1 **A single portion of blueberry (*Vaccinium corymbosum L.*) improves protection against DNA damage but not vascular function in healthy male volunteers.**

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27 **Abbreviations**

28 ACNs, anthocyanins

29 ANOVA, analysis of variance

30 BB, blueberries

31 BMCs, blood mononuclear cells

32 BMI, body mass index

33 CydG, cyanidin 3-glucoside

34 CJ, control jelly

35 e-NOS, endothelial nitric oxide synthases

36 HDL, high-density lipoprotein

37 FMD, flow mediated dilation

38 FPG, formamidopyrimidine DNA glycosylase

39 GAE, gallic acid equivalent

40 LDL, low density lipoprotein

41 LSD, least significant difference

42 MNBCs, mononuclear blood cells

43 NO, nitric oxide

44 RHI, reactive hyperemia index

45 TFA, trifluoroacetic acid

46 TG, triglycerides

47 TSC, total serum cholesterol

48 UHPLC-MS/MS, ultra-high-performance liquid chromatography/mass spectrometry

49

ABSTRACT

It has been suggested that anthocyanin-rich foods may exert antioxidant effects and improve vascular function as demonstrated mainly in vitro and in the animal model. Blueberries are rich sources of anthocyanins and we hypothesized that their intake could improve cell protection against oxidative stress and affect endothelial function in humans.

The aim of the study was to investigate the effect of one portion (300 g) of blueberries on selected markers of oxidative stress and antioxidant protection (endogenous and oxidatively-induced DNA damage) and of vascular function (changes in peripheral arterial tone and plasma nitric oxide levels) in male subjects.

In a randomized cross-over design, separated by a wash out period ten young volunteers received one portion of blueberries ground by blender or of a control jelly. Before and after consumption (at 1 h, 2 h and 24 h), blood samples were collected and used to evaluate anthocyanin absorption (through mass spectrometry), endogenous and H_2O_2-induced DNA damage in blood mononuclear cells (through the comet assay) and plasma nitric oxide concentrations (through a fluorometric assay). Peripheral arterial function was assessed by means of Endo-PAT 2000.

Blueberries significantly reduced ($p<0.01$) H_2O_2-induced DNA damage (-18 %) 1 h after blueberry consumption compared to control. No significant differences were observed for endogenous DNA damage, peripheral arterial function and nitric oxide levels after blueberry intake.

In conclusion, one portion of blueberries seems sufficient to improve cell antioxidant defense against DNA damage, but further studies are necessary to understand their role on vascular function.

Keywords: Blueberry, DNA damage, FPG sensitive sites, vascular function, healthy subjects

74 **1. Introduction**

75

76 Blueberries (BB) contain bioactive compounds such as phenolic acids and in particular
77 anthocyanins (ACNs), a group of water-soluble pigments responsible for the blue, red and purple
78 color of fruits and vegetables [1-2]. Several *in vitro* and *in vivo* studies have documented the
79 bioactivity of ACNs suggesting anti-inflammatory properties, improvement of lipid profiles,
80 modulation of detoxifying enzymes, reduction of blood pressure, and platelet aggregation [2; 3-8].
81 Some of these biological activities and protective effects can be attributed to their antioxidant
82 activity against reactive oxygen species. In particular, blueberry ACNs have been documented to
83 reduce H_2O_2-induced reactive oxygen species in endothelial and red blood cells and decrease liver
84 DNA damage in rats [9-10]. Concerning the health effects of berries in human interventions studies,
85 results are still scarce and inconclusive [11]. Duthie et al. [12] documented that the intake of 750
86 mL/day of cranberry juice did not affect endogenous DNA damage, oxidized pyrimidines and H_2O_2
87 sensitivity in a group of female volunteers. Ramirez-Tortosa et al. [13] showed no change in
88 baseline DNA strand breaks when volunteers consumed a 200 g of berry dessert (grape, cherry,
89 blackberry, black currant) and raspberry juices for 2 weeks. On the contrary, we documented that 6
90 weeks of a wild blueberry drink significantly reduced the levels of formamidopyrimidine DNA
91 glycosylase (FPG)-sensitive sites and H_2O_2-induced DNA damage in subjects with risk factors for
92 cardiovascular diseases [14]. Additionaly, Wilms et al. [15] documented a reduction of the levels of
93 H_2O_2-induced DNA damage after 4 weeks of supplementation with 1L/day of a mixture of
94 blueberry and apple juices in healthy female volunteers.

95 Berries and ACNs are also believed to improve endothelial-dependent vasodilation. Most of
96 the beneficial evidence of berries on the modulation of endothelial function derives from *in vitro*
97 and *ex vivo* studies [16-20]. We have demonstrated that 7-week consumption of a wild blueberry
98 rich-diet improved the mechanical properties of the aorta in an animal model [20]. In humans, the
99 results are still unconvincing. For example we have recently documented that a 6-week wild

100 blueberry drink intervention did not significantly affect peripheral arterial function determined
101 through the EndoPAT 2000 device in humans [14]. Consequently we hypothesized that, if the
102 modulation of this function is strictly related to the increased ACN circulating levels, the lack of
103 effect may be due to the rapid absorption and elimination of ACNs (generally within the first 3-4
104 hours). In fact, in our long term study, no ACNs were detectable in plasma following wild blueberry
105 consumption, since blood samples were taken 12 h after the blueberry drink. Thus, we hypothesized
106 that modulation of vascular function may be observed shortly after 1 h from BB intake.
107 To test the hypothesis, we designed an acute study to investigate the effect of BB both on oxidative
108 stress and vascular function. In particular, we evaluated the effect of a single portion of blueberry
109 *(Vaccinium corymbosum)* (300 g, providing about 348 mg ACNs) on endogenous FPG-sensitive
110 sites and oxidatively (H_2O_2)-induced DNA damage (primary endpoints) with the aim in
111 establishing, whether the short-term increase in ACN circulating levels following the intake of
112 blueberry, could affect peripheral arterial function and modulate nitric oxide (NO) plasma levels in
113 a group of healthy volunteers.
114

115 **2. Methods and materials**

116

117 **2.1 Study subjects**

118

119 Ten healthy male subjects, ages 20.8 ± 1.6 y with body mass index (BMI) 22.5 ± 2.1 kg/m^2, were
120 recruited from the student population of the University of Milan according to the following
121 inclusion criteria: no smokers; no history of cardiovascular, diabetes, hepatic, renal, or
122 gastrointestinal diseases; not consuming any dietary supplement, drug, or medication for at least
123 one month before the beginning of the study. Subjects were selected on the basis of an interview to
124 evaluate their dietary habits and ensure that they were as homogeneous as possible, in particular for
125 fruit and vegetable consumption. This was obtained by means of a food frequency questionnaire
126 previously published and specifically revised to focus on food sources rich in antioxidants [21].
127 Exclusion criteria were: hypertension (systolic blood pressure > 140 mm Hg and/or diastolic blood
128 pressure > 90 mm Hg), high total serum cholesterol (TSC) (≥5.17 mmol/L), low high-density
129 lipoprotein (HDL)-cholesterol (<1.03 mmol/L), high low density lipoprotein (LDL)-cholesterol
130 (≥3.36 mmol/L), high triglycerides (TG) (≥1.69 mmol/L), overweight (BMI ≥ 25 kg/m^2). Other
131 exclusion criteria were as follows: high (> 5 portions/day) or low (< 2 portions/day) intake of fruit
132 and vegetables and alcohol consumption (< 3 drinks per week were acceptable). Volunteers who
133 followed a specific diet (e.g. vegetarian, vegan, or macrobiotic) and those who had a specific
134 aversion for blueberry consumption were excluded. All participants gave informed consent and the
135 study was approved by the Ethics Committee of the University of Milan.

136

137 **2.2 Blueberry and placebo preparation**

138

139 Blueberries (*Vaccinium corymbosum* L. "Brigitta") from a single batch were purchased, sorted and
140 immediately frozen by Individually Quick Freezing technique in a tunnel (Thermolab, Codogno,

141 Italy) and stored at −20°C until use. For the study, BB were partially thawed (3 h at 20°C) and
142 homogenized in a commercial food processor (Moulinex, Paris, France). They were packed in
143 portions of 300 g, thermally sealed under partial vacuum (Minipack-Torre S.P.A., Dalmine,
144 Bergamo, Italy) and stored at −20°C for few days. The evening before the experiment, the BB
145 portions were placed at + 4°C for defrosting. The BB was gelatinous in texture; for this reason, a
146 control jelly (CJ) was utilized as placebo. The CJ was prepared by suspending 20 g of food grade
147 gelatin (Universal, Peru) and adding the same amount of BB sugars (about 27.1 g total, 16.4 g
148 fructose and 10.7 g glucose) in 200 mL of hot water. The CJ containing a food colorant was
149 prepared the day before the experiment and stored at +4° C to solidify.
150
151 **2.3 Experimental design**
152
153 Subjects were deprived of ACN-food sources 10 days before experimentation. Volunteers received
154 a complete list of ACN-rich foods to be avoided; the list included ACN-rich foods such as berry
155 fruits, red wine and red/purple fruits and other colored products. Subjects were randomly divided
156 into two groups of 5 subjects each: group 1 was assigned to the sequence BB/wash-out/CJ, whereas
157 group 2 followed the sequence CJ/wash-out/BB. The study was scheduled at different days to avoid
158 interference between withdrawal times and the study of vascular function. Each analysis was
159 separated by 10 days of wash-out period. Lunch and dinner was standardized and subjects were
160 asked to exclude all ACN-containing foods and maintain their regular lifestyle. Moreover basal
161 levels of peripheral vasoreactivity (RHI, reactive hyperemia index) were measured on a group of
162 fasted volunteers (n=9) early in the morning, in two different days, to ensure within-subject
163 repeatability. Average data obtained (2.05 ± 0.29 RHI for day 1 and 2.04 ± 0.27 RHI for day 2) did
164 not demonstrate an inter-day effect on vascular function as also recently reported [22].
165 Thus for the present study, peripheral arterial function was measured in two consecutive days.
166 Baseline levels were assessed the first day, while the second day peripheral arterial function was

167 evaluated 1h after the intake of BB or CJ. This protocol was chosen to avoid multiple measurements
168 (involving 5 min arterial occlusion through cuff inflation) in a short time-period, since it could
169 promote vasodilation through NO production [23].
170 The subjects fasted overnight before the ingestion of one portion of thawed BB (providing 348 mg
171 of ACNs) or CJ (without ACNs). The products were consumed early in the morning and blood was
172 collected by a phlebotomist at time 0 (before the consumption of the products) and 1 h, 2 h and 24 h
173 after BB or CJ consumption. Samples were drawn into evacuated tubes with heparin as
174 anticoagulant. One day-food records were kept by subjects in each experimental session, 2 days
175 before and 1 day after the intake of the BB product to check compliance to the dietary instructions.
176 Moreover, a direct interview by a registered dietitian was scheduled.
177
178 **2.4 Sugars, total phenolics and vitamin C determination in BB and CJ**
179
180 A duplicate sample (50 g) of BB was homogenized and suspended in water, centrifuged at 3000 *x g*
181 for 1 minute, filtered and injected for the analysis. Glucose and fructose were quantified by ultra
182 performance liquid chromatography (UPLC). The LC consisted of an Alliance model 2695 (Waters,
183 Milford, MA) equipped with a model 2996 photodiode array detector (Waters), coupled with mass
184 spectrometry (Micromass, Beverly, MA). The separation was carried out on BEH Amide column
185 (150 x 2.1 mm, 1.7 µm, Waters) at 35°C. Solvents were triethanolamine 0.2% and acetonitrile:
186 triethanolamine at a ratio of 74:26 (v/v). The elution gradient was linear and the amount of
187 triethanolamine was increased from 0% to 35% in 11 minutes at set up flow rate of 0.45 mL min^{-1}.
188 The calibration curve was obtained from 5 mg L^{-1} to 100 mg L^{-1} for both sugars. The percentage
189 relative standard deviation was calculated after injecting standard solutions of glucose and fructose
190 at increasing concentration (2 mg L^{-1}, 10 mg L^{-1} and 50 mg L^{-1}) in quintuplicates. Phenolic
191 compounds were extracted in duplicate from BB by applying a formic acid-water (5:95 v/v)
192 extracting media, according to Brambilla et al. [24]. Total phenolic compounds of the extracts were

193 analyzed by Folin-Ciocalteau assay [25] and expressed as gallic acid equivalents (GAE mg/100g)
194 while chlorogenic acid and individual anthocyanin compounds were analyzed by gradient reverse
195 phase-high-performance liquid chromatography (RP-HPLC) and diode array detection and were
196 quantified by measuring detector response to the commercial standards (Polyphenols Laboratory
197 Sandes, Norway) [24]. All ACN monoglycosides were expressed as cyanidin 3-glucoside (CydG)
198 equivalents. Vitamin C was extracted and determined by HPLC analysis as previously described by
199 Riso et al. [26].

200

201 **2.5 Analysis of biochemical parameters**

202

203 Blood samples were drawn and immediately centrifuged at 1000 *x g* for 15 minutes for plasma and
204 serum separation and stored at -80°C until analysis. A general laboratory clinical assessment was
205 performed in serum including evaluation of lipid profile (TG, TSC, LDL-C and HDL-C) and
206 glucose. All these parameters were determined using a cobas® 6000 analyzer series (Roche
207 Diagnostics, North America). Plasma concentration of total NO was calculated by measuring the
208 products of oxidation (nitrate and nitrite) by a Fluorometric Assay Kit (Cayman Chemical, Ann
209 Arbor, MI).

210

211 **2.6 Anthocyanin extraction and analysis in plasma**

212

213 Two aliquots of plasma (1 mL) were acidified with trifluoroacetic acid (TFA, 1%), vortexed, and
214 centrifuged for 1 min at 4500 *x g* and the supernatant was stored at -80°C until analysis.
215 Anthocyanins were extracted from plasma using a Micro-Plate solid phase extraction HLB Oasys
216 Cartridge preactivated with methanol (500 μL) and washed with 500 μL acidified water (1% TFA).
217 Plasma (400 μL) was diluted with 140 μL of acidified water (1% TFA) and 60 μL of water

218 containing the Internal Standard (50 ng/mL of CydG). Plasma was vortexed, centrifuged and loaded
219 onto the cartridge.
220 The samples were drained under gravity and the cartridge washed with acidified water (100 µL; 1%
221 TFA) and 100 µL of water-methanol (80:20 v/v) acidified with TFA (0.1%). The ACNs were eluted
222 from the cartridge using 50 µL of methanol (70%) containing TFA (0.1%). The filtered sample was
223 injected into Ultra performance liquid chromatography-mass spectrometry (UHPLC-MS/MS)
224 system for analysis according to a method previously published [27].
225

226 **2.7 Evaluation of endogenous DNA damage and cell resistance against H_2O_2-induced DNA**
227 **damage**
228

229 Mononuclear blood cells (MNBCs) were separated from whole blood by density gradient
230 centrifugation [29]. The FPG-sensitive sites (oxidized purines) and cell resistance against H_2O_2
231 (500 µmol/L, 5 min) induced DNA damage were evaluated by the comet assay as previously
232 described in detail [28-29].
233

234 **2.8 Evaluation of peripheral arterial function**
235

236 Endothelial-dependent vasodilation in the small finger arteries was assessed by a non-invasive
237 plethysmographic method (Endo-PAT 2000, Itamar Medical Ltd., Caesarea, Israel) based on the
238 registration of pulsatile blood volume in the fingertips of both hands.
239 The Endo-PAT equipment consists of two finger-mounted probes, which include a system of
240 inflatable latex air-cushions within a rigid external case; pulsatile volume changes of the fingertip
241 are sensed by a pressure transducer, located at the end of each probe, and transferred to a personal
242 computer where the signal is band pass-filtered (0.3 to 30 Hz), amplified, displayed, and stored. For
243 the evaluation, subjects were in the supine position and both hands on the same level in a

244 comfortable, thermoneutral environment. Arterial systolic and diastolic blood pressure and heart
245 rate frequency were measured before starting the test. A blood pressure cuff was placed on one
246 upper arm (study arm), while the contralateral arm served as a control (control arm). After a 10-min
247 equilibration period, the blood pressure cuff on the study arm was inflated to 60 mmHg above
248 systolic pressure for 5 min. The cuff was then deflated to induce reactive hyperemia (RH) while the
249 signals from both PAT channels (Probe 1 and Probe 2) were recorded by a computer. The RHI, an
250 index of the endothelial-dependent flow-mediated dilation (FMD), was derived automatically in an
251 operator independent manner, as the ratio of the average pulse wave amplitude during hyperaemia
252 (60 to 120 s of the post-occlusion period) to the average pulse wave amplitude during baseline in
253 the occluded hand, divided by the same values in the control hand and then multiplied by a baseline
254 correction factor. A RHI value of 1.67 provides a sensitivity of 82% and a specificity of 77% for
255 diagnosing endothelial dysfunction [30].
256
257 **2.9 Statistical analyses**
258
259 Sample size has been calculated taking into account the expected variation in the primary endpoint
260 considered as evaluated in our previous study [14]. In particular, ten subjects were calculated to be
261 more than sufficient to evaluate a difference of DNA damage after the wild blueberry drink of 8.6
262 (standard deviation 0.9), with alpha=0.05 and a statistical power of 80%. This number of subjects is
263 comparable to those used in previous acute studies [31-33] for the evaluation of vascular function
264 modulation (secondary endpoint). Moreover, the "repeated measure" experimental design used, in
265 which each subject acts as its own control, reduces the error variance, thus increasing statistical
266 power.
267 Statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK,
268 US). Data were analyzed by ANOVA for repeated measures design. ANOVA with treatment (BB
269 vs CJ) and time (before and after each treatment) as dependent factors was applied to evaluate the

270 effect of BB on the variables under study. Differences were considered significant at $P \leq 0.05$; post-
271 hoc analysis of differences between treatments was assessed by the Least Significant Difference
272 (LSD) test with $P \leq 0.05$ as level of statistical significance. To evaluate the relationship between
273 variation in ACN plasma levels following BB intake and those in DNA damage, linear correlation
274 analysis was performed. Data are presented as means ± standard deviation (SD).
275
276 **3. Results**
277
278 **3.1 Composition and characteristic of the blueberry portion**
279
280 The nutritional composition of BB is reported in **Table 1**. One portion (300 g) of the BB provided
281 about 27 g of sugars (fructose and glucose), 348 mg of ACNs (malvidin-galactoside, delphinidin-
282 galactoside and malvidin-arabinoside making up more than 50% of the total ACN content), 727 mg
283 of total phenolic acids, 90 mg of chlorogenic acid and 2.4 mg of vitamin C. The CJ provided the
284 same amount and type of sugars but not of bioactive compounds.
285
286 **3.2 Baseline characteristics of the subjects**
287
288 Baseline anthropometric and clinical characteristics of the subjects are reported in **Table 2**. All data
289 were within the range of normality.
290
291 **3.3 Plasma concentration of ACNs following BB and CJ intake**
292
293 Anthocyanins were not detectable in plasma at baseline while a significant ($P<0.001$) increase was
294 observed 1 h (13.7 ± 10.7 nmol/L) and 2 h (18.7 ± 6.4 nmol/L) after BB intake. Twenty four hours

295 after BB intake, ACNs were not detected in plasma; CJ intake resulted in undetectable plasma
296 ACNs.
297
298 **3.4 Effect of BB and CJ intake on the levels of DNA damage in MNBCs**
299
300 Results on DNA damage in MNBCs are reported in **Table 3**. Oxidized purines evaluated through
301 quantification of FPG-sensitive sites were not significantly different following BB or CJ intake. The
302 levels of H_2O_2-induced DNA damage decreased 1 h after the BB intake (from 51.7 ± 4.9% to 42.7 ±
303 8.7%, $P \leq 0.01$), while no effect was observed after CJ (from 53.2 ± 2.8% to 52.0 ± 7.6%, $P = 0.84$).
304 However, the protective effect was transient and the level of H_2O_2-induced DNA damage returned
305 to baseline 2 h after BB consumption. There was no correlation between the decrease in H_2O_2-
306 induced DNA damage and the increase in ACNs observed at 1 h.
307
308 **3.5 Effect of BB and CJ intake on peripheral arterial function and plasma nitric oxide levels**
309
310 Peripheral arterial function (reactive hyperemia response), blood pressure, heart rate and plasma NO
311 levels, before and after BB and CJ consumption, are reported in **Table 4.** According to the repeated
312 measures ANOVA, after either the WB or the CJ intake no significant changes were observed for
313 all the variables under study. The mean percent change in RHI index between the pre-to-post
314 intervention was +0.5% (95% CI: -7.3%, +8.4%) after the BB and -4.5% (95% CI: -13.9%, +6.4%)
315 after the CJ intake. On the whole, a high inter-individual variability was observed in the percent
316 changes of RHI index (**Figure 1**).
317

318 **4. Discussion**

319

320 Several human studies have demonstrated that the intake of single portions of fruits such as
321 kiwifruits, apples and orange juice was associated with decreased intrinsic levels of oxidatively
322 damaged DNA and increased resistance to H_2O_2-generated DNA damage [34-37]. In the present
323 study we also documented that the intake of one portion of BB significantly reduced the levels of *ex*
324 *vivo* H_2O_2-induced DNA damage in healthy male volunteers as hypothesized. The protective effect
325 was shown 1 h after the consumption of BB but not after 2 h, while no significant effect was
326 observed after CJ intake. The protection against oxidative stress may be related to other bioactives
327 absorbed, apart from ACNs (e.g. phenolic acids, vitamin C), acting alone or synergistically.
328 Moreover, these compounds could have indirectly activated signaling mechanisms of defense (e.g.
329 antioxidant enzymes through gene expression modulation) [37] even though the effect is not
330 maintained at 2 h.
331 No significant effect was observed on oxidized DNA bases after BB or CJ intake. This result as
332 previously reported [34] is not surprising, since the levels of FPG-sensitive sites measured,
333 represent the steady-state levels of oxidatively damaged DNA. In fact, in cultured cells, the repair of
334 FPG-sensitive sites has a half-life of 1-5 hours [38-40]. Since the removal of DNA damage is not
335 instantaneous, a long-term supplementation may be required to establish the possibility of *in vivo*
336 efficacy of BB intake on endogenous levels of oxidatively damaged DNA [34]. In fact, we have
337 recently documented that a 6 week intervention period with a wild BB drink reduced the level of
338 oxidized DNA bases in subjects with cardiovascular risk factors [14].
339 Additionally beneficial effects have been observed following the intake of one portion of
340 cranberry juice [41], dark chocolate [42-43] or flavonol-rich cocoa drink [31;44], green tea [45-46]
341 or red wine [47] in healthy and unhealthy subjects on endothelial function. The protocols generally
342 used, are based on multiple measurements of vasoreactivity (through both FMD and EndoPAT
343 2000) in a short time-period after the intake of the test products. However conflicting opinions on

344 this procedure are reported in literature. The International Brachial Artery Reactivity Task Force
345 suggested that multiple measurements may promote vasodilation through NO production [23]. This
346 effect may possibly mask improvement of vascular function due to the intervention (i.e.
347 overestimation). In this regard, Liu et al. [48] documented a significant increase in the RHI when
348 the PAT was measured at 0.5-hour intervals (for 2.5 h) indicating a crossover effect, but not at 1 h
349 intervals (for 4 h) and 2 h intervals (for 12 h) in healthy male subjects. In addition, Forchhammer et
350 al. [49] demonstrated an intra-day reproducibility in a group of healthy subjects whose vascular
351 function was measured on four different occasions (in the morning, before and after lunch and in
352 the afternoon) within the same day. Thus it seems that the time-period among measurements is an
353 important variable in this type of assessment and it should be seriously considered to avoid cross-
354 over effects. In the present study, we measured peripheral arterial function in two consecutive days
355 after demonstration of inter-day reproducibility as demonstrated by others [22]. We failed to
356 demonstrate an effect of BB on peripheral vascular function one reason being that most of the
357 subjects in the present study had RHIs in the normal range (RHI $\geq$ 1.67). It seems plausible that
358 improvements may be easier demonstrated in subjects with reduced vascular function (e.g. elderly
359 or subjects who are at risk of developing cardiovascular diseases) or after vascular function
360 challenges (e.g. following smoking or a meal rich in saturated fats).
361 The lack of the BB effect in modulating vascular function may be also attributed to the
362 length of time between the BB intake and the measurement of peripheral arterial function (1 h). In
363 fact, more time may be necessary to detect an effect on endothelial function following the exposure
364 to BB and their bioactives. In this regard, Dohadwala et al. [41] documented an improvement of
365 vascular function at 2 h and 4 h after the intake of a single portion of cranberry juice.
366 In the present study, the observations on vascular function are consistent with the non-
367 significant changes in plasma total NO, indicating that the short-term consumption of BB did not
368 exert any changes on this marker. Plasma NO concentration is mainly related to systemic
369 inflammation, whereas the endothelium-derived nitric oxide synthase (eNOS) production of NO is a

370 minor contributor to alterations in its plasma concentration. Nevertheless, we cannot exclude that a
371 modulation of NO occurred at the endothelial level without influencing total plasma levels. In fact,
372 some authors reported that the consumption of red wine polyphenols and flavonoids may affect
373 vascular function by increasing the half-life of endothelial NO [50]. Future studies with larger
374 numbers of subjects or with established vascular dysfunction may contribute to our understanding
375 of the beneficial effects of BB consumption on vascular function and modulation of plasma NO
376 levels.
377 Possible study limitations are the small sample size of healthy subjects considered for the
378 demonstration of an effect on vascular function at one time-point after the ingestion of BB. Even
379 though the statistical power increases with the number of subjects, the effect size is not dependent
380 on the sample size. Our results suggest that BB did not produce any short-term protective effects in
381 healthy subjects with uncompromised vascular function but we cannot exclude that improvements
382 in the vascular function occur at later time points than 1 h after a single BB consumption. In
383 addition, regular BB intake may protect against the development of vascular dysfunction in patients
384 with cardiovascular risk factors.
385 In conclusion this study documented that one portion of blueberries (300 g) can improve cell
386 resistance against H_2O_2-induced DNA damage, and this is in accordance with previous observations
387 with other fruits provided in single portions thus supporting the importance of consuming vegetable
388 foods regularly.
389

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391

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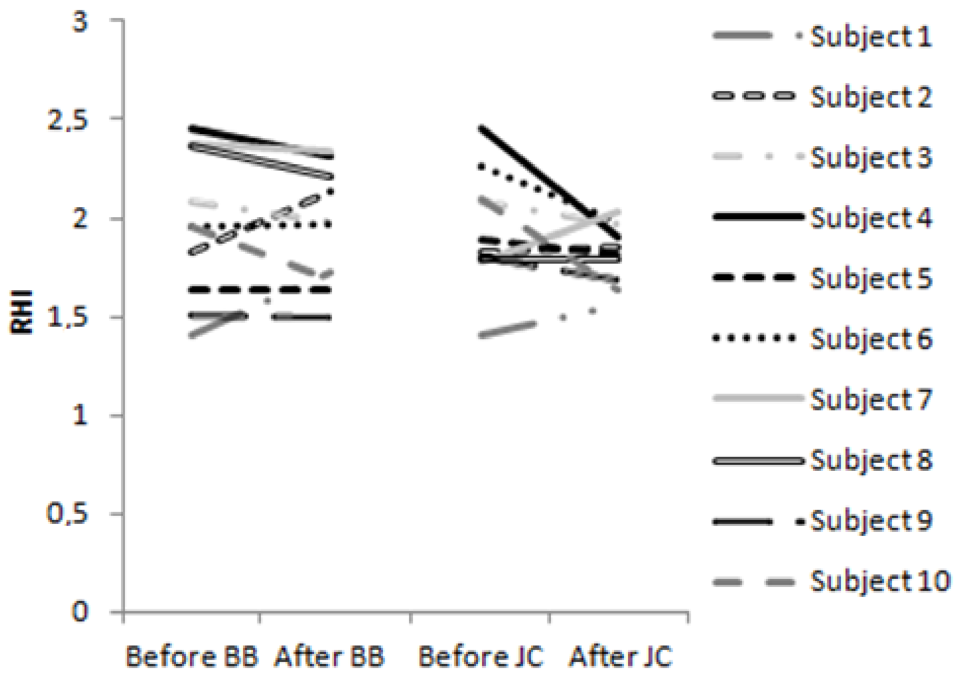
Figure legend

FIGURE 1 Individual values of reactive hyperemia index measured by Endo-PAT2000 (Itamar Medical Ltd., Caesarea, Israel) and registered before and after BB (Blueberry) and CJ (Control jelly) intake in the group of volunteers (n = 10).

529



530

531 Table 1 Nutritional composition of Blueberry (BB) and Control jelly (CJ)

	BB	CJ
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	-
Vitamin C (mg/100g)	0.8 ± 0.1	-

532 Data are expressed as means ± SD.

533

534 Table 2 Subject characteristics at the beginning of the study

	Value
Age (y)	20.8 ± 1.6
Body weight (kg)	72.4 ± 7.9
BMI (kg/m^2)	22.5 ± 2.1
Systolic pressure (mmHg)	119.5 ± 8.8
Diastolic pressure (mmHg)	76.5 ± 6.2
Heart rate (beat/min)	62.2 ± 15.3
Glucose (mmol/L)	3.92 ± 0.26
TG (mmol/L)	1.69 ± 0.49
TSC (mmol/L)	4.4 ± 0.64
HDL-C (mmol/L)	1.44 ± 0.33
LDL-C (mmol/L)	2.43 ± 0.37

Data (n=10) are expressed as means ± SD.

BMI, body mass index; TG, triglycerides; TSC, total serum cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

535

536

537

538 Table 3 Effect of one portion of Blueberry (BB) or Control jelly (CJ) on background, FPG sensitive
539 sites and H_2O_2-induced strand breaks

	T 0h	T 1h	T 2h	T 24h
BB consumption				
Background SBs (% DNA in tail, EB)	5.9 ± 0.6	5.9 ± 0.6	5.9 ± 0.7	6.0 ± 0.4
Net FPG-sensitive sites (% DNA in tail)	12.9 ± 2.1	13.1 ± 1.4	12.4 ± 1.4	13.1 ± 1.4
Background SBs (% DNA in tail, PBS)	8.1 ± 1.0	7.9 ± 1.5	8.00 ± 1.2	8.3 ± 0.8
Net H_2O_2-induced DNA damage (% DNA in tail)	51.7 ± 4.9	42.7 ± 8.7*	50.1 ± 9.1	51.8 ± 6.1
CJ consumption				
Background SBs (% DNA in tail, EB)	6.1 ± 0.6	5.9 ± 0.6	5.9 ± 0.3	5.8 ± 0.2
Net FPG-sensitive sites (% DNA in tail)	14.5 ± 3.6	14.1 ± 2.7	13.5 ± 1.4	14.8 ± 0.8
Background SBs (% DNA in tail, PBS)	8.5 ± 0. 7	8.6 ± 0.9	8.7 ± 0.1	8.4 ± 0.3
Net H_2O_2-induced DNA damage (% DNA in tail)	53.2 ± 2.8	52.0 ± 7.6	54.0 ± 4.3	49.3 ± 3.3

540 Data (n=10) are expressed as means ± SD.

541 SBs, strand breaks; PBS, phosphate-buffered saline; EB, endonuclease buffer; FPG,formamidopyrimidine DNA
542 glycosylase.

543 *Significantly different from each other time point in the same row and different with respect to each other time point
544 for the CJ group; $p \leq 0.01$.

545

546 Table 4- Effect of one portion of Blueberry (BB) or Control jelly (CJ) intake on peripheral arterial

547 function and total plasma nitric oxide (NO)

Variables	before BB	after BB	before CJ	after CJ
Systolic blood pressure (mmHg)	119.5 ± 8.8	118.6 ± 8.7	122.5 ± 10.4	121.3 ± 8.5
Diastolic blood pressure (mmHg)	76.5 ± 6.2	73.9 ± 6.1	76.3 ± 4.8	76.3 ±10.3
Heart rate (beat/min)	62.2 ± 15.3	61.6 ± 17.1	63.5 ± 16.4	64.0 ± 19.8
RHI	1.96 ± 0.39	1.95 ± 0.30	1.94 ± 0.30	1.82 ± 0.16
Total NO (µmol/L)	64.6 ± 22.5	64.7 ± 18.6	72.2 ± 21.8	72.5 ± 18.7

548 Data (n=10) are expressed as means ± SD.

549 RHI; reactive hyperemia index; NO, nitric oxide.

550