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ORIGINAL ARTICLE

Dark chocolate reduces endothelial dysfunction after successive breath-hold dives in cool water

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Abstract
Objective The aim of this study is to observe the effects of dark chocolate on endothelial function after a series of successive apnea dives in non-thermoneutral water.
Methods Twenty breath-hold divers were divided into two groups: a control group (8 males and 2 females) and a chocolate group (9 males and 1 female). The control group was asked to perform a series of dives to 20 m adding up to 20 min in the quiet diving pool of Conflans-Ste-Honorine (Paris, France), water temperature was 27 °C. The chocolate group performed the dives 1 h after ingestion of 30 g of dark chocolate. Flow-mediated dilatation (FMD), digital photoplethysmography, nitric oxide (NO), and peroxynitrite $ONOO^-$) levels were measured before and after each series of breath-hold dives.
Results A significant decrease in FMD was observed in the control group after the dives (95.28 ± 2.9 % of pre-dive values, $p < 0.001$) while it was increased in the chocolate group (104.1 ± 2.9 % of pre-dive values, $p < 0.01$). A decrease in the NO level was observed in the control group (86.76 ± 15.57 %, $p < 0.05$) whereas no difference was shown in the chocolate group (98.44 ± 31.86 %, $p > 0.05$). No differences in digital photoplethysmography and peroxynitrites were observed between before and after the dives.
Conclusion Antioxidants contained in dark chocolate scavenge free radicals produced during breath-hold diving. Ingestion of 30 g of dark chocolate 1 h before the dive can thus prevent endothelial dysfunction which can be observed after a series of breath-hold dives.

Keywords Free radicals · Nitric oxide · Peroxynitrites · Flavonoids · Flow-mediated dilation

Abbreviations

AT II	Angiotensin II
BH_2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin
cGMP	Cyclic guanosine monophosphate
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FMD	Flow-mediated dilation
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
$ONOO^-$	Peroxynitrites
PI3K	Phosphatidylinositol-3-kinase
PO_2	Oxygen pressure
PPT	Peak-to-peak time
RAAS	Renin–angiotensin–aldosterone system
rpm	Revolutions per minute
sGC	Soluble guanylate cyclase
SI	Stiffness index
t test	Student's test

Introduction

Summertime in warmer climate regions is the ideal time for breath-hold divers and snorkelers to return to their favorite activities in a natural environment. Although these

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occasional breath-hold divers do not in general dive very deep and for long, professionals such as spear-fishermen, pearl collectors or competitions divers do (Schipke et al. 2006). These divers sometimes stay several hours in the sea where the temperature does not exceed 27° and sometimes without thermal protection. To our knowledge all studies on endothelial function in relation to breath-hold diving were performed in thermoneutral conditions (Theunissen et al. 2013a). Endothelial dysfunction accompanied by an increase in circulating nitric oxide (NO) had been noted after a series of successive breath-hold dives at 20 m depth. This NO increase had been associated with the cardiovascular effort required by breath-hold diving. By contrast, endothelial dysfunction observed by a decrease of flow-mediated dilation (FMD) was explained by a lesser availability of NO to participate in the FMD, either by its combination to superoxide anions or by another phenomenon independent from NO such as an autonomous nervous system action. Breath-hold diving is indeed associated with a change in sympathetic- and parasympathetic activity (Foster and Sheel 2005).

The cold inhibits NO synthase expression and provokes a vasoconstriction (Sun 2011). Intaking antioxidants, however, induces a vasodilation by NO release from endothelial cells (Cruz et al. 2006; Nakayama et al. 2013) and/or by enhancing its biodisponibility (Zhang et al. 2009). Endothelial dysfunction can thus be reduced by supplementing antioxidants for 4 weeks (Obad et al. 2007b). Dark chocolate has been proven to have positive effects on endothelial function (Grassi et al. 2012; Monahan 2012). This study therefore has two principal aims: (1) verify whether the underlying mechanisms to endothelial dysfunction are maintained in cold water conditions below 27° and (2) observe whether eating dark chocolate before a series of successive breath-hold dives can limit the endothelial dysfunction generally observed post-apnea.

Methods

Study population

After written informed consent, 20 non-smoking experienced (at least 4 years of experience) breath-hold divers volunteered for the study. Prior to entering the study, they were assessed fit to dive by a qualified diving physician. None of the subjects had a history of previous cardiac abnormalities and none of them were on any cardio-active medication. All participants were asked to refrain from strenuous exercise and nitrate-rich food for 48 h before the tests and not to dive for 72 h before testing. They were divided into a chocolate group (9 males and 1 female) and a control group (8 males and 2 females).

Dive profile and timeline of measurements

Each breath-hold diver performed successive dives to a depth of 20 m for a cumulative breath-hold time of 20 min. Dives were organized in pairs allowing each diver to act as the other's safety buddy. The total time in the water was around 1 h. At the surface, the divers were asked not to do any cardiovascular effort. All dives were performed (after breathing air) in a calm diving pool (Conflans-Ste-Honorine, France). The air temperature was 30° and the water temperature was 27 °C. The divers did not use wet suits.

The chocolate group performed the same dives in the same conditions as the control group 1 h after ingestion of 30 g of a commercially available Belgian dark chocolate with 86 % cocoa. The amount of polyphenols was 135.8 ± 2.9 μmol of catechin equivalents per gram.

Measurements

Endothelial function

Arterial endothelial function was assessed before and after each series of breath-hold dives by measuring the FMD of the brachial artery (Raitakari and Celermajer 2000). Since it is the relative FMD variation which is of interest here, the FMD measurement was assessed according to the methodology used by Brubbakk et al. (2005) for the same kind of in-field analysis following a standardized protocol and guidelines (Corretti et al. 2002). The FMD was measured with a 5–10 MHz transducer (Mindray DP 6600, Mindray, China). The brachial artery diameter was measured from longitudinal images with the lumen–intima interface visualized on both (anterior and posterior) walls. Boundaries for diameter measurement were identified automatically by means of a boundary tracking software (FMD-I software, FLOMEDI, Belgium) and optically controlled by an experimenter. Once the basal measurements were obtained, the sphygmomanometric cuff, placed above the ultrasound probe, was inflated and held at 50 mmHg above systolic pressure for 5 min. Occlusion up to 5 min produces a transient artery dilation attributable to NO synthesis (Lieberman et al. 1996). After ischemia the cuff was rapidly deflated and the brachial artery was monitored for an additional 4 min automatically. A single experienced experimenter performed and read all vascular studies. The FMD was computed as the percentage change in brachial artery diameter measured at peak dilation.

Digital photoplethysmography

Arterial stiffness of large arteries was estimated from pulse wave obtained at the finger by an infrared sensor (Pulse Trace PCA 2, Micro Medical, UK). This non-invasive

method is very easy to use and non-experimenter-dependent (Allen 2007). The waveform depends on vascular tone in the arterial tree. The contour of the wave exhibits two peaks. The first peak is formed by pressure transmitted along a direct path from the left ventricle to the finger. The second peak is formed in part by pressure transmitted along the aorta and large arteries to sites of impedance mismatch in the lower body (Millasseau et al. 2002). The peak-to-peak time (PPT) is the time taken for pressure to propagate along the aorta and large arteries to the major site of reflection in the lower body and back to the root of the subclavian artery. The waveform volume in the finger is thus directly related to the time it takes for the pulse waves to travel through the arterial tree. This PPT is proportional to subject height, and the stiffness index (SI) was formulated as *h*/PPT where *h* corresponds to the height expressed in m and PPT is the Peak-to-peak time expressed in seconds. Large artery stiffness decreases the time taken for pressure waves reflected from the periphery of the circulation to return to the aorta. Reflected waves arrive earlier in the cardiac cycle and may in part explain the change in pulse contour (Nicholas et al. 1990).

Blood analysis

Blood samples were collected before diving and 15 min after the series of dives. The samples were drawn from an antecubital fossa vein into an EDTA tube and centrifuged according to the protocol (1,000 rpm for 15 min for the NO and 3,500 rpm for 10 min for the peroxinitrites ($ONOO^-$) at 4 °C) in order to separate blood cells and plasma. The plasma was then stored at −80 °C and all analyses were performed within the following 6 months on the same microplate (one for each test) in order to analyze all the samples at the same time and avoid variance bias.

Plasma levels of nitrite and nitrate, NO metabolites, were determined by a colorimetric method (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions. The NO_2/NO_3 Assay Kit contains dyes, nitrate reductase, enzyme cofactor, buffer solution and NO_2, NO_3 solutions as standards. Total NO metabolites are thus detectable. Nitrite/nitrate (NO*x*) concentration in different dilutions of plasma ultrafiltrate was determined by colorimetry based on the Griess reaction (Theunissen et al. 2013a).

Peroxynitrites were measured according to the manufacturer's instructions using the OxiSelect^{TM} Nitrotyrosine ELISA kit.

All experimental procedures were conducted in accordance with the Declaration of Helsinki and were approved by the Academic Ethical Committee of Brussels. All methods and potential risks were explained to the participants in detail and they gave their written informed consent before the experiment.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 (La Jolla, CA, USA). Data are given as a percentage of pre-dive values. The difference between the percentage of pre-dive values and 100 % was compared by a two-tailed one-sample *t* test when normality of the sample was reached as assessed by the Kolmogorov–Smirnov test. Otherwise, the non-parametric Wilcoxon Rank Sum test was used. Statistical significance level was set at $p < 0.05$.

Results

When comparing control and chocolate divers, as far as age (47.3 ± 12.5 years vs. 49.7 ± 8.3 years, $p = 0.63$), height (178.4 ± 7.6 cm vs. 179.8 ± 6.1 cm, $p = 0.65$), weight (76.1 ± 11.5 kg vs. 77.3 ± 7.6 kg, $p = 0.78$) are concerned; both groups are comparable. The divers were asked to perform a series of breath-hold dives to 20 m in order to dive a cumulative breath-hold time of 20 min.

The mean number of breath-hold repetitions was 10 ± 2 dives in the control group with an immersion time of 19.57 ± 2.41 min and 9 ± 2 dives with a mean immersion time of 18.53 ± 3.5 min in the chocolate group. The mean recovery period between successive breath-hold dives was 4.55 ± 1.19 min. The duration time in the water was about 1 h. All divers completed the study and no one developed symptoms of decompression sickness.

Brachial artery diameter and flux-dependent dilation

No increase in pre-occlusion brachial artery diameter was observed for either the control group (102.1 ± 5.5 % of pre-dive values, $p > 0.05$) or the chocolate group (99.9 ± 1.5 % of pre-dive values, $p > 0.05$).

An FMD decrease was however observed in the control group between pre- and post-dive (95.3 ± 2.9 % of pre-dive values, $p < 0.001$) whereas the FMD was increased in the chocolate group (104.1 ± 2.9 % of pre-dive values, $p < 0.01$). The difference between the control group and that having eaten chocolate an hour before diving is statistically significant ($p < 0.001$). FMD changes are presented in Figs. 1, 2.

Digital photoplethysmography

In the microcirculation, we do not find any variation between the values of pre- and post-dive in the two groups of the PPT (100.8 ± 21.5 % of pre-dive values, $p > 0.05$ for the control group and 108.7 ± 25.1 %, $p > 0.05$ for the chocolate group). Furthermore, no variation is observed for the arterial rigidity (SI is 103.1 ± 20.9 % of pre-dive

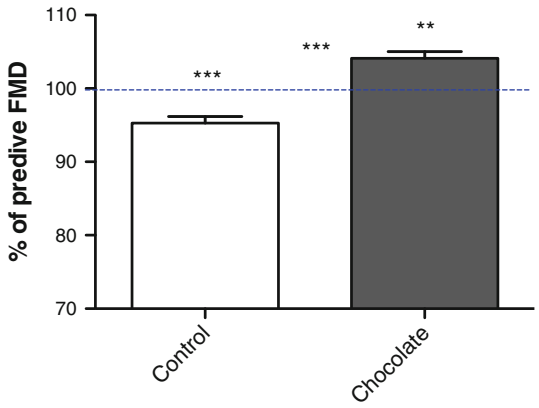

Fig. 1 Evolution of flow-mediated dilation (FMD) in the control group and in the group of breath-hold divers having eaten 30 g of dark chocolate an hour before the dives. Results are given in mean and standard error of the mean (SEM). The FMD of the control group is significantly reduced post-dives compared to its pre-dive value (***), whereas that of the divers having eaten chocolate significantly increases (**). The difference of FMD between the two groups is highly significant (***) (**$p < 0.01$, ***$p < 0.001$)

values, $p > 0.05$ after a series of successive dives and 96.2 ± 20.6 %, $p > 0.05$ postdive with chocolate ingestion).

Circulating nitric oxide and peroxinitrites

A reduction in circulating NO is observed after a series of successive dives to 20 m totaling a cumulative breath-hold time of 20 min (86.8 ± 15.6 % of pre-dive values, $p < 0.05$) whereas no NO variation is observed after the series of successive breath-hold dives preceded by chocolate ingestion (98.4 ± 31.9 % of pre-dive values, $p > 0.05$).

Looking at the oxidative stress markers, no variation in $ONOO^-$ is observed, neither for the control group (112.8 ± 40.1 % of pre-dive values, $p > 0.05$) nor for the chocolate group (105.1 ± 34.6 % of pre-dive values, $p > 0.05$).

The absolute values of the different parameters cited above are given in Table 1.

Discussion

In order to achieve a total of 20 min of cumulative breath-hold time, the divers participating in this study had to stay around 1 h almost still in water at a temperature of 27°. These conditions were chosen in order to be comparable to those a diver doing breath-hold diving would encounter in the sea during summer. Indeed these conditions caused most of our divers to feel cold, as would be the case at sea. Our results point towards the same mechanisms than earlier studies (Theunissen et al. 2013a, b) but the cold could have decreased the NO-related values.

As a reminder, diving comprises of three phases: First a descent phase when the divers have to make an effort to descend during the first meters. Then a hyperoxic phase at 20 m, due to an increase in PaO_2. This phase is rapidly followed by a hypercapnic hypoxic phase due to the oxygen (O_2) consumption, which induces carbon dioxide (CO_2) production. Finally, during the return to the surface, the divers have to make a physical effort until they recover their neutral buoyancy. This hypercapnic hypoxic phase increases with the pressure decrease.

The activation of endothelial nitric oxide synthase (eNOS) to form NO is mediated by the protein kinase Akt (Lehoux and Tedgui 2004), in turn photosphorylized by the phosphatidylinositol-3-kinase (PI3K) (Dimmeler et al. 1998). The NO thus produced by eNOS activates the soluble guanylate cyclase (sGC), an enzyme catalysing guanosine triphosphate into cyclic guanosine monophosphate (cGMP), second messenger of vascular relaxation (Pellegrin et al. 2009). Previous studies (Theunissen et al. 2013a, b) have shown an increase of NO production during breath-hold diving. This increase has been associated to the cardiovascular effort done during this type of diving. It is indeed known that physical exercise increases NO production (Pellegrin et al. 2009; Wang et al. 1993) and that, under normal conditions, physical training enhances endothelial function (Pellegrin et al. 2009), which is directly linked to an increase in biodisponibility of NO in vascular smooth muscle (de Moraes et al. 2008). One could also consider the NO availability and the efficiency of eNOS in the pulmonary vascular system having a protective effect on the onset of edema in breath-hold divers (Cialoni et al. 2012) hence the eventual benefits of dark chocolate supplementation.

This phenomenon was not found in this study: we have instead found a decrease in NO in the control group. We hypothesize that the NO production induced by exercise was counterbalanced by several factors:

1. Despite the increase in NO production, exercise produces also an oxidative stress (Davies et al. 1982; Duthie et al. 1990). A bigger oxidative stress, however, increases the amount of superoxide anions. These can quickly interact with NO to form $ONOO^-$, thus decreasing NO availability in the vascular smooth muscle. Theunissen et al. (2013b) have already demonstrated the presence of oxidative stress post breath-hold dive.
2. The hyperoxia brought by the last phase of the descent portion of the breath-hold dive also results in an increase of superoxide anions production, free radicals. This can be transformed in H_2O_2, a powerful oxidant,

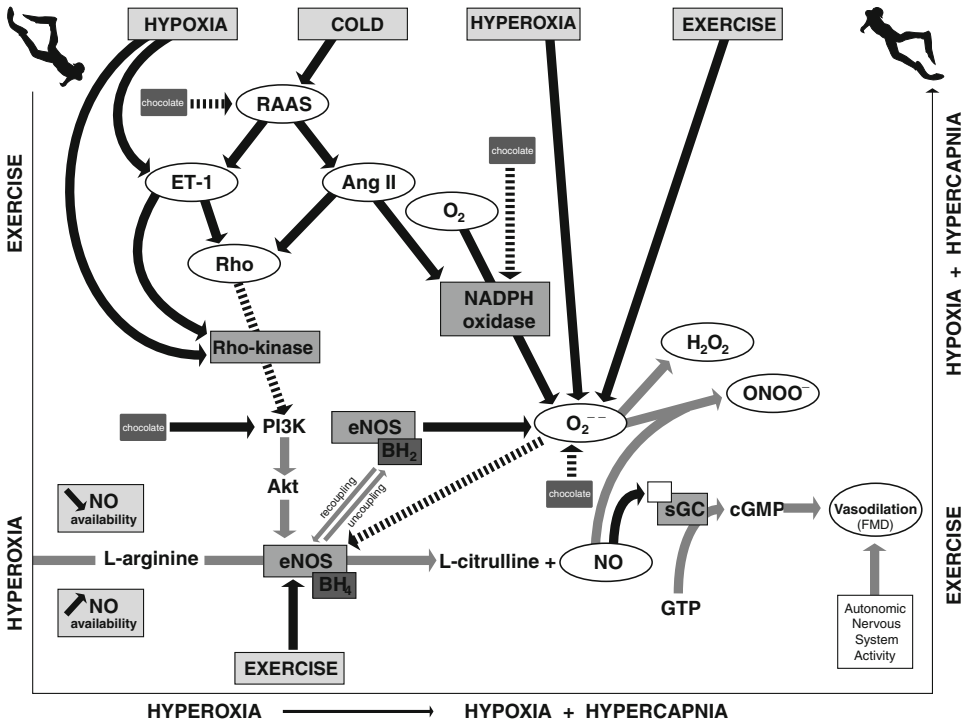

Fig. 2 Influence of exercise, hyperoxia, hypoxia, cold exposure and antioxidants on nitric oxide (NO) bioavailability. In normal conditions NO is produced from L-arginine by the endothelial nitric oxide synthase (eNOS). This enzyme needs a cofactor, tetrahydrobiopterin (BH_4). The NO produced by eNOS activates the soluble guanylate cyclase (sGC), which catalyzes the formation of cyclic guanosine monophosphate 3,5 (cGMP) from guanosine triphosphate (GTP) inducing vasodilation as observed by flow-mediated dilation (FMD). Exercise contributes to an increased NO production through eNOS activation but is also associated with oxidative stress, including production of superoxide anions (O_2^{-}). These are formed by NADPH oxidase from oxygen. Hyperoxia also induces superoxide anions production. The latter oxidize BH4 into dihydrobiopterin (BH_2) reducing BH_4 levels. This induces an uncoupling of eNOS leading to production of superoxide anions by eNOS instead of NO. This enhances oxidative stress. Indeed, superoxide anions can combine with NO to form peroxynitrite ($ONOO^{-}$) or be converted into hydrogen peroxide (H_2O_2). Exposure to cold activates the renin–angiotensin–aldosterone system (RAAS) increasing production of endothelium-1 (ET-1) and angiotensin (Ang II). Both increase the amount of Rho. This induces an increased activity of Rho-kinase, resulting in decreased eNOS activation and therefore reduced NO production, due to the inactivation of phosphatidylinositol-3-kinase (PI3K). Furthermore, Ang II increases the presence of NADPH oxidase and therefore promotes formation of superoxide anions. This consequently increases oxidative stress leading to a reduced bioavailability of NO. Hypoxia activates ET-1 production and Rho-kinase activity leading to a downregulation of eNOS. NO produced by exercise is thus counterbalanced by the combination of eNOS inhibitions and reduced NO availability by oxidative stress. When eating dark chocolate antioxidants reduce the RAAS system and NADPH oxidase activity, activate PI3K production and scavenge superoxide anions. All these effects lead to a better activity of eNOS. *Continue black lines* activation/production, *Discontinue black lines* inhibition/scavenging, *Gray lines* transformation

or reacts with NO to form $ONOO^{-}$ (Jamieson et al. 1986; Sureda et al. 2009), thus creating oxidative stress and decreasing NO biodisponibility (Demchenko et al. 2002). The hypothesis that the increase of PO_2 favors oxidative stress, and therefore endothelial dysfunction, is generally accepted (Demchenko et al. 2000). Hyperoxia is also associated with a decrease in tetrahydrobiopterin (BH_4), a major cofactor of eNOS (Fismen et al. 2013). The lack of BH_4 in endothelial cells exposed to an oxidative stress can induce a decoupling of eNOS leading to a production of superoxide anions by the eNOS instead of NO (Vasquez-Vivar et al. 1998). This induces a reduced activity of eNOS and a lesser release of NO (Fismen et al. 2013; Forstermann 2010; Ilan et al. 2005). This reduced NO production is observed by a decrease in vasodilation shown by the FMD reduction. Nevertheless, we can consider that free radicals produced during deep breath hold diving perhaps are not completely bad and actually may contribute to a "training effect" for breath hold divers since recent studies are reporting beneficial effect of oxidative stress and, on the contrary, a more mitigated effect for antioxidant intake (Nikolaidis et al. 2012).

3. Hypoxia also increases the expression and activity of Rho-kinase (Takemoto et al. 2002). Rho-kinase however facilitates the downregulation of the eNOS expression induced by hypoxia (Takemoto et al. 2002), thus reducing the endothelial production of NO. Furthermore, hypoxia increases endothelin-1

Table 1 Absolute values of the pre-occlusion diameters of the brachial artery, of the flow-mediated dilation, of the photoplethysmographic and haematological parameters before (pre-dive) and after (post-dive) a series of successive breath-hold dives to 20 m depth totalling a cumulative breath-hold time of 20 min for the control and the chocolate groups

	Control ($n = 10$)				*t* test
	Pre-dive		Post-dive		
	Mean	SD	Mean	SD	
Pre-occlusion brachial diameter (mm)	4.4	0.7	4.4	0.6	NS
Flow-mediated dilation (%)	113.9	4.3	108.5	5.2	***
Peak-to-peak time (ms)	177.6	50.8	177.1	53.7	NS
Stiffness index (m/s)	10.8	3	11.1	3.8	NS
Nitric oxide (NO) (μM)	8.4	4	7.6	4.6	*
Peroxinitrites ($ONOO^-$) (μM)	23.8	6.5	25.7	6.2	NS
	Dark chocolate ($n = 10$)				
Pre-occlusion brachial diameter (mm)	4.6	0.4	4.6	0.4	NS
Flow-mediated dilation (%)	112.7	6.6	117.3	6.6	**
Peak-to-peak time (ms)	192.5	39.4	204.4	40.7	NS
Stiffness index (m/s)	9.7	2.1	9.1	2.1	NS
Nitric oxide (NO) (μM)	10.5	4.5	10.1	4.5	NS
Peroxinitrites ($ONOO^-$) (μM)	22.7	7.8	23.4	8.4	NS

Results are given in mean and standard deviation (SD). The Student's *t* test compares the values of pre- and post-dive for each group (NS $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$)

(ET-1) levels (Pisarcik et al. 2013) having an effect on the eNOS inhibition via the Rho-kinase activation. Exercise and/or, at least partially, intermittent hyperoxia and subsequent hypoxia (McQuillan et al. 1994) during the dive could explain the decrease in the endothelial response since hyperoxia and hypoxia both lead to a reduction in NO biodisponibility. The increase in PO_2 during breath-hold diving is very short, nevertheless it has been shown that even very short hyperoxic (Balestra et al. 2006) or hypoxic (de Bruijn et al. 2008) situations played a powerful triggering role provoking physiological responses found after successive breath-hold dives.

4. The cold could also intervene in this decrease of circulating NO observed. Exposure to cold indeed activates the renin–angiotensin–aldosterone system (RAAS), which in turn reduces the expression of eNOS and the formation of NO (Sun 2011). Indeed, RAAS regulates the production of ET-1 due to the cold (Sun 2011). ET-1 as well as angiotensin II (AT II), increases the amount of Rho (Budzyn et al. 2005). This activation induces an increase in Rho-kinase, which by PI3K inactivation results in a decrease in the activity of eNOS (Budzyn et al. 2005). Bailey (2005) and Dubraca (2005) show also an increase in Rho-kinase activity when vessels are exposed to a particular physiological stress, such as a change in the status of redox (Thompson-Torgerson et al. 2007).

The studies of Theunissen et al. (2013a, b) present an increase in circulating NO after a series of successive breath-hold dives. It is noteworthy that the conditions of the dives, with the exception of the water temperature, were the same as those in that study. Therefore oxidative stress, hyperoxia and hypoxia, parameters all going against NO biodisponibility and linked to breath-hold diving, were already present in these previous studies. We could therefore consider that here the decrease in circulating NO in the control group is due to the additional effect of cold. The increase in NO production linked to the cardiovascular effort would here not be enough to compensate the combined effect of oxidative stress, hyperoxia at the start of the dive, hypoxia of the end of the dive and the cold.

In addition a portion of the remaining NO is inactivated due to its transformation in $ONOO^-$. This decrease leads to a vasoconstriction and alters the NO-dependent vasoconstriction, which agrees with recent discoveries on breath-hold divers (Theunissen et al. 2013a; Zhilyaev et al. 2003).

In this study our results do not show a modification in $ONOO^-$ amounts. The $ONOO^-$ are coming from NO combination to superoxide anions. If a reduction of NO is observed, the reaction forming $ONOO^-$ is thus reduced. This could therefore explain the stability of $ONOO^-$ amount despite an increase in oxidative stress, but questions the use of $ONOO^-$ as a marker of oxidative stress. Other markers have indeed shown a change after breath-hold diving, such as an increase in thiobarbituric acid

reactive substances (Joulia et al. 2002), or of the activity of superoxide dismutase and glutathione peroxidase (Rousseau et al. 2006).

In the group supplemented with dark chocolate, NO and $ONOO^-$ values stay constant, pre- and post-dive. Taking flavonoids has antioxidant effects on endothelial cells by reducing, on the one hand, the expression and activity of NADPH oxidase and, on the other hand, by diminishing oxidative stress (Andriantsitohaina et al. 2012), thus limiting the available quantity of free radicals in blood, including that of superoxide anions. Flavonoids are indeed associated with a direct decrease in NADPH oxidase (source of superoxide anions), to a decrease in RAAS activity (activating system for NADPH oxidase) and to an increase in the expression of antioxidative enzymes and PI3K amount (which intervenes in the activation of eNOS) (Andriantsitohaina et al. 2012).

Consequently the amounts of BH_4 and PI3K, as well as enzymatic activity of eNOS, are increased. However the divers were cold, which induced the mechanisms already described above for the control group reducing the activity and expression of eNOS. The combined effect of exercise with taking antioxidants has therefore induced an increased activity and expression of the eNOS. However this increase seems, from our results, to have been counteracted by hyperoxia, hypoxia, cold and exercise-induced oxidative stress, all of which reduce the activity of this enzyme. Given that the amount of NO is stable and that taking antioxidants has reduced the amount of superoxide anions present in the blood, the equilibrium of the reaction forming the $ONOO^-$ has not been perturbed, which explains why the $ONOO^-$ amount stayed stable.

In this study signs of endothelial dysfunction, indicated by an FMD reduction, were found in the control group after a series of successive breath-hold dives. Conversely, a significant increase in FMD was found in the group which was supplemented with chocolate.

In the control group the reduction of the FMD confirms the results obtained previously (Theunissen et al. 2013a). The FMD decrease seems to confirm that breath-hold diving has an influence on endothelial function and could be partially explained by the reduced NO biodisponibility.

Conversely the divers supplemented with dark chocolate presented an increase in FMD. This could be explained by the antioxidative characteristics of dark chocolate. However, the increase in FMD despite an amount of NO which was not modified points towards the latter being due to another factor than the NO-cGMP pathway. This agrees with the results of Theunissen et al. (2013a) after a series of successive breath-hold dives in 33° water where the FMD was reduced despite a significantly increased circulating NO. Breath-hold diving is accompanied by a change in the sympa- and para-sympathic activities (Foster and Sheel 2005). In our study, however, the diameters of pre-occlusion are not modified, neither in the control group nor in the group that ate chocolate. We could therefore hypothesize that the autonomous nervous system effect is not a main factor responsible for the increase in FMD in divers having been supplemented with chocolate. Nevertheless, other authors point out that hyperoxia leads to cardiovascular function and autonomous nervous system alterations during the exposition and after the return to normoxic breathing (Gole et al. 2011). The increased FMD could then be due to a relaxation of the vascular smooth muscle by the antioxidant effects of chocolate. Indeed, it has been suggested that in the process of vascular dysfunction during diving, functional changes in the vessel wall may not be limited to the endothelium and may be mediated by alterations in vascular smooth muscle (Lambrechts et al. 2013). But the vascular responses to oral nitroglycerin administration are not altered by chocolate/cocoa (Heiss et al. 2007; Monahan et al. 2011), what proves that the increase FMD after chocolate intake is not due to a greater reactivity of the vascular smooth muscle. The antioxidant characteristics of chocolate are then maybe responsible for the FMD increase without circulating NO increase after the dive, but the underlying mechanisms remain to be determined.

Contrary to FMD, photoplethysmography did not show any changes in either group. Previous studies have already observed a different reaction between flux-dependent dilation and photoplethysmography (Cornelissen et al. 2012; Obad et al. 2007a; Theunissen et al. 2013a). Llauradó et al. (2013) report that a change in endothelial function is not associated with an arterial stiffness in diabetic patients of type 1. The stiffness of arterial trunk comprises complex modifications of the structural organization of the different components of the arterial wall (Zieman et al. 2005) which lead to a reduction of its distensibility. They suggest that endothelial dysfunction precedes arterial rigidity (Llauradó et al. 2013). Diving provokes an oxidative stress which attacks the endothelium and could therefore increase arterial stiffness over longer time. This could be avoided by the input of flavonoids (Teede et al. 2003; van der Schouw et al. 2002) but this study, because of its limited duration, does probably not allow showing it.

Conclusion

In this study we observe a decrease in FMD and of circulating NO level after a series of successive breath-hold dives. The FMD decrease seems to confirm that breath-hold diving has a negative influence on endothelial function and could partially explain the decrease in NO biodisponibility. We hypothesize that the NO production induced by exercise is counterbalances by several factors,

like hyperoxia, hypoxia, cold and the oxidative stress produced by exercise. However, an increase in FMD without change of NO amount was observed after a series of successive breath-hold dives carried out in the same conditions but for divers having eaten dark chocolate. This could be explained by the ingestion of the dark chocolate. The antioxidants in the dark chocolate could indeed decrease the production of free radicals during the breath-hold dive. The combined effect of exercise and taking antioxidants could therefore have induced an increase in activity and expression of eNOS. However, this increase would seem to have been counteracted by the combined action of hyperoxia, hypoxia, cold and the oxidative stress produced by exercise. The lack of change of NO, together with an FMD modification, suggests that the latter may be due to another factor than the NO-cGMP pathway. Despite an increase in oxidative stress associated to breath-hold diving, the amount of $ONOO^-$ did not change, with or without antioxidants input. The production of $ONOO^-$ being NO dependent, it could have been slowed down by the lack of NO increase. This could therefore explain the stability of $ONOO^-$ amount despite an increase in oxidative stress, but challenges the use of $ONOO^-$ level as a marker of oxidative stress.

This study does not allow observing the effects of flavonoids on arterial stiffness. To do so would require a study over a longer period of time.

Nevertheless it is clear from our study that ingesting 30 g of dark chocolate an hour before the dive could be an effective way of preventing the endothelial dysfunction observed after a series of successive breath-hold dives.

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

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