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A single serving of blueberry (V. *Corymbosum*) modulates peripheral arterial dysfunction induced by acute cigarette smoke in young volunteers: a randomized-controlled trial

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Abbreviations: ACNs, anthocyanins; dAix, digital augmentation index; dAix@75, digital augmentation index normalized for the heart rate; DBP, diastolic blood pressure; ED, endothelial dysfunction; F-RHI, Framingham reactive hyperemia index; HPLC, high performance liquid chromatography; HR, heart rate; NO, nitric oxide; RHI, reactive hyperemia index; SEM, standard error of the mean, SBP, systolic blood pressure; TSC, total serum cholesterol.

Keywords

Blueberry; Reactive hyperemia index; Blood pressure; Smoking; Healthy subjects

Abstract

Cigarette smoking causes oxidative stress, hypertension and endothelial dysfunction. Polyphenol-rich foods may prevent these conditions. We investigated the effect of a single serving of fresh-frozen blueberry intake on peripheral arterial function and arterial stiffness in young smokers.

Sixteen male smokers were recruited for a 3-armed randomized-controlled study with the following experimental conditions: S-smoking treatment (one cigarette); BS- blueberry treatment (300 g of blueberry) + smoking; CS- control treatment (300 mL of water with sugar) + smoking. Each treatment was separated by one week of wash-out period. Blood pressure, heart rate, peripheral arterial function (reactive hyperemia index, RHI and Framingham (F)-RHI), and arterial stiffness (digital augmentation index, dAix; digital augmentation index normalized for a heart rate of 75 bpm, dAix@75) were measured before and 20 min after smoking by Endo-PAT2000.

Smoking impaired blood pressure, heart rate and peripheral arterial function, but did not affect arterial stiffness. Blueberry consumption counteracted the impairment of RHI induced by smoking (-4.4±0.8% BS treatment *vs* -22.0±1.1% S treatment, $p<0.01$) and F-RHI (+28.3±19.2% BS treatment vs -42.8±20.0% S treatment, $p<0.0001$), and the increase of systolic blood pressure (+8.4±0.02% BS *vs* +13.1±0.02% S mmHg, $p<0.05$) after cigarette smoking. No effect was observed for arterial stiffness and other vital signs.

In conclusion, data obtained suggest a protective role of blueberry on RHI, F-RHI, and systolic blood pressure in subjects exposed to cigarette smoking or to smoke of one cigarette. Future studies are necessary to elucidate the mechanisms involved.

Introduction

Several studies have documented that both active and passive cigarette smoke exposure induces endothelial dysfunction, an early phenomenon involved in the atherosclerotic process.[1-3] The mechanism of endothelial dysfunction could be mediated by several substances that constitute the particulate (tar) and gaseous phase of the cigarette[4] and that are involved in the production of radical oxygen species (ROS). In this regard, ROS induce oxidative stress and inflammation with detrimental consequences on bioavailability of nitric oxide (NO), the most important vasodilator produced by endothelial cells.[4] The reduction of NO causes an increase in blood pressure[3] and arterial wall stiffness[5], one of the underlying pathophysiological mechanisms of the cardiovascular process.[5] Arterial stiffness is considered a predictor of cardiovascular events in the general population[6], and its measurement provides information about the functional and structural vascular changes not only at the level of the aorta, but also at microvascular level.[6] In fact, the augmentation index (Aix) is widely used as a surrogate measure of arterial stiffness and a composite index of arterial dysfunction.[7]

Polyphenols, such as anthocyanins (ACNs), present in high amounts in berries, are recognized as potential bioactive compounds able to counteract ROS production by reducing oxidative stress and inflammation.[8-9] Moreover, ACNs have been proposed as mediators of NO production, thus playing a crucial role in the modulation of arterial stiffness, endothelial function and blood pressure.[10-11] Most of the evidence on health and vascular benefits of polyphenols derives from *in vitro* and *ex-vivo* studies[12-13], while in humans the results are still inconclusive.[14-23] On the whole, an improvement of endothelial function has been observed in several studies after a single administration of polyphenol rich-foods and/or bioactive compounds compared to chronic dietary intervention studies.[15;21-23] It is clear that several factors related with the type of population enrolled (e.g. age, sex, dietary habits, physical activity, risk factors and exposure to oxidative stress) could contribute to different results obtained both in short and long term studies. In addition, the specific experimental protocol used, or the different methodologies applied to determine endothelial

function [e.g. peripheral arterial tone (PAT) *vs* brachial artery ultrasound (BAUS)] can be important variables.

We recently developed an *in vivo* experimental model to study peripheral arterial function following a stressor/insult. The experimental protocol involves the evaluation of Reactive Hyperemia Index (RHI) and blood pressure response in smokers exposed to smoke from one cigarette. Through PAT technology measurements, we demonstrated an impairment of peripheral arterial function 20 min after smoking.[24] The same model may be exploited to investigate the vasoactive properties of bioactives when introduced before the stress, causing dysfunction (i.e. smoking one cigarette). Thus, the aim of the present study is to explore the effect of a single serving of fresh-frozen blueberry serving (300 g) on markers of peripheral arterial function and blood pressure in young and healthy smokers.

Methods

Preparation of blueberry and control treatment

Fresh blueberries (*Vaccinium corymbosum L*. "Brigitta") from a single batch were purchased, sorted and immediately frozen by Individually Quick Freezing technique (Thermolab, Codogno, Italy) and stored at −20°C until use. For the study, 300 g of frozen blueberry was thawed at + 4°C overnight and provided to the participants. Since blueberry contained 16 g fructose and 11 g glucose, the control treatment was prepared by suspending the same amount of sugars in 300 mL of water. No bioactive compounds were added to the control.

Sugars, anthocyanins, total phenolics and vitamin C determination in blueberry

Sugar (glucose and fructose) content was quantified by ultra high pressure liquid chromatography-mass spectrometry as previously described.[25] Individual ACNs and chlorogenic were analyzed by high performance liquid chromatography (HPLC) analysis[25], while total phenolic compounds were

analyzed by Folin-Ciocalteau assay and expressed as gallic acid equivalents (mg/100g).[26] Vitamin C (ascorbic acid) was extracted and determined by HPLC analysis as previously described.[27]

Subject recruitment

Sixteen healthy male smokers, 23.6 ± 2.9 average of age and BMI of 23.0 ± 1.9 kg/m^2, were recruited from the student population of the University of Milan according to the following criteria: 20-30 years of age, homogeneous for smoking habit (about 15 cigarette/day), physical activity (25-30 min per day of brisk walk or jog) and alcohol consumption (up to 10-14 drinks per week). Subjects were recruited on the basis of an interview by a dietitian to evaluate their dietary habits. This was obtained by means of a food frequency questionnaire previously published[28] and revised focusing on polyphenol-rich foods with particular attention to berry consumption. Exclusion criteria were: hypertension (systolic blood pressure > 140 mm Hg and/or diastolic blood pressure > 90 mm Hg), fasting hyperglycaemia (>5.5 mmol/L), hypertriglyceridemia (TG ≥1.69 mmol/L) and hypercholesterolemia (total serum cholesterol (TSC) ≥5.17 mmol/L, low HDL cholesterol (HDL-C) <1.03 mmol/L, high LDL cholesterol (LDL-C) ≥3.36 mmol/L), endothelial dysfunction (RHI <1.67) and overweight (BMI ≥ 25 kg/m^2). Other exclusion criteria were: history of cardiovascular, coronary, diabetes, hepatic, renal, or gastrointestinal diseases, traumas of the arms or hand, fingers, atopic dermatitis, thyroid disturbance, depression, anxiety, palpitations and chronic backache. Subjects were excluded if they were taking supplements or medications for at least one month before the beginning of the study. The study was performed in accordance with the ethical standards established in the 1964 Declaration of Helsinki and approved by the Ethics Committee of the University of Milan. Moreover, this study was registered at www.isrctn.org as ISRCTN59129089. All participants signed informed consent form.

123 **Experimental design**

124 Volunteers were selected for a repeated measures 3-armed randomized-controlled study and
125 assigned to 3 different groups: S- Smoking treatment; BS- Blueberry treatment (300 g of blueberry)
126 + Smoking; CS- Control treatment (300 mL of water with sugar) + Smoking. Each protocol was
127 separated by 7 days of wash-out period (**Figure 1**). The control treatment was chosen since it was
128 reported that sugar intake may affect endothelial function.[29] Both blueberry and control products
129 presented similar glycaemic response within the first 15 min following their consumption and
130 dropped to baseline after 1h (data not shown). Subjects were deprived of polyphenol-rich foods 10
131 days before experimentation. Specific attention was devoted to foods such as chocolate, berry fruits
132 (i.e. blueberries, cranberries, raspberries, blackcurrants, and elderberries), red wine and red to blue
133 fruits, and green tea. Volunteers were asked to limit coffees to three per day, as well as caffeine-rich
134 beverages (e.g. energy drinks), to standardize their intake and reduce a potential effect on vascular
135 function. The day before the experiment and during the trial, breakfast, lunch and dinner were
136 standardized. Breakfast consisted of milk and biscuits (i.e. shortbread) while lunch was composed
137 of two sandwiches (one with cooked ham and cheese and one with raw ham). During dinner,
138 subjects could eat pasta or rice with butter and cheese, and a steak with potatoes and two slices of
139 white bread. The dinner was consumed by 9.00 pm. Only one coffee was allowed at the end of the
140 dinner. No alcoholic drinks or soft drinks were permitted. Overall the meals were standardized in
141 order to provide adequate energy/macronutrients intake, limiting polyphenols and taking into
142 account Italian dietary habits. Moreover, all participants were asked to refrain from physical activity
143 from the day before the experiment and to continue smoking the number of cigarettes/day as
144 declared in the questionnaire.
145 For the present study, peripheral arterial function was measured in two consecutive days. This
146 protocol was chosen to avoid multiple measurements (involving 5 min arterial occlusion through
147 cuff inflation) in a short time-period, because it could promote vasodilation through NO production
148 between test and re-test evaluation.[30] In addition, we excluded an inter-day variability

demonstrating a within-subject repeatability of measurement of vascular function[20] as also reported by other authors.[31-32] Therefore, baseline levels were assessed the first day early in the morning in volunteers, fasted overnight. The second day, vascular function was assessed after subjects smoked one cigarette (S) or consumed 300 g blueberry or the control treatment, followed by one cigarette smoking (BS or CS respectively). The cigarette, containing approximately 6 mg of Tar by volume, 0.5 mg of nicotine and 0.9 mg of carbon monoxide, was smoked 100 min after blueberry or control consumption. The protocol is described in **Figure 1** and was designed to measure peripheral arterial function 120 min after blueberry intake (i.e. 20 min after smoking); the protocol was chosen by considering previous observations on the beneficial effect on endothelial function observed at this specific time-point following the intake of a polyphenol-rich food.[15,21] Reactive hyperemia index (RHI), and digital augmentation index (dAix) were tested 20 min after smoking (T= 120 min). Systolic (S), and diastolic (D) blood pressure (BP), and heart rate (HR) were measured before smoking (T= 100min) and 5 min after smoking one cigarette (T=105) and at the end of the endothelial function measurement (T= 120 min).

Determination of peripheral arterial function and arterial stiffness

Endothelial-dependent vasodilation in the small finger arteries was assessed by a non-invasive plethysmographic method (Endo-PAT2000, Itamar Medical Ltd., Caesarea, Israel) based on the registration of pulsatile blood volume in the fingertips of both hands.[33]

Briefly, subjects were in the supine position and both hands on the same level in a comfortable, thermoneutral environment. Arterial systolic and diastolic blood pressure and heart rate frequency were measured before starting the test. A blood pressure cuff was placed on one upper arm (study arm), while the contralateral arm served as a control (control arm). After a 10-min equilibration period, the blood pressure cuff on the study arm was inflated to 60 mmHg above systolic pressure for 5 min. The cuff was then deflated to induce RH while the signals from both PAT channels (Probe 1 and Probe 2) were recorded by a computer. The RHI, an index of the endothelial-

dependent flow-mediated dilation, was derived automatically in an operator independent manner, as the ratio of the average pulse wave amplitude during hyperaemia (60 to 120 s of the post-occlusion period) to the average pulse wave amplitude during baseline in the occluded hand divided by the same values in the control hand and then multiplied by a baseline correction factor. A RHI value of 1.67 provides a sensitivity of 82% and a specificity of 77% for diagnosing endothelial dysfunction.[33] In addition to the RHI we have also reported in our paper the Framingham RHI (F-RHI), which was automatically calculated using, however, a different post-occlusion hyperaemia period (90 to 120 s) without baseline correction factor. The F-RHI, that has been shown to correlate with other CVD risk markers[34-35], was expressed as natural log of the resulting ratio. The EndoPAT device also generates dAix, strongly correlated to aortic Aix, calculated from the shape of the pulse wave recorded by the probes during baseline.[36] Because Aix is influenced in an inverse and linear manner by heart rate, the dAix was automatically normalized by considering a heart rate of 75 bpm (dAix@75).

Biochemical measurements

Blood samples were drawn and immediately centrifuged at 1000 x g for 15 min. for serum separation and stored at -80°C until analysis. A general laboratory clinical assessment was performed in serum, including evaluation of lipid profile (TAG, TSC, LDL-C and HDL-C), and glucose. All these parameters were determined using standard laboratory methods as previously described.[14]

Statistical analysis

Sample size has been calculated taking into account the expected variation of RHI as the primary endpoint considered. Based on our previous observations[14,24], sixteen subjects were calculated to be sufficient to evaluate a difference of RHI after blueberry intake of 0.30 (standard deviation 0.40),

with alpha=0.05 and a statistical power of 80%. Moreover, the "repeated measures" experimental design in which each subject acts as its own control, allows reduction of the error variance.

Statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). The Shapiro-Wilk test was applied to verify the normal distribution of the variables. Data of the variables under study were analyzed by one way ANOVA with time (before and after smoking) or treatment (smoking *vs* consuming a portion of blueberry + smoking *vs* consuming a control drink + smoking) as dependent factors. The variables of the treatment were reported as the percentage change (i.e. [after treatment-before treatment]/ before treatment *100). The mean changes are described as mean with 95% CI. Differences are considered significant at $p \leq 0.05$; post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with $p \leq 0.05$ as level of statistical significance. Data presented as mean values standard error of the mean (SEM).

Results

Baseline characteristics of the subjects

The anthropometric and clinical characteristics of the sixteen subjects enrolled in the study are reported in **Table 1**. Lipid profile (TAG, TSC, LDL-C and HDL-C), glucose, BP, RHI (>1.67) and BMI were in the normal range.

Composition and characteristics of blueberry and control treatments

The fresh-frozen blueberries provided 27 g of total sugars (16.4 g of fructose and 10.6 g of glucose), 309 mg of ACNs (malvidin-galactoside, delphinidin-galactoside, petunidin-galactoside and malvidin-arabinoside were the dominant compounds), 856 mg of total phenolic acids, 30 mg of chlorogenic acid and 2.4 mg of ascorbic acid. The control provided the same amount and type of sugars but no bioactive compounds (**Table 2**).

Effect of smoking on reactive hyperemia index and arterial stiffness

The values of RHI, F-RHI, dAix and dAix@75 before and after smoking are reported in **Table 3**. Peripheral arterial function, measured through the digital hyperemic response by the RHI, was impaired after smoking. Smoking induced a significant reduction of endothelial function and in 9 out of 16 subjects the RHI indicated endothelial dysfunction (RHI<1.67). A significant impairment was also observed for F-RHI. The F-RHI reduction occurred in 13 out of 16 subjects, while a small increase with respect to baseline value was observed in 3 subjects. Regarding dAix, a significant ($p=0.003$) reduction was also observed (**Table 3**), while no significant ($p=0.819$) effect was detected after normalization for heart rate (dAix@75).

Effect of smoking on blood pressure and heart rate

Smoking a single cigarette significantly increased the levels of SBP (from 116.0 ± 1.7 mmHg to 131.7 ± 1.6mmHg; P=0.0001), DBP (from 76.1 ± 2.1 to 83.5 ± 1.9; P=0.005), and HR (from 63.3 ± 2.9 beat/min to 70.7 ± 2.9 beat/min; P=0.047). This effect was transitional and the values dropped to baseline at the last measurement.

Effect of blueberry and control treatments on reactive hyperemia index and arterial stiffness

The mean percentage variation values of RHI (A), F-RHI (B), dAix (C), and dAix@75 (D) for each treatment are reported in **Figure 2(A-D).** Repeated measures ANOVA revealed a significant effect of treatment for the variable RHI ($p=0.0006$), and F-RHI ($p=0.003$) while no effect was observed for dAix and dAix@75 ($p=0.20$ and $p=0.79$, respectively). The mean percentage change pre to post treatment for RHI was -25.2% (95%CI: -34%, -16.2%) following S treatment, -17.5% (95%CI: -26%, -8.9%) following CS treatment and -6.6% (95%CI: -13%, -0.5%) following BS treatment (**Fig 2A**). The mean percentage change pre to post treatment for F-RHI was -42.7% (95%CI: -85.4%, -0.15%) for S treatment, -8.1 % (95%CI: -36.5%, +20.3%) for CS treatment and +28.3% (95%CI: -

12.6%, +69.2%) for BS treatment (**Fig 2B**). Post-hoc analysis (LSD test) revealed that consumption of a single blueberry serving significantly counteracted the reduction of RHI and F-RHI after S treatment (BS *vs* S, p=0.0001 and p=0.0008, respectively). However, the reduction was significantly different with respect to CS treatment (BS *vs* CS, p= 0.01) for RHI, but not for F-RHI (BS *vs* CS, p= 0.06). No effect was observed between S *vs* CS treatment for both the variables (RHI, p=0.09 and F-RHI, p=0.08).

Effect of blueberry and control treatments on systolic and diastolic blood pressure, and heart rate

The mean percentage variation for SBP, DBP and HR for each treatment 5 min after smoking, are reported in **Figure 3(A-C)**. Statistical analysis revealed a significant effect of treatment for SBP (p=0.01). The mean percentage change between the pre to post treatment was +13.1% (95%CI: 10.5%, 15.7%) after S treatment, +12.7% (95%CI: 10.2%, 15.2%) after CS treatment, and +8.4% (95%CI: 5.4%, 11.4%) after BS treatment (**Fig 3A**). Post-hoc analysis (LSD test) showed that the consumption of a single blueberry portion counteracted significantly the increment of SBP after S treatment (BS *vs* S, p=0.008). This effect was also significantly different with respect to CS treatment (BS *vs* CS, p= 0.01) while no significant difference was observed between S and CS (p=0.90). No effect was observed after blueberry intake for the variables DBP and HR among the three treatments (p=0.71 and p=0.50, respectively).

Discussion

In the present study we documented that acute smoking can significantly reduce peripheral arterial function and increase blood pressure and heart rate in healthy male smoker volunteers. The deleterious effects observed are in accordance with those found in several studies[1-3] and with our previous observations.[24] Endothelial dysfunction could be related to multiple compounds following

combustion of tobacco smoke that elevate the levels of vasoconstrictors such as vascular endothelial growth factors and endothelin-1, reduce NO levels, and increase oxidative stress.[4]

We demonstrated that a single 300 g serving of fresh-frozen blueberry could counteract the endothelial dysfunction induced by smoking, when measured 2 h after blueberry consumption. These results are in accordance with Karatzi et al.[37] which documented the capacity of red wine and dealcoholized red wine to counterbalance the endothelial dysfunction, induced after 30 and 60 min from smoking, in young healthy smokers. In addition, our results are also in accordance with the previous observations in which polyphenol-rich foods, such as chocolate and cranberries, demonstrated to affect vascular function 2 hours after consumption.[15,21] These beneficial effects could be dependent of the absorption of bioactive compounds. In a previous study we demonstrated that one serving (300g) of blueberries could increase ACNs plasma levels up to 2 h from intake.[38] Thus, the beneficial effects on endothelial function could be related to the kinetic of absorption of polyphenol compounds. In this regard, many studies demonstrated that ACNs are rapidly absorbed in the blood (generally within 2-3 hours) reaching nanomolar concentrations that tend to disappear within the first 4-6 hours from food intake. In the meantime, ACN metabolite concentrations increase in plasma as an effect of endogenous metabolic pathways already after 2 h from their consumption.[39] Thus, an important parameter to consider, when performing short-term studies, is the length of time between the intake of food/supplement and measurement of peripheral arterial function. In this regard, in a previous study, we failed to demonstrate modulation of endothelial function 1h after 300 g blueberry consumption in non-smoking male subjects.[20]

As far as long term intervention studies are concerned, results are still inconclusive. We recently reported that 6 weeks of wild blueberry drink consumption failed to significantly alter vascular function in subjects with cardiovascular risk factors[14], even though half of the population experienced an improvement. Similar results have been observed by other authors after intervention with cranberries[15] and apples.[16] One possible explanation could be related to different protocols used [different time of exposure to bioactive compounds, markers related to vascular function (flow

mediated dilation *vs* peripheral arterial function), methodologies (PAT *vs* BAUS), and different study populations] as it was previously mentioned. However, we cannot exclude that the conflicting results on modulation of endothelial function can be due to differences in food sources and amount and type of polyphenol considered. In this context positive effects on endothelial function after dark chocolate and/or flavonols intake seem to derive from medium-long intervention studies.[37-38;40-42] Results available suggest that the vasodilatory and vasoprotective mechanisms of polyphenols include improved bioavailability of vasodilators (i.e. NO, endothelium-derived hyperpolarizing factor and prostacyclin), inhibition of the synthesis of vasoconstrictor endothelin-1 in endothelial cells and the inhibition of expression of pro-angiogenic factors such as vascular endothelial growth factor and matrix metalloproteinase-2 in smooth muscle cells.[43-44]

In the present study, we documented that even though smoking reduced dAix, no effect was observed after normalization for heart beats. Our findings are in agreement with several studies where acute smoking did not affect arterial stiffness in young smokers[45]; on the contrary studies performed in older smokers showed an increase in arterial stiffness.[45] Thus, the age of volunteers can be a critical factor in the outcome, since young people have more elastic walls able to counteract the vasoconstriction induced by smoking.[45-46]

It has been suggested that consumption of polyphenol-rich foods may reduce and improve arterial stiffness[47-48]; in the present study the intake of blueberry did not affect this parameter. Our results are in accordance with Mathew et al.[49] in which no effect on arterial stiffness was observed following consumption of a high-fat meal and pomegranate juice extract, in contrast with Karatzi et al.[48] that documented modulation of arterial stiffness following an acute consumption of polyphenol-rich beer.

Short-term smoking can increase blood pressure and heart rate. In the present study, we demonstrated that acute cigarette smoking impaired blood pressure and heart rate. These changes were observed 5 min after smoking and were not apparent 30 min later. This is in accordance with Lekakis et al.[2] and Stefanadis et al.[50], who documented a prompt increment in heart rate and blood

pressure during the first 5 min after smoking attributed to an increase in circulating levels of catecholamines that reach a maximum concentration 5-10 min after smoking, and return to baseline levels after 30 min.[50]

In this context, we have demonstrated that the consumption of blueberry before smoking can counteract the increase of SBP compared to the control, supporting the potential beneficial effect of polyphenol compounds in the modulation of blood pressure.

Several studies indicate that diets rich in antioxidant compounds can improve blood pressure. A recent meta-analysis has reported for the first time that the intake of polyphenol and ACN-rich foods is associated with low levels of blood pressure.[11] Similar results were also observed by Mathew et al.[49] in which the consumption of an active drink (containing a pomegranate extract) resulted in suppression of the postprandial increase in systolic blood pressure following a high-fat meal. On the contrary, two recent dietary intervention studies reported that 4-week consumption of an ACN-extract did not reduce the levels of blood pressure in healthy and pre-hypertensive men.[51-52]

Conclusion

In conclusion, we documented that blueberries may prevent peripheral arterial dysfunction induced by acute cigarette smoking in young volunteers. These results confirm previous observations on the protective role of blueberry in the modulation of vascular function, emphasizing the contribution of berry fruit consumption especially in people exposed to oxidative stress such as smokers. Prospective short-term studies in larger samples are needed to confirm blueberry's beneficial effects and to underline the mechanisms involved in the modulation of vascular function, Moreover, long term interventions are needed to clarify the effect of regular berry fruit consumption justifying possible dietary recommendations.

354 **Author contributions**

355 The authors' contributions are as follows: Cristian Del Bo' and Daniela Fracassetti analyzed,
356 interpreted the data and drafted the manuscript; Marisa Porrini and Patrizia Riso obtained funding,
357 contributed to the study concept and design, supervised the study, and critically revised the
358 manuscript; Jonica Campolo and Dorothy Klimis-Zacas contributed to the study concept and design
359 and critically revised the manuscript. None of the authors had any conflict of interest.

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526 106.
527

Table 1- Anthropometric and clinical characteristics of the subjects at baseline (n=16)

Variables	Mean ± SEM
Age (years)	23.6 ± 0.7
Height (cm)	178.1 ± 1.7
Weight (kg)	73.1 ± 2.3
BMI (kg/m^2)	23.0 ± 0.5
Smoke (cigarettes/day)	15 ± 1
SBP (mm Hg)	116.0 ± 1.7
DBP (mm Hg)	76.1 ± 2.1
HR (beat/min)	63.3 ± 2.9
RHI	2.23 ± 0.07
F-RHI	0.65 ± 0.07
dAix(%)	-8.6 ± 2.0
dAix@75 (%)	-18.4 ± 2.2
TSC (mmol/L)	4.13 ± 0.08
HDL-C (mmol/L)	1.43 ± 0.10
LDL-C (mmol/L)	2.20 ± 0.10
TAG (mmol/L)	1.01 ± 0.08
Glucose (mmol/L)	4.34 ± 0.17

SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; RHI, reactive hyperemia index; F-RHI, Framingham reactive hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm; TSC, total serum cholesterol.

Table 2- Nutritional composition of Blueberry and Control treatment

	Blueberry	Control
Sugars (g/100g)		
Fructose	*5.46 ± 0.10*	*5.46*
Glucose	*3.57 ± 0.18*	*3.57*
Total phenolic compounds (mg/100g)	242.4 ± 23.9	-
Chlorogenic acid (mg/100g)	30.1 ± 1.2	-
Total anthocyanins (mg/100g)	116.1 ± 6.9	-
Mv-3-gal	*31.19 ± 1.55*	
Mv-3-glc	*2.72 ± 0.08*	
Mv-3-ara	*16.71 ± 0.80*	
Dp-3-gal	*19.0 ± 2.04*	
Dp-3-glc	*0.58 ± 0.11*	
Cy-3-gal	*15.50 ± 1.27*	
Cy-3-glc	*0.51 ± 0.02*	
Cy-3-ara	*1.77 ± 0.06*	
Pt-3-gal	*12.31 ± 1.44*	
Pt-3-glc	*2.36 ± 0.10*	
Peo-3-gal	*8.07 ± 0.30*	
Peo-3-glc	*1.26 ± 0.04*	
Vitamin C (mg/100g)	0.8 ± 0.1	-

555 Data are expressed as means ± SD.

556 *Mv-3-gal, malvidin-3-galactoside; Mv-3-glc, malvidin-3-glucoside; Mv-3-ara, malvidin-3-*
557 *arabinoside;Dp-3-gal, delphinidin-3-galactoside; Dp-3-glc, delphidin-3-glucoside; Cy-3-gal,*
558 *cyanidin-3-galactoside; Cy-3-glc, cyanidin-3-glucoside; Cy-3-ara, cyanidin-3-arabinoside;Pt-3-*
559 *gal, petunidin-3-galactoside; Pt-3-glc, petunidin-3-glucoside; Peo-3-gal, peonidin-3-galactoside;*
560 *Peo-3-glc, peonidin-3-glucoside.*

561 **Table 3**- Arterial function and arterial stiffness measured before and 20 min after smoking a
562 cigarette (n=16)[1]
563

	Before smoking	20 min after smoking	p value[2]
RHI	2.23 ± 0.08	1.64 ± 0.07	0.0001
F-RHI	0.65 ± 0. 08	0.31 ± 0.07	0.002
dAix (%)	-7.8 ± 2.1	-14.1 ± 1.8	0.003
dAix@75 (%)	-18.8 ±2.2	-19.1 ± 2.2	0.819

564
565 [1]Data are expressed as mean ± SEM. RHI, reactive hyperemia index; F-RHI, Framingham reactive
566 hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index
567 standardized for heart rate of 75 bpm.

568 [2]Overall P value for one-way ANOVA with STATISTICA (Statsoft Inc., Tulsa, OK, US).
569

570 **Figure 1 Randomized experimental design.**

571 Figure legend

572 dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate
573 of 75 bpm; G, groups; F-RHI, Framingham reactive hyperemia index; HR, heart rate; BP, blood
574 pressure; RHI, reactive hyperemia index

575

576 **Figure 2** Mean percent variation of RHI (A), F-RHI (B), dAix (C), dAix@75(D) measured during
577 each treatment (n=16)[1].

578 Figure legend

579 [1]Data are expressed as mean ± SEM. S, smoking treatment; CS, control + smoking treatment; BS,
580 blueberry + smoking treatment; RHI, reactive hyperemia index; F-RHI, Framingham reactive
581 hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index
582 standardized for heart rate of 75 bpm.

583 [a,b] Graphs with different letters are significantly different from other treatments ($p \leq 0.01$).

584

585 **Figure 3** Mean percent variation of SBP(A), DBP (B) and HR (C) measured during each treatment
586 (n=16)[1].

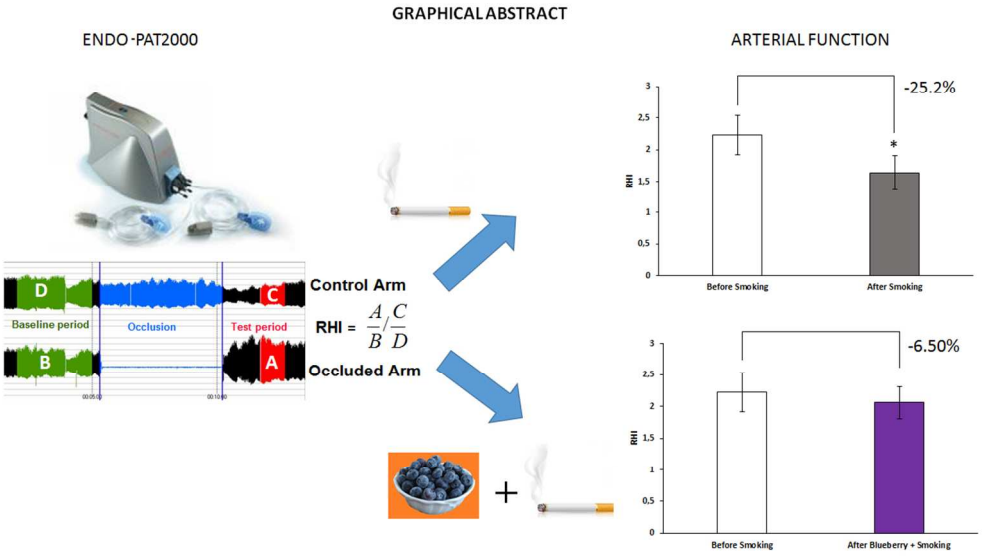
587 Figure legend

588 [1]Data are expressed as mean ± SEM. S, smoking treatment; CS, control + smoking treatment; BS,
589 blueberry + smoking treatment; SBP, systolic blood pressure; DPB, diastolic blood pressure; HR,
590 heart rate.

591 [a,b] Graphs with different letters are significantly different from other treatments ($p \leq 0.05$).

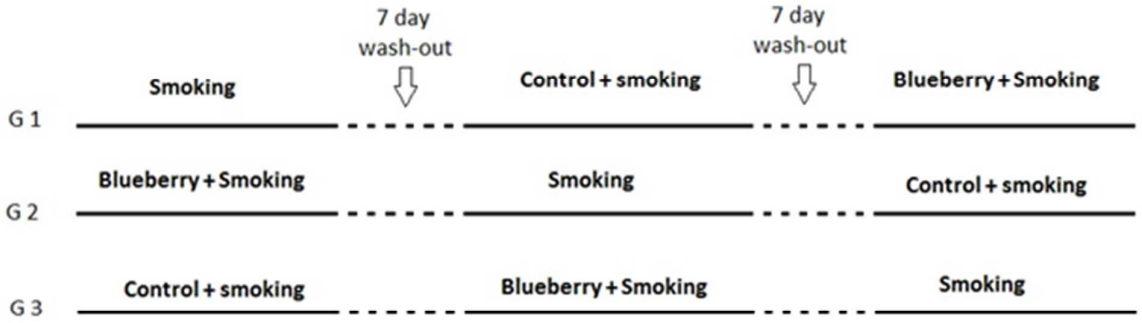
Graphical abstract

The consumption of one portion (300 g) of blueberry is able to counteract peripheral arterial dysfunction induced by smoking in young healthy subjects.



338x190mm (96 x 96 DPI)

Figure 1



TIME	Blueberry treatment	Control treatment	Smoking treatment
T= 0 min	Blueberry intake	Control intake	———
T=100 min	BP; HR; 1 cigarette	BP; HR; 1 cigarette	BP; HR; 1 cigarette
T=105 min	BP; HR	BP;HR	BP;HR
T=120 min	RHI,FRHI, dAIx, dAIx@75	RHI,FRHI, dAIx, dAIx@75	RHI,FRHI, dAIx,dAIx@75

Figure 2

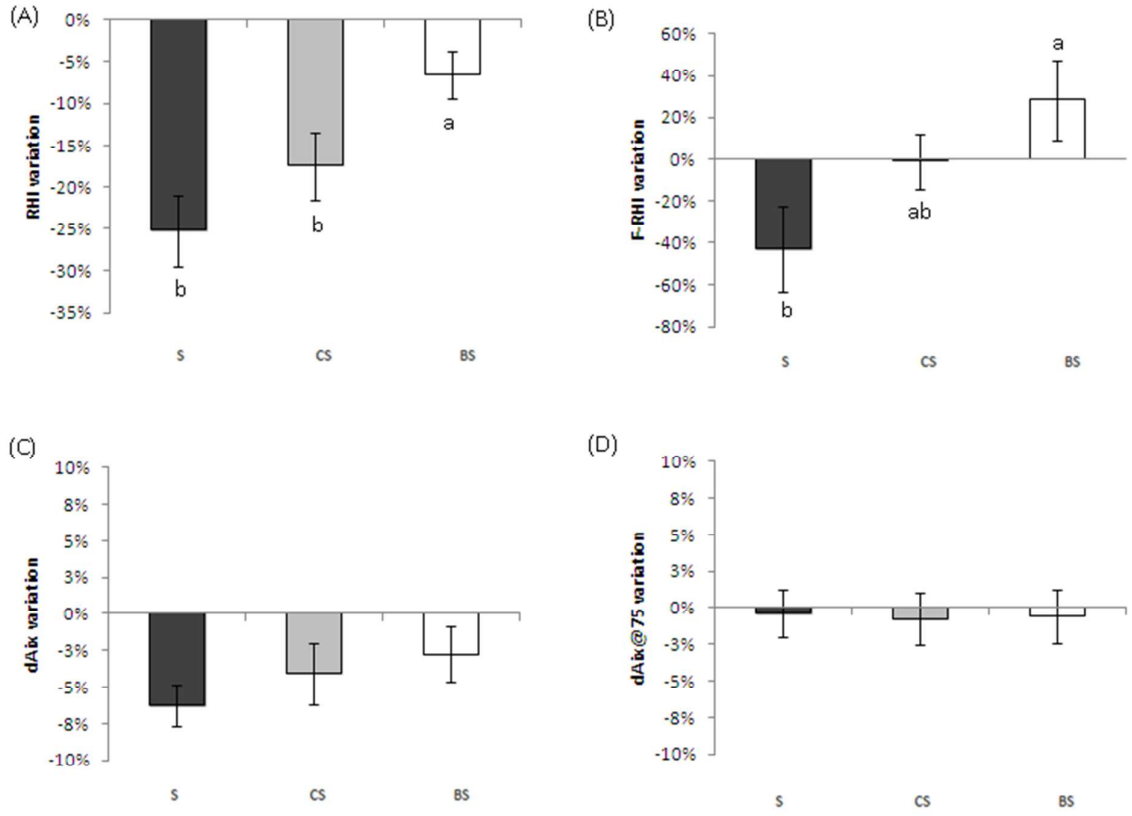
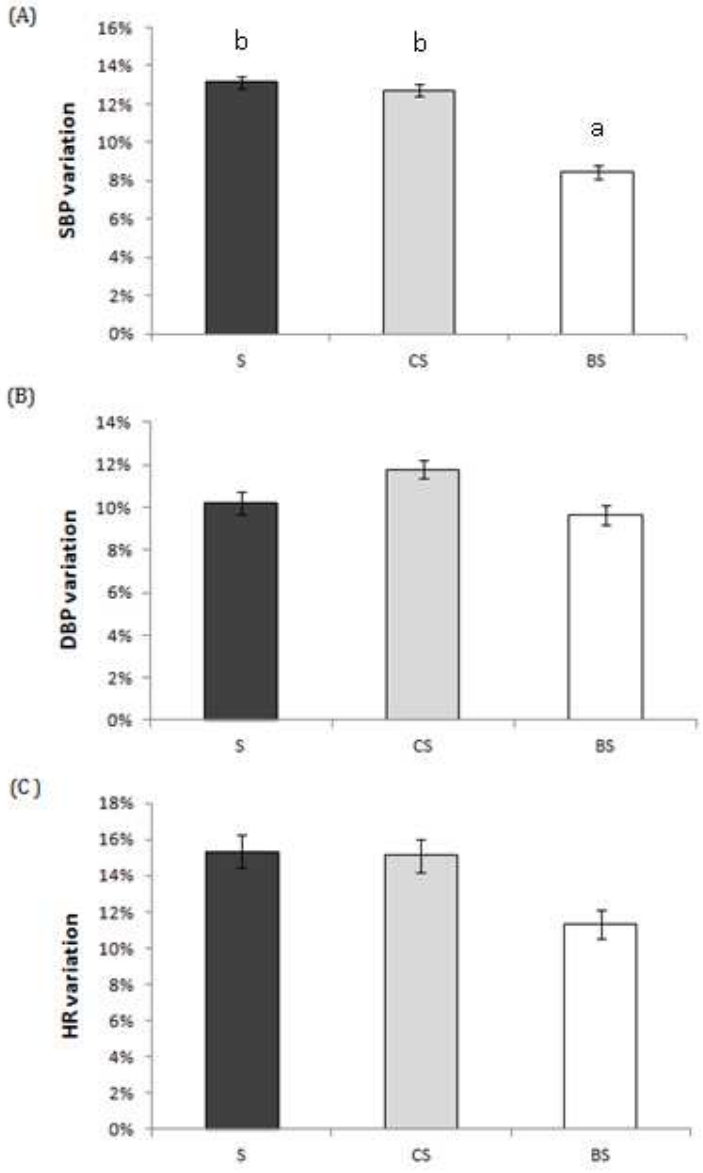

(A)
RHI variation
0%
-5%
-10%
-15%
-20%
-25%
-30%
-35%
b
b
a
S
CS
BS
(B)
F-RHI variation
60%
40%
20%
0%
-20%
-40%
-60%
-80%
b
ab
a
S
CS
BS
(C)
dAix variation
10%
8%
5%
3%
0%
-3%
-5%
-8%
-10%
S
CS
BS
(D)
dAix@75 variation
10%
8%
5%
3%
0%
-3%
-5%
-8%
-10%
S
CS
BS

Figure 3


(A)
SBP variation
16%
14%
12%
10%
8%
6%
4%
2%
0%
b
b
a
S
CS
BS
(B)
DBP variation
14%
12%
10%
8%
6%
4%
2%
0%
S
CS
BS
(C)
HR variation
18%
16%
14%
12%
10%
8%
6%
4%
2%
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CS
BS