Research Article

Bergamot Polyphenolic Fraction supplementation improves metabolic balance, endothelial function and maximal oxygen uptake in athletes

Rocco Mollace[1], Micaela Gliozzi[2], Annamaria Tavernese[2], Vincenzo Musolino[2], Cristina Carresi[2], Miriam Scicchitano[2], Ernesto Palma[2], Saverio Nucera[2], Francesca Bosco[2], Federica Scarano[2], Francesca Oppedisano[2], Alessia Maretta[2], Jessica Maiuolo[2], Saverio Muscoli[1], Santo Gratteri[1], Francesco Romeo[1] and Vincenzo Mollace[2]*

[1]School of Cardiology, University of Roma "Tor Vergata", Italy

[2]Institute of Research for Food Safety and Health, Department of Health Sciences, University Magna Graecia of Catanzaro, Italy

*Address for Correspondence: Vincenzo Mollace, Institute of Research for Food Safety and Health, Department of Health Sciences, University "Magna Graecia" of Catanzaro, Italy, Tel: 009274758007; Email: mollace@libero.it

Abstract

Background: The study aimed to evaluate the effects of a 4-week Bergamot Polyphenolic Fraction (BPF Gold; Bergamet Sport) supplementation on serum nitric oxide (NO), asymmetric dimethyl-arginine (ADMA), Endopat indices of endothelial function and maximal oxygen uptake ($\dot{V}O_{2max}$) of athletes.

Methods: The effects of dietary supplementation (BPF Gold, 650 mg twice a day for 4 weeks) and placebo administration on flow-mediated dilatation (via Endopat measurements), serum markers (NO, ADMA), lipid profile, and $\dot{V}O_{2max}$ were analysed in 30 athletes both before and after dietary protocols.

Results: Significant differences between pre- and post-intervention baseline NO levels were observed after BPF Gold dietary protocol. Higher post-intervention baseline NO level was observed after BPF Gold diet compared with placebo. Moreover BPF Gold Sport increased baseline NO concentration (ΔNO). The positive correlation was observed between baseline post-intervention NO concentration and maximal oxygen uptale and also between ΔNO and $\Delta VO_{2\,max}$ in response to BPF Gold supplementation. There was an association between a higher Edopat values of endothelial function and higher VO_{2max} after Bergamet Sport diet compared with lower values of placebo.

Conclusion: These findings suggest that an increase in NO release in response to BPF Gold Sport supplementation may play a central role in cardiovascular adaptive mechanisms and enhanced exercise performance in athletes.

Introduction

Aerobic capacity and endothelial function represent major determinants of exercise performance in athletes being highly improved by physical training. This improvement is the result of adaptive changes in skeletal muscle, mostly due to their ability to use oxygen to generate energy for muscle work. Exercise training leads to beneficial changes in cardiovascular system including an improvement in left ventricular function, a decrease in myocardial oxygen demand for the same level of external work performed as well as metabolic shift

towards an increased lipid metabolism[1,2]. In a recent study, arterial structural and functional remodeling and a specific pattern for arterial responsiveness was suggested in humans following regular training [3].

These functional responses in arteries are influenced by exercise-induced endocrine and/or neural mechanisms and are probably affected by athletic status [4]. Physical exercise leads to progressive increase in heart rate (HR), which, in turn, increases blood flow and vascular shear stress. The potential mechanisms through which vascular control may be beneficially modified in response to repeated exposure to shear stress include increased endothelium-dependent vasodilatation [5], higher production of endothelial nitric oxide (NO) and endothelial NO synthase (eNOS) expression [6]. Several studies support that functional responses in arteries are influenced by mechanisms that modulate arterial vasomotor tone and can be affected by type of exercise training and/or athletic status [7,8]. It is important to note that power athletes have larger brachial arteries and greater vasodilatory responses; however, in endurance-trained athletes, lower and not higher flow-mediated dilatation (FMD)'s values have been observed in response to larger artery size [3]. It has been hypothesized that higher oxygen extraction fraction in the exercising muscle could be associated with peripheral vascular adaptations in skeletal muscle consisting of longer blood transit time, more homogenous perfusion and enhanced vascular flow capacity [9].

The physiological consequence of intense physical exercise is peripheral vasoconstriction induced by activation of the arterial baroreflex, which results in an increase in sympathetic outflow and blood pressure (BP; 8). In athletes, peripheral vasoconstriction during maximal exercise is prevented by improved endothelium-dependent dilator capacity (as indicated by plasma levels of nitrite oxide; NO), which may increase skeletal muscle oxidative capacity during exercise [10]. Apart from these molecular mechanisms, dietary factors also seem to play an important role in optimizing the effects of training [11].

Evidence exists suggesting that dietary supplementation with polyphenols had a positive impact on vascular function in healthy subjects [12,13] and patients with metabolic [14] and cardiovascular disease [14-16]. In particular, evidence has been accumulated that bergamot-derived polyphenolic Fraction (BPF Gold) leads to significant metabolic and anti-oxidant effects, mostly due to its peculiar composition in polyphenols which exert a significant vaso-protective response in patients. The potential mechanisms by which supplementation with BPF Gold could improve cardiovascular function are currently under investigation, but their action may be related to the improvement in vascular endothelial function (e.g. increased availability of NO) [17] enhancement of vascular reactivity, production of endogenous antioxidant enzymes and anti-inflammatory effects (e.g. cytokines, free radical species etc) [18,19].

There are limited data regarding the effect of BPF Gold supplementation on vascular endothelial function and optimization of physical performance of athletes. We hypothesized that BPF Gold might improve this function via the stimulation of NO production and release. To verify this, we examined the relationships between endothelial function, as measured by EndoPAT analysis, NO concentrations and maximal oxygen uptake ($\dot{V}O_{2max}$) and BPF Gold supplementation in cyclists.

Methods

Subjects

Thirty healthy and well-trained male cyclists (aged: 28.1±5.4 years) who were trained for at least 3 years participated in the study. They were randomly

assigned to both dietary protocols (i.e., placebo or the BPF GOLD (Bergamet Sport) supplementation. Then, after a 2-week break (i.e., washout period), members of each group were crossed over to the opposite treatment protocol and following brachial artery measurement, biochemical investigations and physical exercise test were repeated. All subjects participated in the study during the p r e -season period. The training status of the subjects expressed as maximal oxygen consumption ($\dot{V}O_{2max}$) was 69.8±4.9 ml.kg^{-1}.min^{-1} and the mean individual monthly training volume was 655±53 km. Age, height, body mass and body mass index of the participants (mean±SD) are presented in table 1. Echocardiography (ECG) measurements of cyclists indicate that left ventricular structure and function were within normal range (Table 1).

The exclusion criteria used in order to eliminate factors which might influence the vascular parameters were as follows: evidence of hemodynamic dysfunction, inflammatory diseases in the preceding 3 months and cigarette smoking. All subjects reported that they were not taking citrus fruits, citrus beverages and supplements or any medication that could affect cardiovascular function. They were instructed to abstain from exercise within 24 hours before the ultrasound measurements. No caffeine, antioxidants and alcohol were permitted during 48 hours before the experiment. Three weeks prior to the study, all participants were put on a mixed, isocaloric diet (2800±800 kcal/day) consisting of carbohydrates in the amount of 365.3±152.6 g/day, proteins: 130±45.5 g/day and fats: 109±45 g/day (monounsaturated fats: 35±15 g/day and polyunsaturated fats: 8.2±4 g/day).

The isocaloric diet was continued with BPF Gold or placebo administration and during the washout period. We supplemented our subjects for 4 weeks and before each diet protocol the endopat analysis and biochemical variables were analyzed. The cyclists consumed the prescribed diet for a total of 12 weeks (3 weeks before, during the 4 weeks of both dietary regimens and 2-week break period). To make sure that subjects consumed a diet, they were asked to sign a statement of compliance with diet. Physiologist and cyclist's coach regularly reminded them about the need to comply with dietary regime. The study was approved by the Ethics Committee of the University of Catanzaro and conformed to the standards set by the Declaration of Helsinki.

Supplementation procedures and training protocol

All clinical data, including biochemical parameters and ultrasound examination, were obtained after an overnight fast. M-mode and two-dimensional Doppler-ECG were performed in all subjects using an ARTIDA (Toshiba, Japan) ultrasound system with standard imaging transducers to determine the left ventricular muscle mass, interventricular septum diameter during diastole and left ventricular

Table 1: Anthropometric and Echocardiographic variables of the athletes enrolled for the study (mean±SD).
Variables Subjects (n=30)
Age (years) 28.1±5.4
Body mass (kg) 73.4±4.6
Body height (cm) 183.5±4.2
BMI (kg/m^2) 24.4±1.9
LVM (g) 216.1±33.3
LVMI (g/m^2) 125.6±20.3
IVSDd (mm) 11.1±1.5
LVPWTd (mm) 9.9±1.6
LVEF (%) 60.3±3.2
SV (ml) 81.3±8.3
BMI, body mass index; LVM, left ventricular mass; LVMI, left ventricular mass index; IVSDd, intraventricular septum diameter during diastole; LVPWTd, left ventricular posterior wall thickness during diastole; LVEF, left ventricular ejection fraction; SV, stroke volume.

posterior wall thickness during diastole. The left ventricular mass index (LVMI) was calculated by correcting for body surface area (Table 1).

Endothelial function was assessed using the EndoPAT 2000 technique, which measures PAT using the reactive hyperaemia index (RHI, arbitrary units). Briefly, after 20 minutes' rest in a chair inclined at an angle of about 45° at room temperature, a blood pressure cuff was placed on the non-dominant upper arm (study arm), while the other arm served as the control. The hands were placed on armchair supports with the palm side down, such that the fingers hung freely.

The EndoPAT probes were then placed on the tip of each index finger of both hands. The probes were prevented from touching any other finger or object, and were then electronically inflated. The PAT signal was continuously recorded on a personal computer during the test. Baseline pulse amplitude was measured from each fingertip for five minutes. After baseline recording of five minutes on each arm, arterial flow was then interrupted in the experimental arm by rapidly inflating the cuff to occlusion pressure of 200 mmHg or 60 mmHg plus systolic blood pressure (whichever was higher). After five minutes' occlusion, the cuff pressure was rapidly deflated, and post-occlusion recording continued for another five minutes in the experimental arm as well as the control arm. Pulse amplitude response to hyperaemia was automatically calculated from the hyperaemia in the finger of the experimental arm as a ratio of post-deflation average pulse inger) to obtain the RH–PAT ratio or PAT ratio. The EndoPAT 2000 not only measured endothelial function with the RHI but also assessed arterial stiffness by measuring the peripheral augmentation index (PAIx) from the radial pulse wave analysis. PAIx automatically calculated as the ratio of the difference between the early and late systolic peaks of the waveform relative to the early and late systolic peak of the waveform related to the systolic peak, expressed as percentage.

After initial testing, the BPF Gold (Bergamet Sport) supplemented group received 650 mg/twice a day of Bergamet Sport. The control group received a placebo in the form of gelatin capsules (1.3 g lactose monohydrate). Participants were instructed to take the capsules with meals twice daily for a total of 3 weeks. The participants returned to the laboratory after 4 weeks for post- testing. After a 2-week break, participants were crossed over to the opposite treatment protocol (placebo vs. Bergamet Sport diet).

Measurement and blood collection

At the beginning of the study (pre-intervention) and at the end of each treatment period (post-intervention supplementation or placebo protocol), all subjects reported to the laboratory and had venous blood drawn for the determination of NO and ADMA concentrations and lipid analysis.

All investigated subjects underwent bioelectric impedance analysis (In Body Data Management System) under resting conditions to determine their body mass.

Exercise test

Before and after 4 weeks of each treatment protocol (supplementation or placebo), all subjects performed a standard cycling exercise test (Lode Excalibur Sport Ergometer Bicycle, Groningen, Netherlands) to analyze whether BPF Gold might positively affect exercise performance of cyclists. The test started with a 3-minute warm-up; the intensity was then increased by 40 W every 3 minutes up to maximal exercise intensity. Pulmonary ventilation, oxygen uptake (VO_2) and carbon dioxide output (CO_2) were measured continuously from the sixth minute prior to exercising and throughout each stage of the exercise load using the Oxycon Apparatus (Jaeger, Germany). HR was continuously monitored (PE-3000 Sport-Tester, Polar Inc. Finland) and BP (SBP/DBP) was measured in duplicate with a sphygmomanometer before and immediately after exercise.

Biochemical Analyses

For biochemical analysis, antecubital venous blood samples were drawn always at the same time of day, with the subject in a seated position. Venous blood samples were collected 5 time points (baseline, pre- and post-exercise with placebo and pre- and post- exercise with BPF Gold. Blood was allowed to clot at room temperature and then centrifuged. The resulting serum was aliquoted and frozen at −80°C for later analyses. The measurements of ADMA and total NO and nitrite/nitrate were performed using enzyme-linked immunosorbent assay (R&D System, Inc., Minneapolis, USA). The sensitivity of total NO/nitrite/nitrate assay was 0.25 $\mu mol \cdot l^{-1}$. Intra- and interassay coefficients of variation for total NO/nitrite/nitrate were <2.5% and <4.6%, respectively. ADMA test disclosed values as low as 0.05 $\mu mol \cdot l^{-1}$. The intra- assay coefficient of variation was <9.8%, and the interassay coefficient of variation was <7.5%. Total serum cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL), free fatty acids, triglycerides and serum glucose were measured using commercially available test kits (Novamedical, Italy).

Preparation of BPF Gold

Bergamot Juice (BJ) was obtained from peeled-off fruits by industrial pressing and squeezing as previously described [17]. The juice was oil fraction-depleted by stripping, clarified by ultra-filtration and loaded on suitable polystyrene resin columns absorbing polyphenol compounds of MW between 300 and 600 Da (Mitsubishi). Polyphenol fractions were eluted by a mild KOH solution. Next the fitocomplex was neutralized by filtration on cationic resin at acidic pH. Finally it was vacuum dried and minced to the desired particle size to obtain BPF powder. BPF powder was analysed by HPLC for flavonoid and other polyphenol content showing a 47% concentration of naringine, neohesperidine, neoeriocitrine, bruteridine and melitidine. In addition, toxicological analyses were performed including heavy metal, pesticide, phthalate and sinephrine content which revealed the absence of known toxic compounds. Standard microbiological tests detected no mycotoxins and bacteria. Finally, 650 mg aliquots of the BPF powder supplemented with 50 mg of ascorbic acid as antioxidant were encapsulated with a semi-automatic gelatin encapsulation device by an authorized manufacturer (Bergamet Sport, Nathealth Solution Ltd, USA). Tablets containing 650 mg of maltodextrin supplemented with 50 mg ascorbic acid were prepared for placebo studies.

All capsules were put into bottles containing 60 capsules (for 4 week) and packaged in color-coded plastic bags containing 4 bottles (for 16 weeks) for distribution to participants. An unblinded researcher prepared and masked the samples. Participants were instructed to consume 2 capsules daily 10 min before breakfast and dinner. All procedures have been performed according to the European Community Guidelines concerning dietary supplements.

Statistical analysis

All results are presented as the mean±standard deviation. We analysed differences between pre- and post-interventions (placebo/BPF Gold) baseline and post-exercise variables. The results of the change in pre-intervention and post-interventions baseline NO level (ΔNO) and increase in maximal oxygen uptake (DV O2max) were calculated. The data were analysed by two-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test when appropriate. The statistical analysis includes a two-way ANOVA (placebo *vs.* Bergamet Sport and baseline pre- intervention *vs.* baseline post-intervention). Pearson correlation coefficients were analysed to determine the inter-variable relationships. Statistical significance was set at $P < 0.05$.

Results

The effects of dietary supplementation with BPF Gold (Bergamet Sport 650 mg/twice a day) and placebo administration on total NO and ADMA concentrations, vascular indexes (via EndoPAT analysis) in trained male cyclists were compared after 4 weeks of each treatment protocol.

In particular, significant differences between pre-intervention and post-intervention baseline NO levels were observed after Bergamet Sport dietary protocol (Figure 1). Indeed, significant higher post-intervention baseline NO level was observed after BPF Gold diet compared with placebo (Figure 1). Moreover, the supplementation with BPF Gold increased baseline NO concentration (ΔNO) compared with placebo. Finally, the effect of BPF Gold on NO levels was seen also after cessation of exercise test; Figure 1). No significant effect of BPF Gold diet was observed regarding NO increase (ΔNO) at maximal exercise intensity compared with post-intervention baseline levels. A significant positive correlation was observed between NO level and VO_{2max} both in response to supplementation and placebo. However, importantly, the positive correlation was observed between ΔNO and DVO_{2max} only in response to BPF Gold supplementation.

ANOVA did not reveal any significant effect of BPF Gold on ADMA basal levels. However, significant increase in post-exercise ADMA levels was observed in investigated subjects after both treatment protocols compared with baseline post-interventions levels (Figure 2). After BPF Gold supplementation, significant negative correlation was seen between baseline ADMA level and NO.

The effect on NO release after BPF Gold supplementation correlated with changes seen in the parameters of vascular endothelial function before and after a 4-week period of BPF Gold or placebo administration as presented in Table II. Resting HRs as well as SBP and DBP did not differ between both treatment protocols. In particular, the BPF Gold intervention significantly improved endothelium dependent vasodilatation studied by means of Endo PAT (Table 2) compared to the placebo administration. Finally, BPF Gold supplementation significantly increased maximal oxygen uptake (VO_{2max}) and individual O_2-uptake/HR (VO_{2max}/HR_{max}), which was associated with lower HR at maximal exercise intensity (Table 2).
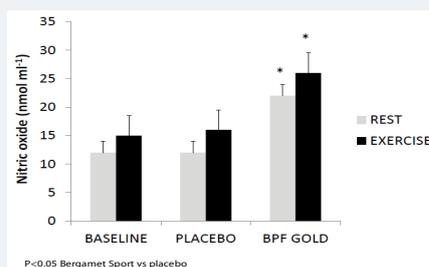

Figure 1: The effect of BPF Gold on nitric oxide production in athletes at rest and after exercise.
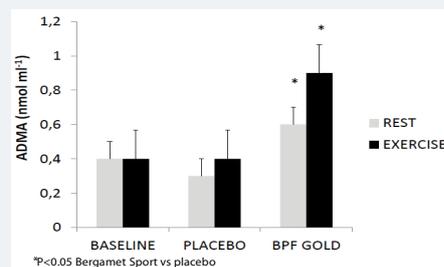

Figure 2: The effect of BPG Gold on asymmetric dimethyl-arginine (ADMA) at rest and after exercise in athletes.

Table 2: Physiologic characteristics, oxygen consumption, lipid profile, glucose concentrations and Endopat indices of endothelial performance in 30 athletes undergoing placebo or Bergamet Sport supplementation twice a day for 4 weeks (mean ± SD).

Variables	Pre-intervention	Placebo	Bergamet Sport
HR (beats·min^{-1})	62.0 ± 9.0	60.0 ± 10.0	54.0 ±10.6
SBP at rest (mmHg)	125.0 ± 12.0	126.0 ±10.0	125.0±13.0
DBP at rest (mmHg)	81.0 ± 10.0	83.0 ± 10.0	81.0 ± 12.5
ASP (mmHg)	103.7 ± 10.1	105.5 ± 12.1	106 ± 12.6
$\dot{V}O_2max$ (	66.8 ± 4.2	68.0 ± 4.6	77.8 ± 5.1
Peak power (watt)	412.0 ± 32.0	416.0 ± 29.0	445.0 ± 28.0*
HR$_{max}$ (beats·min^{-1})	190.0 ± 4.6	190.0 ± 3.8	182.0 ± 4.0 *
$\dot{V}O_2max/HR_{max}$	0.34 ± 0.04	0.35 ± 0.01	0.43 ± 0.01*
Triglycerides (mg·dl^{-1})	126.8 ± 41.8	123.1 ± 45.0	124.1 ± 44.0
Total chol (mg·dl^{-1})	194.1 ±30.5	197.7 ± 30.4	167.1 ± 23.0*
LDL-chol (mg·dl^{-1})	91.6 ± 24.6	96.0 ± 27.0	80.8 ± 27.3*
HDL-chol (mg·dl^{-1})	56.5 ± 11.8	54.4 ± 12.0	68.2 ± 12.4*
Glucose (mg·dl^{-1})	87.6 ± 12.6	84.5 ± 11.8	80.6 ± 8.1
RHI	2,0 ± 0.15	1.98 ± 0.21	1.66 ±1.4*
fRHI)	0,30 ± 0.4	0,31 ± 0.3	0.24 ± 0.3*
AI	-20,3 ± 4.0	-19,8 ± 3	-6.0 ± 2.0*
* P<0.05 Bergamet Sport vs placebo.			

HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; $\dot{V}O_2max$, maximal oxygen uptake; RHI, Reactive Hyperemia Index; fRHI, Framingham Reactive Hyperemia Index; AI, Augmentation Index.

Discussion

The present study was undertaken to investigate whether BPF Gold supplementation might exert a beneficial effect on serum NO and ADMA concentrations, EndoPAT vascular indexes of endothelium-dependent vasodilatation and muscle oxidative capacity in cyclists. Our results have demonstrated that a 4-week BPF Gold supplementation caused elevation of baseline serum NO compared with pre-supplementation levels. An increase in baseline and post- exercise serum NO levels were also observed in contrast to the placebo administration. Moreover, the increased NO production seems to have significant effect on resting endothelium-dependent vasodilatation. The major findings of our study are that incremental exercise is more effective in stimulating NO expression and that this effect is more pronounced in athletes after BPF Gold supplementation. Four weeks of BPF Gold supplementation had a beneficial effect on endothelial function. Endothelial function improvement, might, in turn, have significantly increased individual peak $\dot{V}O_2$ and peak $\dot{V}O_2/HR_{max}$.

Our data are in accordance with previous studies showing that BPF supplementation leads to increase NO bioavailability by protecting NO from oxidative destruction [17]. Given that NO has important roles in cutaneous and skeletal muscle blood, we hypothesized that BPF Gold might reduce cardiovascular strain (i.e., HR, SV, CO) and improve during exercise. Several mechanisms have been reported that may be responsible for the vasoprotective effect of BPF Gold [18,19], including fat oxidation in liver and skeletal muscle [19,20] and maintenance of vasodilatation through the stimulation of NO production [17]. This might explain, at least in part, the beneficial use of BPF Gold in athletes, though the impact in exercise performances still remains to be better clarified. Serum NO increase in response to 4-week BPF Gold supplementation may act as a potent causative factor leading to endothelial function improvement due to its role in the maintenance of vascular function and structure in response to physical exercise. By combining the effects of vasoconstriction, repeated exposure to shear stress and an increase in peripheral resistance, exercise exerts higher pressure load on the vasculature [21]. Increased shear stress and/or up-regulation of eNOS gene expression enhance vascular NO production and stimulate the regulatory mechanisms of the cardiovascular system [22-24], and this effect seems to be enhanced by BPF Gold.

Antioxidant administration has been shown to result in dramatic improvements in exercise performance and compelling evidence has been gathered showing the potential ergogenic properties of antioxidants such as NAC [25]. Pertinent to the present study, a series of studies [25] documented significant improvements in time to fatigue during high intensity cycling. The mechanisms responsible for the improved performance may be associated with increased antioxidant availability and improved K^+ regulation as a result of maintained Na^+/K^+ pump. The present study was designed in order to increase the oxidative stress that is associated with exercise by performing high intensity exercise which is known to induce an excessive elevation in free radical production and subsequent increase in oxidative stress beyond normal conditions. Free radical production appears to play a necessary or regulatory role in many physiological processes and functions including mitochondrial biogenesis, vascular function, and inflammation. Recent findings suggest that healthy individuals subjected to elevated levels of oxidative stress due to eccentric exercise producing muscle damage, weakness or inflammation thereby exhibiting significant improvements in recovery following antioxidant supplementation. Similarly antioxidant supplementation may be warranted in situations in which oxidative stress is chronically elevated (aging, disease, and dysfunction). Under these conditions exogenous antioxidants such as BPF Gold appear to compensate for the inability of the endogenous antioxidant systems to combat the chronic increase in oxidative stress.

Thus, supplementation of athletes with BPF Gold leads to antioxidant effects which, in turn, improves endothelium-dependent vasodilatation via enhanced NO release. This leads to increased scheletal muscle blood flow and improved oxygen consumption, suggesting a significant support of exercise performance in athletes receiving BPF Gold supplementation.

Acknowledgements

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