Original articles

The effect of pre-dive ingestion of dark chocolate on endothelial function after a scuba dive

Sigrid Theunissen, Costantino Balestra, Antoine Boutros, David De Bels, François Guerrero and Peter Germonpré

Abstract

(Theunissen S, Balestra C, Boutros A, De Bels D, Guerrero F, Germonpré P. The effect of pre-dive ingestion of dark chocolate on endothelial function after a scuba dive. *Diving and Hyperbaric Medicine*. 2015 March;45(1):4-9.)

Objective: The aim of the study was to observe the effects of dark chocolate on endothelial function after scuba diving.

Methods: Forty-two male scuba divers were divided into two groups: a control (n = 21) and a chocolate group (n = 21). They performed a 33-metres deep scuba-air dive for 20 minutes in a diving pool (Nemo 33, Brussels). Water temperature was 33^{O}C. The chocolate group ingested 30 g of dark chocolate (86% cocoa) 90 minutes before the dive. Flow-mediated dilatation (FMD), digital photoplethysmography and nitric oxide (NO) and peroxynitrites ($ONOO^{-}$) levels were measured before and after the scuba dive in both groups.

Results: A significant decrease in FMD was observed in the control group after the dive (91 ± 7% (mean ± 95% confidence interval) of pre-dive values; $P < 0.001$) while it was increased in the chocolate group (105 ± 5% of pre-dive values; $P < 0.001$). No difference in digital photoplethysmography was observed between before and after the dives. No variation of circulating NO level was observed in the control group whereas an increase was shown in the chocolate group (154 ± 73% of pre-dive values; $P = 0.04$). A significant reduction in $ONOO^{-}$ was observed in the control group (84 ± 12% of pre-dive values; $P = 0.003$) whereas no variation was shown after the dive with chocolate intake (100 ± 28% of pre-dive values; *ns*).

Conclusion: Ingestion of 30 g of dark chocolate 90 minutes before scuba diving prevented post-dive endothelial dysfunction, as the antioxidants contained in dark chocolate probably scavenge free radicals.

Key words

Antioxidants, cardiovascular, hyperoxia, nitric oxide, diving research, scuba, circulation

Introduction

Endothelial dysfunction after scuba diving was first described in 2005, measured by flow-mediated dilatation (FMD).[1] Other authors have confirmed endothelial dysfunction after a scuba dive.[2] That FMD is nitric oxide-dependant is the commonly accepted assumption.[3] Endothelial nitric oxide (NO) production is triggered by endothelial nitric oxide synthase (eNOS), the latter requiring several major cofactors such as tetrahydrobiopterin (BH_4). Endothelial-NOS is dependant on various activators (physiological and nutritional) such as polyphenols (red wine, cocoa or green tea) or Akt (also known as protein kinase B, PKB).[4]

Polyphenols contained in dark chocolate have the power to improve vascular health by stimulating the formation of vasoprotective factors such as NO, leading to vasodilatation. They also improve vascular smooth muscle function by reducing oxidative stress. Reduction of oxidative stress could reduce the NO degradation through superoxide anions and thus prevent vasoconstriction. Akt increases eNOS activity thus stimulating NO production through phosphatidylinositol kinase (PI3K)-dependent mechanisms.[5] Peroxynitrites ($ONOO^{-}$) have been used as a marker of oxidative stress following diving.[6]

Endothelial dysfunction is associated with poor cardiovascular outcome, leading to research into prevention measures. The aim of the study was to measure the effects of dark chocolate ingestion before a scuba dive on endothelial function.

Methods

STUDY POPULATION

All experimental procedures were conducted in accordance with the Declaration of Helsinki (2008 revision) and were approved by the Academic Ethical Committee of Brussels (B200-2009-039). All methods and potential risks were explained in detail to the participants. After written, informed consent, 42 non-smoking, experienced (at least four years of experience), male scuba divers volunteered for the study. All subjects needed to fulfil exercise criteria (at least 30 minutes of exercise two to three times per week). Prior to entering the study, they were assessed as 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 (21 subjects) and a control group (21 subjects).

DIVE PROTOCOL

The subjects performed a 33-metre deep scuba dive for 20 minutes without a decompression stop in a calm, 8-m diameter pool (Nemo 33, Brussels, Belgium). Water and air temperature were 33^{O}C and 29^{O}C respectively. The chocolate group performed the identical dive in the same conditions as the control group 90 minutes after ingestion of 30 g of dark chocolate (86% cocoa). No exercise was undertaken during the dives.

ENDOTHELIAL FUNCTION

Arterial endothelial function was assessed before and after diving by measuring brachial artery FMD following a standardized protocol and guidelines.[7] FMD was measured with a 5–10 MHz transducer (Mindray DP 6600, Mindray, China). The brachial artery diameter was measured on longitudinal images with the lumen/intima interface visualized on both the anterior and posterior walls. Boundaries for diameter measurement were identified automatically by means of boundary-tracking software (FMD-I software, FLOMEDI, Belgium) and manually adjusted by the same technician who performed all the vascular measurements and was blinded to the group assignment of the subjects, Once the basal measurements were obtained, the sphygmomanometer cuff, placed above the ultrasound testing region was inflated and held at 50 mmHg above systolic blood pressure for 5 min. Occlusion up to 5 min produces a transient arterial dilatation attributable to NO synthesis.[8] After ischaemia, the cuff was deflated rapidly and the brachial artery was monitored for an additional four minutes. The FMD was computed as the percentage change in brachial artery diameter measured at peak dilatation.

ARTERIAL STIFFNESS

Arterial stiffness of small arteries was estimated from the pulse wave obtained at the finger by an infra-red sensor (Pulse Trace PCA 2, Micro Medical, UK). This non-invasive method is easy to use and reproducible.[9] 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.[9] 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 metres and PPT is the peak-to-peak time expressed in seconds. Small artery stiffness decreases the time taken for pressure waves reflected from the periphery to return to the aorta. Reflected waves arrive earlier in the cardiac cycle and may in part explain the change in pulse contour.

BLOOD ANALYSES

Blood samples were collected before diving and 15 minutes after the dive. Samples were drawn from an antecubital fossa vein into an EDTA tube and centrifuged according to a standard protocol (1,000 rpm for 15 min for NO and 3,500 rpm for 10 min for $ONOO^-$ at 4^{O}C) in order to separate blood cells and plasma. The plasma was then stored at -80^{O}C and all analyses were performed within the following six months on the same microplate (one for each test) in order to analyse all the samples at the same time to 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. Peroxinitrites were measured using the OxiSelect™ Nitrotyrosine ELISA kit (Bio-Connect BV, The Netherlands).

STATISTICAL ANALYSIS

For logistical reasons, a repeated measures study design was not possible. Power analysis for a 10% change in FMD, based on previous studies with a SD of approximately 7%, indicated a need for 18–20 subjects per group. Statistical analyses were conducted using GraphPad Prism 5 (La Jolla, California, USA). Data are reported 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 Student's *t*-test after normality of distribution of the sample was determined 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

All divers completed the study and no-one developed symptoms of decompression sickness. There were no statistical differences in demographics between the two groups. Mean age was 37 ± 6 years in the control group and 35 ± 6 years in the chocolate group. Height and BMI were respectively 178 ± 6 cm and 24 ± 1 kg·m^{-2} in the control group and 176 ± 5 cm and 24 ± 2 kg·m^{-2} in the chocolate group.

BRACHIAL ARTERY DIAMETER AND FLOW-MEDIATED DILATATION

An increase in pre-occlusion diameter of the brachial artery was observed after the dive in the control group (105 ± 9% of pre-dive values, $P = 0.04$) whereas that of the chocolate group did not change (99 ± 3% of pre dive values). FMD was significantly reduced after the dive in the control group (91 ± 7% of pre-dive values, $P < 0.001$) but significantly increased in the chocolate group (105 ± 5%, $P < 0.001$). The

Table 1

Absolute values of the pre-occlusion diameters of the brachial artery, of the flow-mediated dilatation, of the photoplethysmographic and haematological parameters before (pre-dive) and after (post-dive) a scuba dive to 33 metres' depth for 20 minutes for the control and the dark chocolate groups (mean ± 95% confidence intervals (95% CI); * $P = 0.04$; † $P = 0.003$; ‡ $P < 0.001$)

	Pre-dive		Post-dive		
	Mean	CI_{95}	Mean	CI_{95}	*n*
Control					
Pre-occlusion diameter (mm)	4.8	(4.6–5.1)	5.0	(5.8–5.3)	21
Flow-mediated dilatation (%)‡	110	(105–115)	100	(97–103)	21
Peak-to-peak time (ms)	199	(180–217)	210	(188–232)	21
Stiffness index ($m \cdot s^{-1}$)	9.3	(8.5–10.1)	8.9	(8–9.9)	21
Nitric oxide ($\mu M \cdot L^{-1}$)	1.4	(0.7–2)	1.4	(0.8–2)	10
Peroxinitrites ($\mu M \cdot L^{-1}$) †	188	(157–218)	160	(124–196)	10
Dark chocolate					
Pre-occlusion diameter (mm)	4.9	(4.7–5.1)	4.8	(4.6–5.1)	21
Flow-mediated dilation (%) ‡	107	(105–109)	113	(110–115)	21
Peak-to-peak time (ms)	198	(186–210)	201	(189–213)	21
Stiffness index ($m \cdot s^{-1}$)	9.1	(8.5–9.6)	8.9	(8.4–9.4)	21
Nitric oxide ($\mu M \cdot L^{-1}$) *	1.5	(0.9–2.2)	2.0	(1.4–2.5)	10
Peroxinitrites ($\mu M \cdot L^{-1}$)	192	(158–227)	190	(147–233)	10

difference between the control group and the chocolate group was statistically significant ($P < 0.001$). FMD changes are presented in Figure 1.

DIGITAL PHOTOPLETHYSMOGRAPHY

No variation in PPT between pre- and post-dive values was found in either group (106 ± 15% of pre-dive values in the control group versus 103 ± 11% in the chocolate group, n.s.). No variation was observed in the SI (96 ± 15% of pre-dive values in the control group vs. 99 ± 11% in the chocolate group, n.s.).

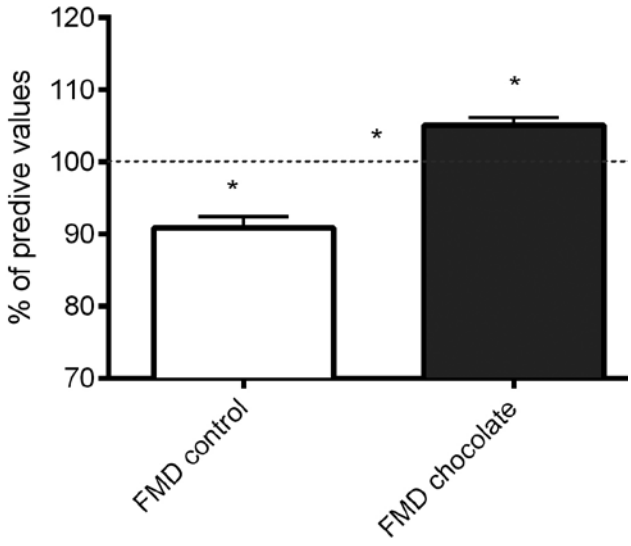
CIRCULATING NO AND $ONOO^-$

No variation in circulating NO concentration was observed in the control group (103 ± 18% of pre-dive values) whereas a significant increase was seen in the chocolate group (154 ± 73%, $P = 0.04$). A significant reduction in plasma concentration of $ONOO^-$ was observed in the control group (84 ± 12% of pre-dive values, $P = 0.003$) whereas no variation in $ONOO^-$ is shown in the chocolate group (100 ± 28%).

The absolute values of the various parameters measured are summarised in Table 1.

Figure 1

Post-dive flow-mediated dilation (FMD, expressed as % of pre-dive value) after scuba dives to 33 metres' depth for 20 minutes in two group of divers ($n = 21$ in each) with or without ingestion of 30 g of dark chocolate 90 minutes before the dive (mean ± 95% confidence intervals); * $P < 0.001$ in both groups and for the post-dive difference between the groups



Discussion

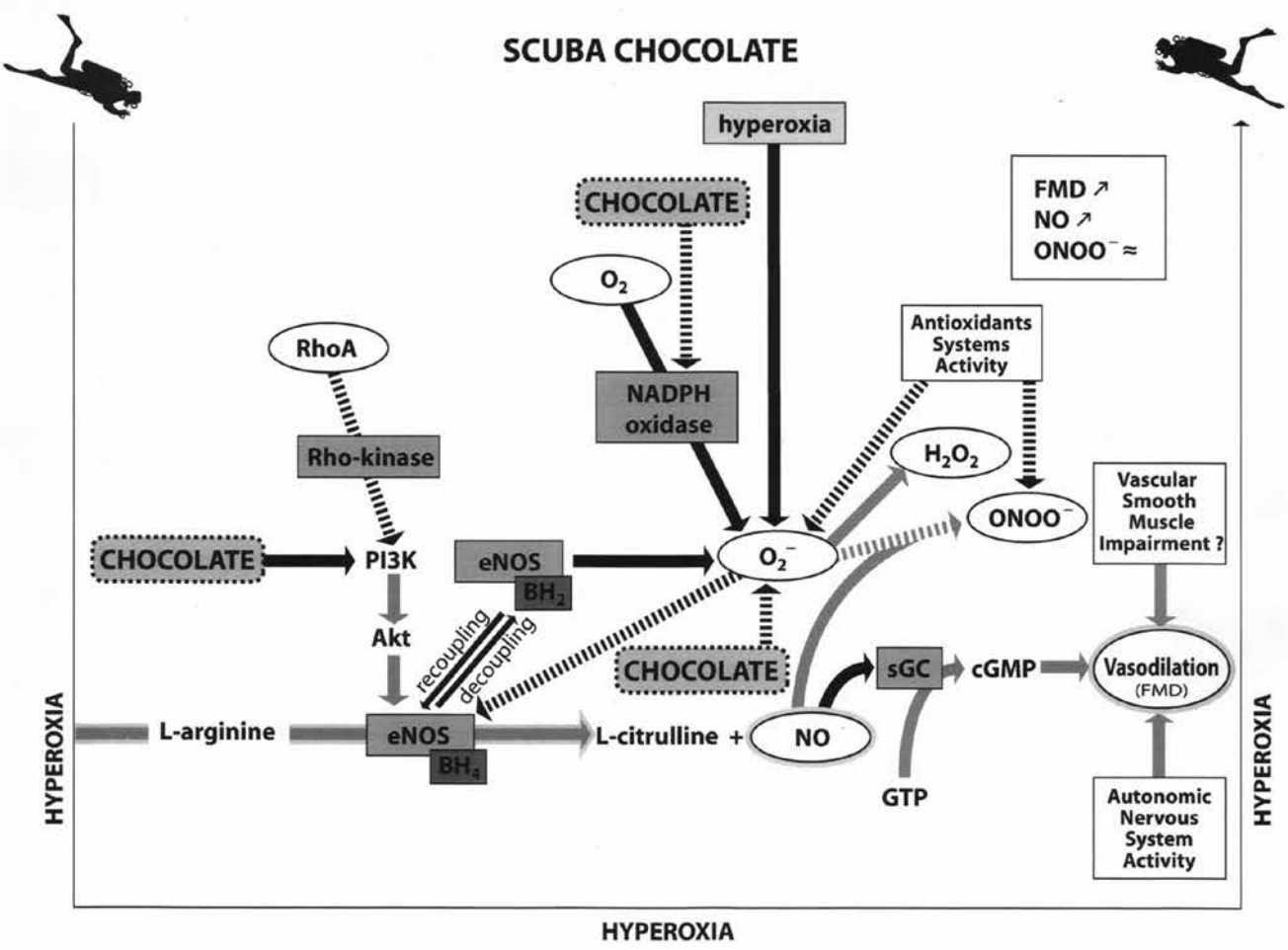
All the dives occurred in thermoneutral waters (33°C) to blunt the physiological mechanisms induced by cold. Our results show a decrease in FMD after a standard scuba dive, consistent with the literature,[1,10] whereas FMD increased post-dive after eating dark chocolate before diving.

STANDARD SCUBA DIVE

One of the accepted hypotheses is that hyperoxia could be responsible for the decrease in FMD by increasing oxidative stress via superoxide anion production.[11] Furthermore, reactive oxygen species (ROS) not only react with NO to reduce its bioavailability, but also oxidize BH_4, a major co-

Figure 2

Theoretical model of the influence of hyperoxia and antioxidants on nitric oxide (NO) bioavailability; chocolate decreases the amount of superoxide anions (O_2^-), decreasing the activity of NADPH oxidase and increasing the level of PI3K; these actions increase the activity of eNOS and thus the production of NO. Since superoxide anions are scavenged by the antioxidants of chocolate, it is possible that the amount of superoxide anions combined with NO may not be enough to increase the level of $ONOO^-$. Continuous black lines: activation/production; discontinuous black lines: inhibition/scavenging; gray lines: transformation



factor of eNOS, which reduces NO production.[12] Indeed, the depletion of BH_4 in endothelial cells exposed to oxidative stress can lead to eNOS decoupling, leading to superoxide anion (free radical) production instead of NO.[12]

No varations were found in NO levels, indicating that eNOS activity was not modified. It has been reported that FMD is NO-dependant,[3] so we should have seen a decrease in NO levels after a standard scuba dive. We believe that superoxide anions produced during diving interact with NO to produce $ONOO^-$ thereby decreasing its availability to contribute to FMD,[13] thus producing vasoconstriction.[14] If so, we should have seen an increase in $ONOO^-$ after diving, whereas our study showed a decrease in $ONOO^-$ possibly suggesting that NO was not transformed to $ONOO^-$. NO production was, thus, neither reduced nor was it transformed to $ONOO^-$. This could explain why there was no NO variation in the control group, a result confirmed in the literature.[15]

Our $ONOO^-$ measures did not seem to confirm the presence of oxidative stress in diving. This could be explained in three ways. Firstly, the levels of NO were not high enough to induce production of $ONOO^-$. Secondly, diving-induced antioxidant systems neutralised $ONOO^-$.[16] Thirdly, oxidative stress was not present during the dives. The last hypothesis conflicts with previous studies demonstrating of oxidative stress during diving.[10,17] This has been demonstrated with markers other than $ONOO^-$, such as thiobarbituric acid reactive substances,[16] superoxide dismutase (SOD) or glutathione peroxidase activity.[18] For these reasons, $ONOO^-$ may not be the best marker to study diving-induced oxidative stress, especially if a deficit in NO is suspected. Nevertheless, $ONOO^-$ levels indicated that NO was probably not inactivated by oxidative stress.

During a scuba dive, FMD is decreased without any NO variation,[15] as in our control group, and this could be due to cardiovascular adaptations,[19] to change in vascular smooth

muscle[20] and/or to autonomic nervous system activity. Indeed ortho- or parasympathetic nervous system activity has been demonstrated during diving.[21]

CHOCOLATE DIVE

Antioxidants contained in dark chocolate are able to scavenge superoxide anions and therefore reduce oxidative stress, leading to reduced eNOS inhibition.[22] Several studies have shown that an acute or chronic intake of dark chocolate reduced arterial stiffness and was thus beneficial for the vascular system.[21,22] Also, accumulation of intracellular free radicals was reduced by a pretreatment containing cocoa procyanidins.[23] Pure cocoa contains between 12 and 18% polyphenols. An intake of 38 to 125 g of chocolate a day significantly increases the diameter of the brachial artery.[24] A small intake of dark chocolate rich in polyphenols as part of nutrition reduces arterial hypertension and promotes NO formation.[25]

The antioxidants in dark chocolate are capable of reducing diving-induced oxidative stress. In autonomous scuba diving, chocolate acts directly on superoxide anions as well as on NADPH oxidase, reducing its activity thus enabling transformation of oxygen into superoxide anions. This leads to a decrease in BH_4 oxidation permitting eNOS to form NO. FMD follows the rise in NO concentrations. In our control group, we saw a reduction in FMD without any variation in NO, possible mechanisms for which are described above. Even if NO and FMD variations go in the same direction after dark chocolate ingestion, it does not mean that changes in vascular smooth muscle and/or to autonomic nervous system activity do not occur. Indeed, some studies link scuba diving and increased vagal activity associated with a decrease in the sympathetic tone of the heart.[21] On the contrary, sympathetic activity is raised during the recovery phase,[21] explaining why FMD does not always follow NO concentrations. The unchanged $ONOO^-$ levels during scuba diving after chocolate intake could be explained by superoxide anions being trapped by antioxidants present in dark chocolate. This could sufficiently reduce their concentration, rendering combination with NO impossible and thus leaving unchanged $ONOO^-$ concentrations. The possible mechanisms associated with chocolate intake in scuba diving are shown in Figure 2.

MICROCIRCULATION

There was no change in the stiffness index (SI) in small vessels in either group, whereas FMD decreased after a scuba dive but increased after the post-chocolate dive. An increase in endothelial function when measured by FMD, but without change in a tonometry-measured pulse wave, has been observed previously after cardiovascular training.[26] The use of post-occlusion reactive hyperaemia may be a better way of assessing short-term changes in endothelial function than the use of photoplethysmography which relies on endothelial structure, the latter remaining unchanged during diving. Indeed, post-occlusion reactive hyperaemia has been shown to vary after a similar air dive.[2]

Extending this research to other domains such as to an older population, in whom increased oxidative stress and alterations in endothelial function occur, could be interesting. The literature shows interesting perspectives on the effects of dark chocolate reducing oxidative stress and thereby cardiovascular risks.[27]

Conclusions

Dark chocolate inhibits post-dive endothelial dysfunction, suggesting the presence of oxidative stress. Peroxinitrites may not be the best biomarkers to evaluate this stress in the current setting. The generally accepted hypothesis is that FMD is NO-dependent, but we showed that FMD variations do not necessarily follow those of circulating NO. It seems that there are many potential factors that could contribute to variations in FMD. Dark chocolate could be an easy, inexpensive and tasty way to reduce the impact of diving on the cardiovascular system.

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Acknowledgements

The authors wish to thank the divers for participating in this study. We also wish to thank Nemo 33 for permission to use their diving pool for our experiments.

Funding

The research was funded from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Program FRP/2007-2013/ under REA grant agreement no. 264816.

Conflict of interest: nil

Submitted: 28 May 2014; revised 08 November and 31 December 2014
Accepted: 02 January 2015

Sigrid Theunissen[1,2], Costantino Balestra[1,3], Antoine Boutros[1], David De Bels[4], François Guerrero[2], Peter Germonpré[5]
[1] Haute Ecole Paul-Henri Spaak, Environmental and Occupational and Aging Physiology Laboratory, Brussels, Belgium
[2] Université de Bretagne Occidentale, UFR Sciences et Techniques, Brest
[3] DAN Europe Research, Brussels, Belgium
[4] Brugmann University Hospital, Department of Intensive Care Medicine, Brussels, Belgium
[5] Center for Hyperbaric Oxygen Therapy, Military Hospital Queen Astrid, Brussels, Belgium

Address for correspondence:
Sigrid Theunissen, PhD
Environmental, Occupational and Aging Physiology Department
Haute Ecole Paul Henri Spaak - ISEK
91 Av C Schaller
B 1160 Brussels
Belgium
Phone: *+32-(0)2-660-2027*
Fax: *+32-(0)2-660-0334*
E-mail: *<theunissen@he-spaak.be>*