory factors have recently been used as biomarkers to predict decompression quality, even in the absence of DCS events.[6]

Different types of breathing gas are used in scuba diving: air and non-air mixtures. The latter contain mainly oxygen, nitrogen and helium that form nitrox (nitrogen + oxygen), heliox (helium + oxygen), and trimix (oxygen, helium and nitrogen) with different advantages and features.[7] Nitrox is mainly used for shallow recreational dives, heliox for deep diving, and trimix for deep but short dives, in order to avoid neurological side effects.[7] The use of trimix in scuba diving has recently become more widespread in order to reduce nitrogen narcosis compared to air diving.[8]

The aim of the present study was to assess the effect of two breathing gas mixtures on the inflammatory mediators in deep seawater dives, and to investigate the differences between trimix and air in physically fit divers.

Methods

All experimental procedures were performed in accordance with the Declaration of Helsinki. The Institutional Review Board of Sapienza University, Rome, Italy approved all protocols (CE 2035/2015). Written informed consent was obtained from all subjects.

STUDY POPULATION

This observational study enrolled 33 experienced, certified divers. Each diver was randomly allocated into one of two groups: air or trimix. The air group (A) was composed of 17 subjects, 16 men and one woman, while the trimix group (T) was composed of 16 subjects, 15 men and one woman. All subjects were physically fit to dive according to the International Diving Medicine Expert Board fitness to dive criteria (http://www.edtc.org/EDTC-Fitnesstodivestandard-2003.pdf): They were non-smokers and performed regular cardiac aerobic and muscle strengthening activity such as brisk walking, running, jogging and swimming during their week and deep dives 4–5 times a month. Body mass index (BMI) was calculated for all divers. All divers were instructed not to consume alcohol for 72 h or coffee for 6 h before the experimental dive.

DIVE EXPOSURE

Each diver performed an open water dive (east coast of Giannutri Island, Italy) during summer wearing a dry suit, to a depth of 50metres of sea water (msw) with a square profile and 20-min bottom time. The water temperature was 15°C at the bottom. The entire dive time was within 1 h. V-planner decompression software was used to calculate the decompression profile for both air and trimix groups (the 'V-planner' is freely available at: https://v-planner.soft.com). Different conservatism settings were used for the air and trimix profiles to result in decompression schedules with identical total decompression time (Figure 1).

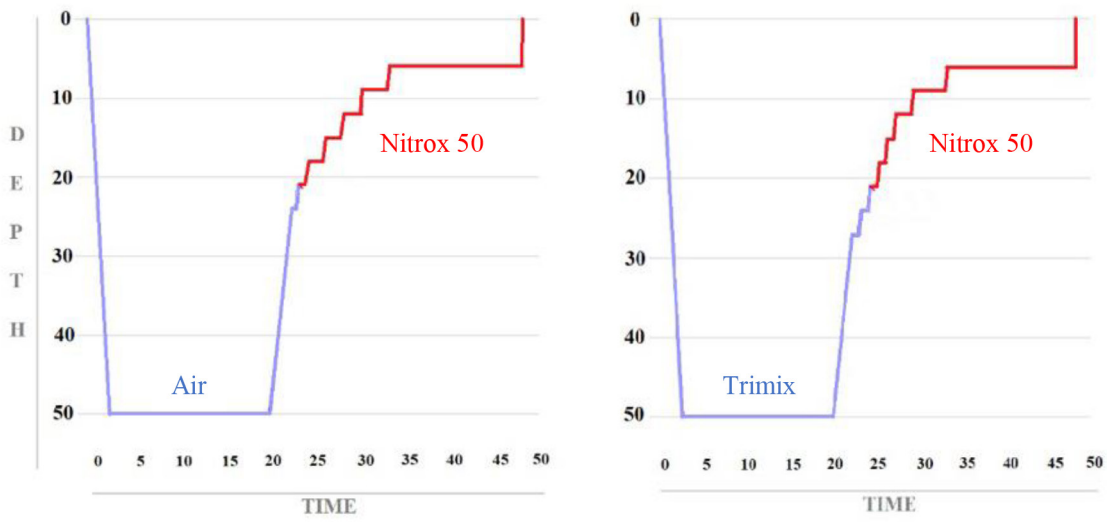
The divers in group A breathed compressed air (21% oxygen, 79% nitrogen) using open-circuit scuba equipment. The divers in group T breathed trimix 21/35 (21% oxygen, 35% helium, 44% nitrogen) also using open-circuit scuba. Both groups used a nitrox mixture (50% oxygen and 50% nitrogen) for decompression starting at 21 msw during the ascent until surfacing (Figure 1). Divers were requested to swim slowly and avoid effort as much as possible. We attempted to wait for constant environmental variables, such as water temperature (15 ± 2°C), same dive path using previously positioned markers on the seabed, and weather variables. The subjects were accompanied on the dive by safety divers who set the swimming pace.

LABORATORY ANALYSIS

A venous blood sample was taken from each diver 30 min before the dive and 2 h after surfacing, to evaluate pro-inflammatory interleukins (IL-1α, IL-1β, IL-6, IL-8), anti-inflammatory cytokines (IL-2, IL-4, IL-10), tumour necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF), interferon-γ (IFNγ), monocyte chemoattractant protein 1 (MCP-1) and epidermal growth factor (EGF) variations induced by diving.

The concentrations of these factors were simultaneously assessed using cytokine and growth factor arrays (Evidence Investigator Biochip Array technology®, Randox Laboratories, Crumlin, UK) in accordance with the

Figure 1
Air and trimix dive profile. Red lines signify Nitrox 50% used by both groups. Depth in metres of seawater and time in minutes



manufacturer's instructions. The sensitivities of the test kits were as follows: IL-1α – 0.8 pg·mL^{-1}; IL-1β – 1.6 pg·mL^{-1}; IL-6 – 1.2 pg·mL^{-1}; IL-8 – 4.9 pg·mL^{-1}; IL-2 – 4.8 pg·mL^{-1}; IL-4 – 6.6 pg·mL^{-1}; IL-10 – 1.8 pg·mL^{-1}; IFNγ – 3.5 pg·mL^{-1}; TNFα – 4.4 pg·mL^{-1}; VEGF – 14.6 pg·mL^{-1}; EGF – 2.9 pg·mL^{-1}; and MCP-1 – 13.2 pg·mL^{-1}.

The reference ranges for healthy adults for these molecules reported with the test kits are duplicated in Table 1.

The primary endpoint was to determine differences in blood cytokines before and after diving in volunteers breathing either air or trimix.

STATISTICAL ANALYSIS

Statistical analysis was performed using Sigmaplot (Systat Software, San Jose, CA, USA). Data normality was assessed using the Kolmogorov-Smirnov test. Data were expressed as means with standard deviation (SD) when normally distributed and as medians and 95% confidence intervals when non-normally distributed. Descriptive analysis was performed using percentages for binary variables. The Wilcoxon matched pair test was used to assess statistical significance before and after diving for non-parametric variables and repeated measures ANOVA was used for parametric variables. Variables that showed differences before and after diving were compared between air and trimix groups. Data were evaluated using *t*-test for independent data with alpha values of 0.05 and Welch's correction.

Results

All subjects completed the study. None of the divers developed DCS or pathological symptoms and signs. All divers performed their dives at the same sites with the same environmental conditions in terms of water temperature and visibility. There were no differences between the two groups in terms of demographic data or diving experience. There were no between group differences in mean age 46.1 (SD 4.9) years versus 47.6 (5.2), $P = 0.40$; BMI 22.6 (2.1) kg·m^{-2} versus 23.5 (2.4), $P = 0.26$; and the median number of yearly dives 50 (95% CI 29–80) versus 75 (49–101), $P = 0.65$. Pre-dive and post-dive results are shown in Table 2. The levels of IL-2, IL-4, IL-10, VEGF, IFNγ, TNFα, IL-1α, IL-1β, and MCP-1 did not significantly change after diving in both groups. IL-8 (Figure 2) and EGF (Figure 3) levels decreased after diving in both groups and IL6 increased (Figure 4). The increase in IL6 was smaller in the trimix dives than in the air dives, but this was not significant ($P = 0.67$). The decreases in IL8 and EGF were not different ($P = 0.47$ and $P = 0.72$, respectively). All molecules were within the normal ranges given in Table 1 both pre- and post-dive. No normal range was defined for MCP-1 and EGF.

Table 1
Reference ranges in healthy adults for inflammation markers assayed. EGF – epidermal growth factor; IFNγ – interferon gamma; IL – interleukin; MCP-1 – monocyte chemoattractant protein 1; TNFα – tumour necrosis factor alpha; VEGF – vascular endothelial growth factor

Inflammation marker	Reference (pg·ml^{-1})
IL-1α	0–3.9
IL-1ß	< 5.0
IL-6	< 7.0
IL-8	0–50.0
IL-2	< 50.0
IL-4	< 38.7
IL-10	< 5.7
VEGF	62.0–707.0
IFNγ	0–15.6
TNFα	< 8.1
MCP-1	undefined
EGF	undefined

Discussion

In the present study we observed a significant increase of IL6 and a decrease of IL8 and EGF levels in both groups which nevertheless remained within the respective normal ranges after a 50 msw dive. The gas mixture (air or trimix) did not influence the inflammatory response of the subjects studied. In the last decades, trimix has been introduced in scuba diving as an alternative to air to reduce gas density and the risk of nitrogen narcosis[7,8] and to explore deeper depths for longer durations using different decompression algorithms, which are still the subject of debate.[9]

Scuba diving triggers pro-inflammatory reactions in blood, with increased expression of adhesion molecules, activation of coagulation, and elevated circulating microparticles.[10–14] Such responses are triggered by oxidative stress and play important roles in maintaining physiological homeostasis. Bubbles, microparticles and circulating agents are, at least in part, responsible for inciting the so-called 'endothelial dysfunction' that, in turn, causes activation of the inflammatory response;[14] thus, monitoring post-dive circulating inflammatory molecules could provide biomarkers of decompression and DCS risk.

These data showed post-dive alterations in only IL-6, IL-8, and EGF levels. Specifically, IL-6 showed an increase, while IL-8 and EGF decreased, in both groups. Although they were

Table 2

Air and trimix groups pre- and post-50 msw dives, showing evaluation of: pro-inflammatory interleukins (IL-1α, IL-1β, IL-6, IL-8); anti-inflammatory cytokines (IL-2, IL-4, IL-10); and other factors, tumour necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF), interferon-γ (IFNγ), monocyte chemoattractant protein 1 (MCP-1) and endothelial growth factor (EGF). NA* – not applicable, the values for pre- and post-dive assays are too close to zero to produce a *P*-value

Marker	Pre-air pg·ml^{-1}	Post-air pg·ml^{-1}	*P*-value	Pre-trimix pg·ml^{-1}	Post-trimix pg·ml^{-1}	*P*-value
Inflammatory factors						
IL-1α median (95% CI)	0 (0, 0)	0 (0, 0)	1	0 (0, 0)	0 (0, 3.34)	0.125
IL-1ß median (95% CI)	0 (0, 0)	0 (0, 0)	0.812	0 (0, 0)	0 (0, 0.33)	0.125
IL-6 median (95% CI)	0.98 (0.85, 1.76)	1.90 (1.27, 3.90)	0.015	0.83 (0.71, 1.19)	1.17 (0.85, 2.71)	0.0002
IL-8 mean (SD)	20.08 (11.23)	11.88 (6.88)	0.0235	22.94 (11.59)	12.68 (7.52)	0.005
Anti-inflammatory factors						
IL-2 median (95% CI)	0 (0, 0)	0 (0, 4.27)	0.187	0 (0, 0)	0 (0, 0)	NA*
IL-4 median (95% CI)	0 (0, 4.42)	1.25 (0, 1.37)	0.105	0 (0, 1.27)	0 (0, 1.25)	0.812
IL-10 median (95% CI)	0.00 (0.00, 1.05)	0 (0, 0.96)	1	0 (0, 0)	0 (0, 0)	NA*
Other factors						
VEGF mean (SD)	203.62 (16.38)	224.39 (23.08)	0.336	189.90 (18.63)	188.40 (18.84)	0.912
INFγ median (95% CI)	0 (0, 0)	0 (0, 0)	NA*	0 (0, 0.25)	0 (0, 0.24)	0.875
TNFα median (95% CI)	2.00 (1.62, 2.18)	1.78 (1.58, 2.01)	0.427	1.81 (1.47, 2.44)	1.72 (1.47, 2.13)	0.135
MCP-1 mean (SD)	337.53 (23.33)	383.75 (28.25)	0.076	361.41 (32.59)	361.52 (42.84)	0.997
EGF mean (SD)	170.57 (60.82)	81.11 (71.84)	0.0005	144.68 (45.41)	58.48 (59.31)	0.0001

statistically significant changes, the values remained within the reference values suggesting dubious clinical relevance and lack of support for any difference between air and trimix. We could argue that the dives were not long enough to manifest a difference in nitrogen and helium gas uptake and washout, or that the dive profile was not 'strenuous' enough to observe significant differences in inflammation. Endothelial physiology, besides on individual genetics, is certainly linked to the inflammatory response but the severity of these reactions increases with increasingly stressful decompression.[13,15]

The increase in IL6 observed after diving was not of a magnitude associated with inflammatory disease, but more in line with increases associated with physical exercise.[16] Several studies have reported that physical activity can induce an acute phase response characterised mostly by an increase in IL6.[17,18] A cytokine cascade induced by exercise markedly differs from that induced by infections in lacking the classical surge in proinflammatory cytokines as TNFα and IL-1β. It is plausible, besides, that the post-exercise EGF reduction, is finalised to facilitate defence mechanisms against oxidative stress and may be linked to the role of EGF in reactive oxygen species (ROS) production.[19] The alterations of IL and EGF in our study, although significant, however, were negligible, and most probably the result of an average physical effort.

The properties of helium (one of the trimix gases) have also been studied extensively outside of diving medicine, arousing much interest in the field of ventilation and organ protection[20] as well on the human or animal immune response.[21–23] There have been conflicting results. One human study of cardiac preconditioning by inhaled helium suggested a mild anti-inflammatory effect.[21] Another study of acute lung injury in newborn pigs breathing heliox or

Figure 2
IL-8 levels before and after dives using air or trimix. Box and whisker plots show median, interquartial range, and highest and lowest values

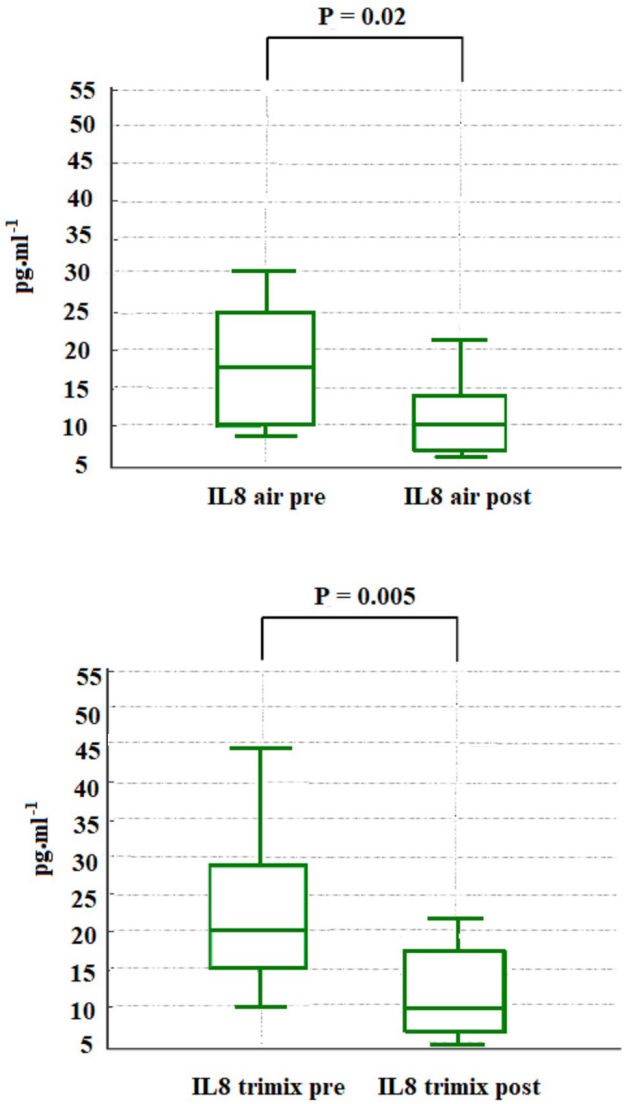
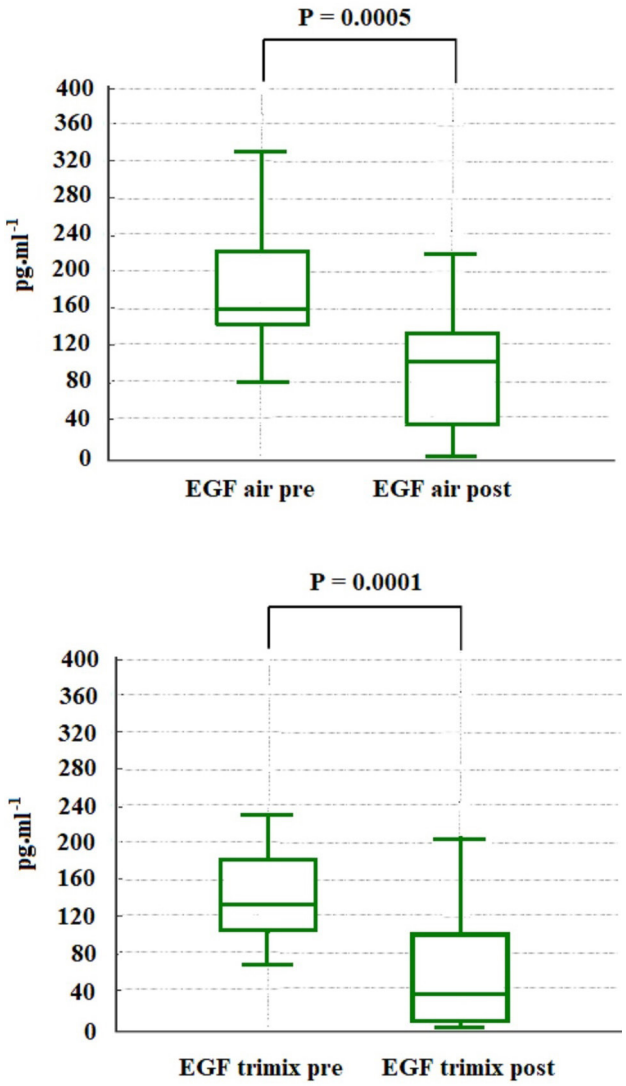


Figure 3
EGF level changes before and after dives using air or trimix. Box and whisker plots show median, interquartial range, and highest and lowest values

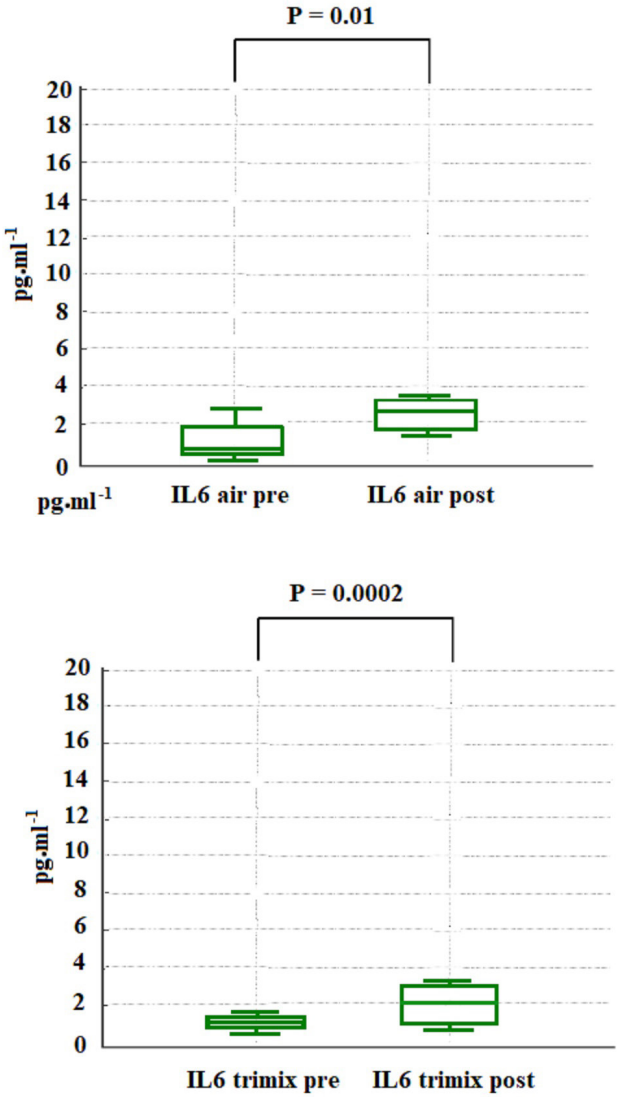


nitrox showed less lung inflammation reflected by lower tissue IL-6 and IL 8 in the heliox group.

Another study using human divers[6] compared a 30 msw no-stop air dive and 50 msw trimix dives using either a ratio decompression profile (RDP) or a compartment decompression model (CDM) decompression. The shorter no-stop air dive to 30 msw and the 50 msw RDP dive were more pro-inflammatory (in terms of chemokines – C-C motif chemokine ligand 2 [CCL2] and C-C motif chemokine ligand 5 [CCL5]) than the 50 msw CDM dive. The authors proposed a protective effect of helium on the endothelium to explain the apparently paradoxical worse outcome for the shallower air profile compared to one of the deeper trimix decompression profiles. There was no difference in CCL2 or CCL5 after a comparable level of surface swimming exercise, suggesting that in their experimental setting decompression and not physical exercise induced the changes in these inflammatory markers. It was hypothesised that divers who performed CDM were exposed to helium for a longer period of time than the other trimix group. It seemed likely that this gas was able to increase nitrous oxide (NO) and to induce the activation of nuclear factor erythroid 2-related factor 2 and the consequent reduction of systemic inflammation and oxidative stress.[23]

These experimental studies, although conflicting, invoke a possible anti-inflammatory role of helium. Our study found no difference in most of the inflammatory factors (including MCP-1) measured between the trimix and air groups casting doubt on a protective effect of helium unless, as noted above, our dives were not 'strenuous' enough during decompression to observe a significant anti-inflammatory role of helium.

Figure 4
IL-6 level changes before and after dives using air or trimix. Box and whisker plots show median, interquartial range, and highest and lowest values



LIMITATIONS OF THE STUDY

There are some limitations in our study. The presence of VGE in these divers was not evaluated and their possible correlation with inflammatory factors studied in order to identify a further indicator of decompression stress. Furthermore, systemic inflammatory markers were evaluated only at 2 h after diving and measures were not subsequently repeated; therefore, we can only suggest the activation-deactivation mechanism of the inflammatory cascade, as described above.

In addition, the study had a small but selected sample size and only included divers certified to perform technical dives at 50 msw, with a similar age and BMI. We attempted to minimise bias due to environmental parameters (same temperature and same dive path, previously decided) and procedural conditions (low physical exertion controlled by an external team of scuba divers). The two study groups did not perform both dives (crossover study); thus, it remains possible that an unknown factor was different in the two groups.

Lastly, the dive profile was not 'strenuous' enough to observe significant inflammatory changes. Further studies should select a dive profile known to induce inflammatory responses, at least using air or nitrox breathing gas.

Conclusions

In physically fit divers, no differences in inflammatory factors were found after deep diving using trimix versus air as the breathing gas. However, the dive profiles induced only small changes in inflammatory markers. The changes observed were within the normal range and were consistent with exercise induced changes.

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Conflicts of interest and funding

This study was funded by PRIN (Italian National Scientific Research Programme) 2012. No conflicts of interest, financial or otherwise are declared by the authors.

Submitted: 01 March 2020
Accepted after revision: 15 January 2021