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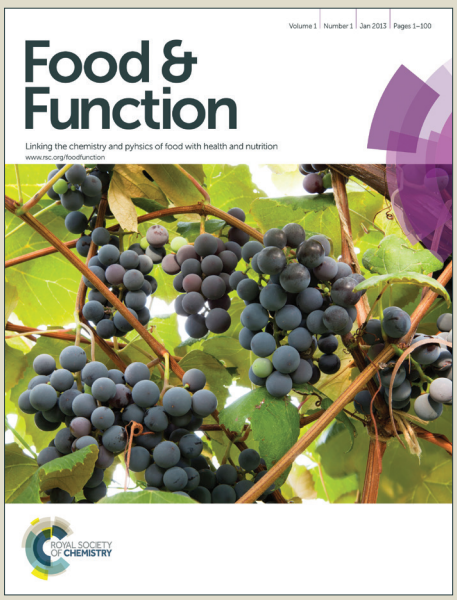

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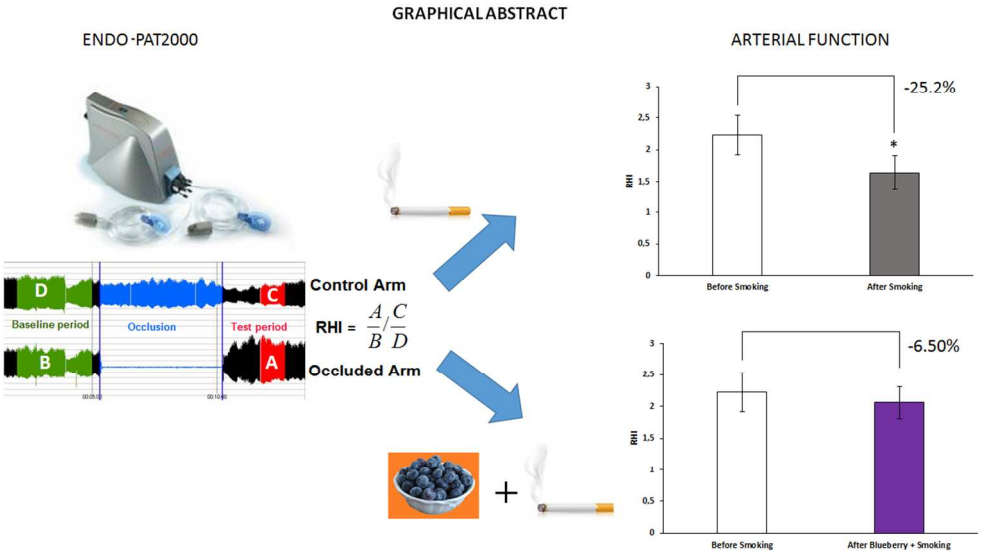

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A single serving of blueberry (V. *Corymbosum*) modulates peripheral arterial dysfunction induced by acute cigarette smoke in young volunteers: a randomized-controlled trial

Cristian Del Bo'[a], Marisa Porrini[a], Daniela Fracassetti[a], Jonica Campolo[b], Dorothy Klimis-Zacas[c] and Patrizia Riso[a*]

[a]Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences, Division of Human Nutrition, Milano, Italy

[b]CNR Institute of Clinical Physiology, CardioThoracic and Vascular Department, Niguarda Ca' Granda Hospital, Milan, Italy

[c]Department of Food Science and Human Nutrition, University of Maine, Orono, Maine, USA

***Corresponding author**: Dr. Patrizia Riso, PhD - DeFENS - Department of Food, Environmental and Nutritional Sciences, Division of Human Nutrition - Università degli Studi di Milano, via G. Celoria 2, 20133 Milano, Italy; **E-mail**: patrizia.riso@unimi.it; **Phone**: +39-02-50316726; **Fax**.: +39-02-50316721;

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: -smoking treatment (one cigarette); - blueberry treatment (300 g of blueberry) + smoking; - 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 and Framingham reactive hyperemia), and arterial stiffness (digital augmentation index, digital augmentation index normalized for a heart rate of 75 bpm) 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 reactive hyperemia index induced by smoking (-4.4±0.8% blueberry treatment *vs* -22.0±1.1% smoking treatment, $p<0.01$) and Framingham reactive hyperemia (+28.3±19.2% blueberry treatment vs -42.8±20.0% smoking treatment, $p<0.0001$), and the increase of systolic blood pressure (+8.4±0.02% blueberry treatment *vs* +13.1±0.02% smoking treatment 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 reactive hyperemia, Framingham reactive hyperemia, and systolic blood pressure in subjects exposed 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, 270 packs containing 20 cigarettes each/year), physical activity (25-30 min per day of brisk walk or jog) and alcohol consumption (up to 10-14 drinks of wine or beer 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 (e.g. chocolate, green tea) 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, drugs 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.

Experimental design

Volunteers were selected for a repeated measures 3-armed randomized-controlled study and assigned to 3 different groups: S- Smoking treatment; BS- Blueberry treatment (300 g of blueberry) + Smoking; CS- Control treatment (300 mL of water with sugar) + Smoking. Each protocol was separated by 7 days of wash-out period (**Figure 1**). All subjects (n=16) completed the three treatments. The control treatment was chosen since it was reported that sugar intake may affect endothelial function.[29] Both blueberry and control products presented similar glycaemic response within the first 15 min following their consumption and dropped to baseline after 1h (data not shown). Subjects were deprived of polyphenol-rich foods 10 days before experimentation. Specific attention was devoted to foods such as chocolate, berry fruits (i.e. blueberries, cranberries, raspberries, blackcurrants, and elderberries), red wine and red to blue fruits, and green tea. Volunteers were asked to limit coffees to three per day, as well as caffeine-rich beverages (e.g. energy drinks), to standardize their intake and reduce a potential effect on vascular function. The day before the experiment and during the trial, breakfast, lunch and dinner were standardized. Breakfast consisted of milk and biscuits (i.e. shortbread) while lunch was composed of two sandwiches (one with cooked ham and cheese and one with raw ham). During dinner, subjects could eat pasta or rice with butter and cheese, and a steak with potatoes and two slices of white bread. The dinner was consumed by 9.00 pm. Only one coffee was allowed at the end of the dinner. No alcoholic drinks or soft drinks were permitted. Overall the meals were standardized in order to provide adequate energy/macronutrients intake, limiting polyphenols and taking into account Italian dietary habits. Moreover, all participants were asked to refrain from physical activity from the day before the experiment and to continue smoking the number of cigarettes/day as declared in the questionnaire.

For the present study, peripheral arterial function was measured in two consecutive days. This protocol was chosen to avoid multiple measurements (involving 5 min arterial occlusion through cuff inflation) in a short time-period, because it could promote vasodilation through NO production

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= 100 min) and 5 min after smoking one cigarette (T= 105 min) 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**). As reported for smoking (see previous paragraph), a similar reduction of RHI was also

observed in 14 out of 16 subjects following CS treatment, while a small increase compared to baseline was documented in 2 subjects. Reduced impairment of endothelial dysfunction was observed in 11 out of 16 subjects following BS treatment compared to baseline, while in 5 subjects RHI increased.

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] In the present study

circulating levels of ACNs or phenolic compounds were not measured thus, we cannot postulate a casual effect of the above compounds in the modulation of RHI.

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

328 are in accordance with Mathew et al.[49] in which no effect on arterial stiffness was observed
329 following consumption of a high-fat meal and pomegranate juice extract, in contrast with Karatzi et
330 al.[48] that documented modulation of arterial stiffness following an acute consumption of
331 polyphenol-rich beer.

332 Short-term smoking can increase blood pressure and heart rate. In the present study, we
333 demonstrated that acute cigarette smoking impaired blood pressure and heart rate. These changes
334 were observed 5 min after smoking and were not apparent 30 min later. This is in accordance with
335 Lekakis et al.[2] and Stefanadis et al.[50], who documented a prompt increment in heart rate and blood
336 pressure during the first 5 min after smoking attributed to an increase in circulating levels of
337 catecholamines that reach a maximum concentration 5-10 min after smoking, and return to baseline
338 levels after 30 min.[50]

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

342 Several studies indicate that diets rich in antioxidant compounds can improve blood pressure. A
343 recent meta-analysis has reported for the first time that the intake of polyphenol and ACN-rich
344 foods is associated with low levels of blood pressure.[11] Similar results were also observed by
345 Mathew et al.[49] in which the consumption of an active drink (containing a pomegranate extract)
346 resulted in suppression of the postprandial increase in systolic blood pressure following a high-fat
347 meal. On the contrary, two recent dietary intervention studies reported that 4-week consumption of
348 an ACN-extract did not reduce the levels of blood pressure in healthy and pre-hypertensive men.[51-
349 52]

350

351 **Conclusion**

352 In conclusion, we documented that blueberries may prevent peripheral arterial dysfunction induced
353 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. However, we should point out that blueberry consumption cannot be considered a means of preventing health consequences due to smoking; this can only be realized by smoking cessation and/or prevention. 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 required to clarify the effect of regular berry fruit consumption justifying possible dietary recommendations.

Author contributions

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

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537

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	-

565 Data are expressed as means ± SD.

566 *Mv-3-gal, malvidin-3-galactoside; Mv-3-glc, malvidin-3-glucoside; Mv-3-ara, malvidin-3-*
567 *arabinoside;Dp-3-gal, delphinidin-3-galactoside; Dp-3-glc, delphidin-3-glucoside; Cy-3-gal,*
568 *cyanidin-3-galactoside; Cy-3-glc, cyanidin-3-glucoside; Cy-3-ara, cyanidin-3-arabinoside;Pt-3-*
569 *gal, petunidin-3-galactoside; Pt-3-glc, petunidin-3-glucoside; Peo-3-gal, peonidin-3-galactoside;*
570 *Peo-3-glc, peonidin-3-glucoside.*

571 **Table 3-** Arterial function and arterial stiffness measured before and 20 min after smoking a
572 cigarette (n=16)[1]
573

	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

574
575 [1]Data are expressed as mean ± SEM. RHI, reactive hyperemia index; F-RHI, Framingham reactive
576 hyperemia index; dAix, digital augmentation index; dAix@75, digital augmentation index
577 standardized for heart rate of 75 bpm.

578 [2]Overall P value for one-way ANOVA with STATISTICA (Statsoft Inc., Tulsa, OK, US).
579

Figure 1 Randomized experimental design (n=16)[1]

Figure legend

[1]Sixteen subjects for each group; dAix, digital augmentation index; dAix@75, digital augmentation index standardized for heart rate of 75 bpm; G, groups; F-RHI, Framingham reactive hyperemia index; HR, heart rate; BP, blood pressure; RHI, reactive hyperemia index

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

Figure legend

[1]Data are expressed as mean ± SEM. S, smoking treatment; CS, control + smoking treatment; BS, blueberry + smoking treatment; 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.

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

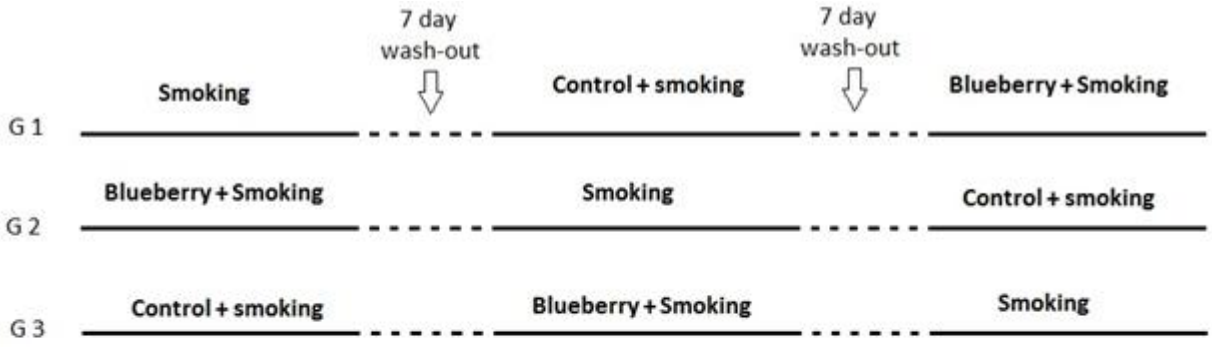
Figure 3 Mean percent variation of SBP(A), DBP (B) and HR (C) measured during each treatment (n=16)[1]

Figure legend

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

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

603 **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

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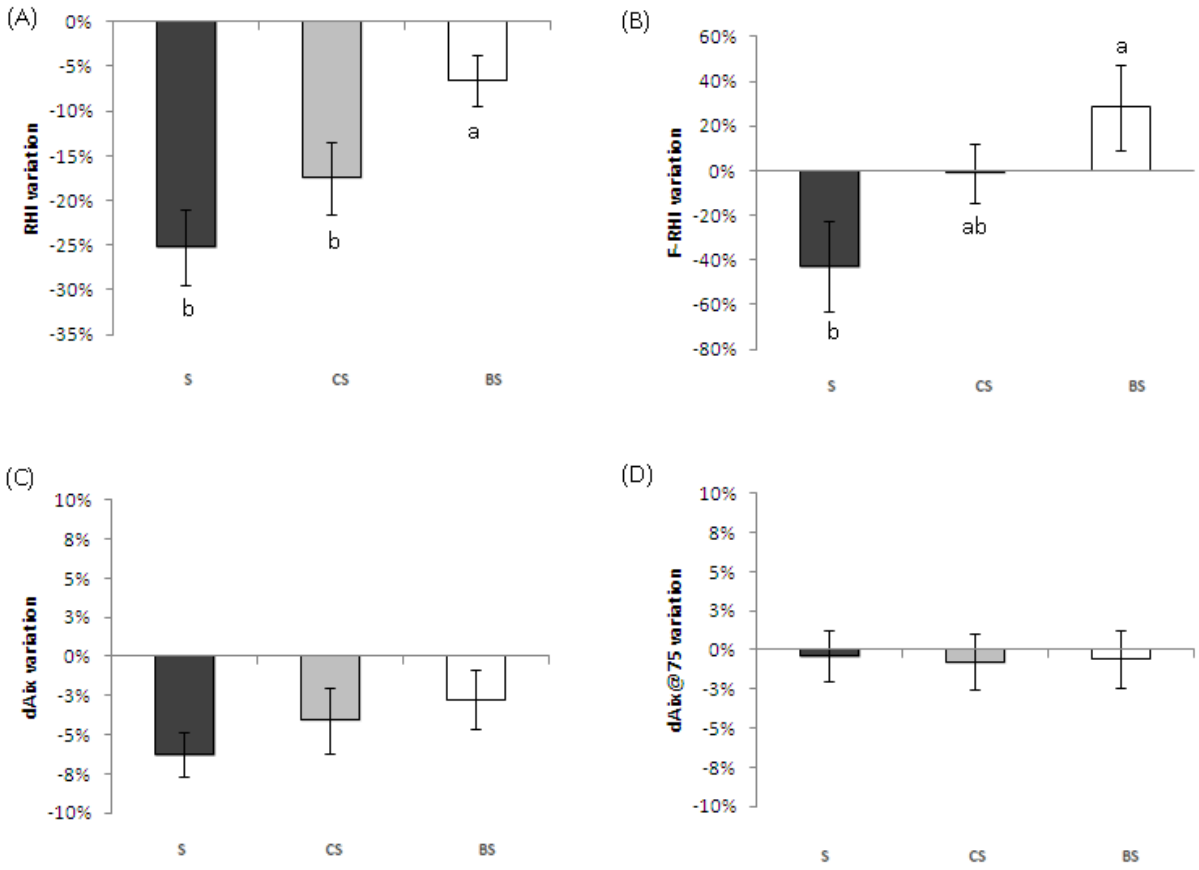
605

606 **Figure 2**
607



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610 **Figure 3**
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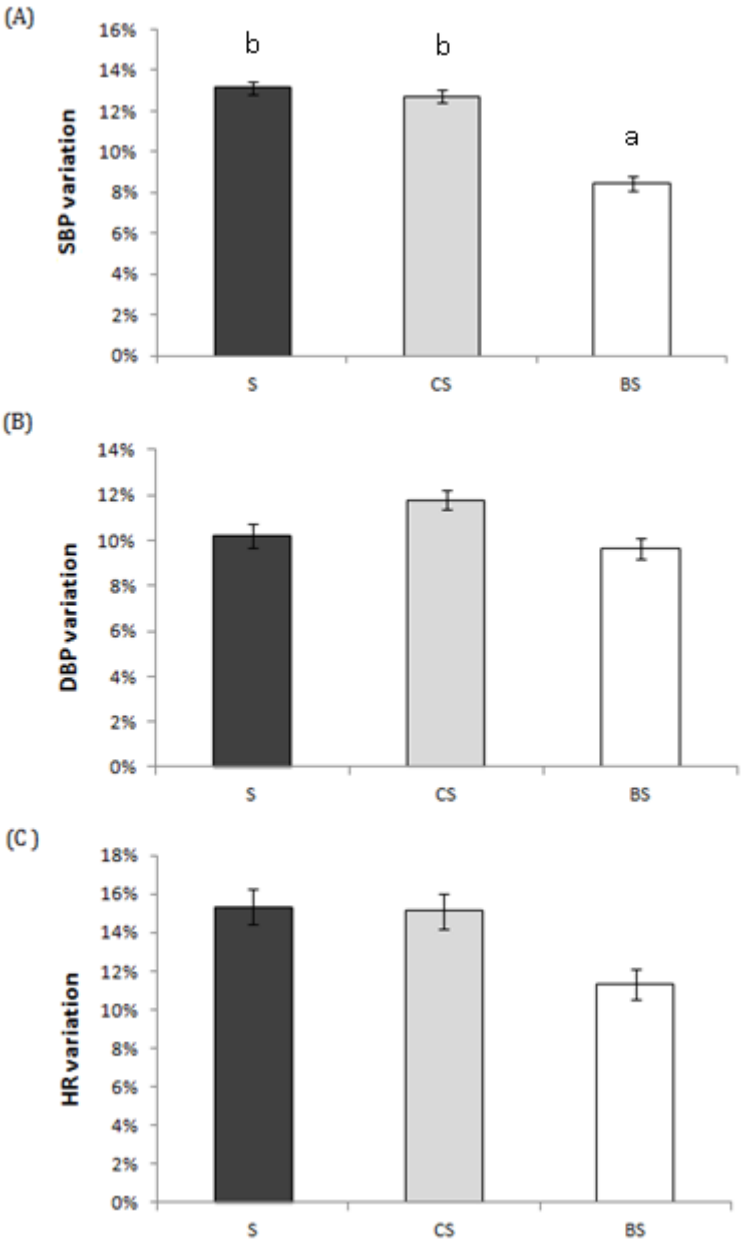

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