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ORIGINAL ARTICLE

Effects of polyphenolic antioxidants on exercise-induced oxidative stress

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Summary Polyphenols are of increasing interest to consumers and food manufacturers for several reasons. Commonly referred to as antioxidants (they are the most abundant antioxidants in our diets), they may prevent various oxidative stress-related diseases, such as cancer, cardiovascular disease, inflammation and others. Physical activity is known to induce oxidative stress in individuals after intensive exercise. In this study, the effect of the flavonoid contents (which are the most abundant polyphenols) was investigated, as the only antioxidant in a replacement drink designed for sportsmen on various oxidative stress biomarkers after two identical trials of sub-maximal aerobic exercise, in a group of 30 sportsmen. In one of the trials, the cyclists consumed the antioxidant supplement (with 2.3 g polyphenols/trial), and in another they consumed a placebo. Blood samples were collected both at rest and after exercise immediately and 45 minutes (min) later, for measurements of plasmatic indices of oxidative stress: lipid oxidation (TBARS), total antioxidant status (TAS); protein oxidation (carbonyl groups, CO) and the lactate dehydrogenase (LDH) and creatine kinase (CK) enzymes for each trial. All values were adjusted for changes in plasma volume. No changes were detected in plasma TAS and LDH after exercise or after the polyphenolic supplement. CK and TBARS increased after exercise in both tests. However, in response to strenuous exercise, the polyphenol-supplemented test showed a smaller increase in plasma TBARS and CK than the placebo test. CO increased by 12% in response to the placebo test, whereas it decreased by 23% in the polyphenol-supplement test. This may indicate that the antioxidant supplement offered protection against exercise-induced oxidative stress.
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Introduction

Flavonoids are polyphenolic compounds found in rich abundance in all land plants.[1] Owing to their polyphenolic nature, flavonoids often exhibit strong antioxidant properties, akin to α-tocopherol, which they structurally resemble and can replace in some model systems.[1,2] Although animals do not directly synthesize flavonoids, their diet contains them in large amounts. One estimate has the average daily consumption of flavonoids by humans as 1 g, an amount far greater than that of other dietary antioxidants such as ascorbate or α-tocopherol.[3] Given the prevalence of these substances in our diet, and their demonstrable antioxidant activity, it is only reasonable to suppose that animals have evolved the capacity to take advantage of the beneficial properties of flavonoids. Bioavailability studies have shown that the concentrations of intact flavonoids in human plasma rarely exceed 1 μM when the quantities of polyphenols ingested do not exceed those commonly ingested with our diets. These maximum concentrations are most often reached 1–2 h after ingestion[4,5] except for polyphenols, which are absorbed only after partial degradation by the microflora in the colon. For most flavonoids absorbed in the small intestine, the plasmatic concentration decreases rapidly (half-life period of 1–2 h).

During strenuous exercise, there is a dramatic increase in oxygen uptake in various organs, particularly in the skeletal muscle.[6] Oxygen-centered radicals are produced in intermediate metabolism. The resting body is equipped with both non-enzymatic and enzymatic antioxidant reserves to prevent the potential harmful effects of reactive oxygen species (ROS).[7] The fine physiological balance between oxidative reactions and antioxidant capacity may be perturbed by intense physical activity. ROS released causes lipid peroxidation of polyunsaturated fatty acids in biological membranes and blood, disturbing cell functions.[8] Malondialdehyde (MDA), a by-product of lipid peroxide, is the most frequently studied marker of oxidative tissue damage during exercise. MDA levels have been found to increase both in different tissues and plasma during exercise.[6,8,9] It has been reported that strenuous physical exercise produces a decrease in antioxidants levels and an increase in the markers of lipid peroxidation in target tissues and blood.[10] Data on changes in total antioxidant capacity (TAC) in humans are conflicting,[11] as is the information on the effect of exercise on lipid peroxidation.[8,12] Evaluation of TAC is one of the most common procedures employed to evaluate the hydrosoluble antioxidant status of biological fluids.[13] After strenuous physical exercise, a significant increase in TAC may occur, although the opposite effect might be anticipated.[14] There is ample evidence of the antioxidant activity of flavonoids both in vivo and in vitro. The results described by Ghiselli et al.[14] indicate that polyphenol-rich beverages are able to transfer their antioxidant capacity to body fluids. The aim of the present study is to follow through the effects of the ingestion of a drink of polyphenolic antioxidants (flavonoids) on lipid and protein oxidation and the TAC of plasma in cyclists under oxidative stress.

Materials and methods

Subjects

A group of 31 sportsmen aged (23.6 $\pm$ 0.9 years) was selected. The subjects were cyclists, engaged in a controlled physical training program consisting of 3 h of training per day, the last week before the tests (20 h of training per week for at least 1 year). Before entry into the study, the subjects' health status was assessed from their medical history, hematological evaluation and exercise electrocardiogram. The individuals were selected consecutively among those fulfilling the inclusion criteria. The inclusion criteria involved an absence of hypertension, diabetes, cardiovascular disease, organic brain disease, alcohol or drug dependence, and any other deviation from normal food habits. The general characteristics of the participants are shown in Table 1.

All details of the study were explained to the participants, who gave their written consent. The protocol was approved by the Ethical Committee of the San Antonio Catholic University of Murcia Spain.

Dietary assessment

All volunteers were explicitly asked to follow their normal lifestyle, particularly with regard to diet and level of physical activity during the last year. A

Table 1 General characteristics of participants.

General characteristics of participants	Values
Age (years)	23.7 $\pm$ 0.9
Weight (kg)	69.7 $\pm$ 1.3
Height (cm)	175.7 $\pm$ 2.4
BMI (kg/m^2)	22.9 $\pm$ 0.8
$VO_{2\,max}$ (l/min)	4.2 $\pm$ 0.1

BMI, Body mass index; $VO_{2\,max}$, Maximal oxygen uptake. Values expressed as mean $\pm$ SEM.

Table 2 Daily nutritional intakes of the 31 sportsmen.

	Mean	SEM
Energy intake (cal)	3188	152
Protein (g)	125	8
Carbohydrate (g)	408	21
Fat (g)	127	9
Fiber (g)	23	2
Cholesterol (mg)	255	19
Vitamin A (μg)	2539	287
Vitamin C (mg)	133	11
Vitamin E (mg)	10	1
Zn (mg)	19	2
Mn (g)	3	0.3
Mg (mg)	390	24

Values expressed as mean±SEM of two 24 h surveys of each subject.

face-to-face interview was conducted with every participant and a detailed food frequency questionnaire was completed in order to obtain information about their dietary habits. The quality, quantity and frequency of consumption of red meat, chicken, fish, eggs, vegetables, fruits, dairy products and soft drinks were similar in all of the subjects. In a pre-test conducted under the direction of a qualified dietician, the subjects were instructed on how to record food intakes using standard household measures. After the food diary was completed, the dietician examined it to clarify the records and eliminate inconsistencies with the help of the subjects. Intakes were recorded twice, before each exercise-test. No statistical difference was found between the 2 day for any of the variables studied $P<0.05$, for 7 variables: energy, protein, fat, carbohydrates, cholesterol, fiber and antioxidant vitamins (Table 2). The energy intake and dietary composition were subsequently analyzed using the AyD dietary analysis computer program.[15]

Analysis of antioxidant capacity of drink with polyphenols

The beverage supplied by Hero-Spain had an energy value of 42 Kcal/100 ml, and contained mainly carbohydrates from fruits concentrates 21% (black grape, raspberry and red currant), known to contain high levels of phenolic compounds;[16–18] the beverage also contained maltodextrin, pectin, sodium citrate and vitamin C (20 mg/l), whey protein hydrolyze, sodium chloride, aroma, sweeteners sodium cyclamate and saccharine and vitamin B1 (15% Recommended Dairy Intake—RDI).

Modal to the placebo, there was prepared by the same ingredients that the drink of study but without contributing any source of carbohydrates. His composition is basically water with sweeteners, fragrant, natural colorant, vitamin C and pectin.

The antioxidant capacity of the beverage (of the same lot of manufacture) was analyzed in three data groups, each of which was kept in darkness at different temperatures: 23, 30 and 37 °C. The measurements of the antioxidant capacity of the drink (Fig. 1) were realized for 1 year of storage, in triplicate, in a spectrophotometer Varian (mod. Cary Bio-50 UV-Vis). The capacity scavenger of radical free of the drink was evaluated by making the sample with polyphenolic antioxidants react with the radical ABTS*.[19] The Trolox Equivalent Antioxidant Capacity (TEAC) assay is based on the scavenging of the 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical ABTS* converting it into a colorless product. The degree of discoloration induced by a compound is related to that induced by Trolox, giving the TEAC value. Results were expressed as TEAC, defined as the millimolar mM concentration of a Trolox solution with antioxidant capacity equivalent to 1.0 mM solution of the substance under investigation.

Quantitative analysis was also carried out the concentration of total phenols of the drink was determined spectrophotometrically with the reagent Folin–Ciocalteau, using gallic acid as standard, as was qualitative analysis of the polyphenolic compounds of the drink (Table 3), by liquid chromatography in an HPLC Merck-Hitachi with L-7100 pump and using a Lichrochart 100 RP-18 reverse phase column Merck, Darmstadt, Germany (25 × 0.4 cm, with 5 μm particle size). For the mobile phase HPLC water with 5% of formic acid

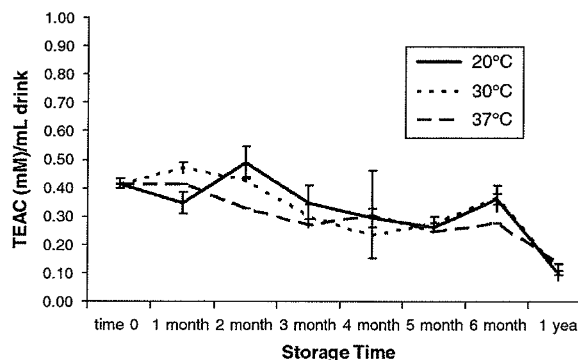

Figure 1 Changes in antioxidant capacity (TEAC) of the replacement drink with polyphenols for a year of storage in darkness at different temperatures. Values expressed as Mean±SEM.

Table 3 Polyphenolic antioxidants contained in the replacement drink.

Polyphenolic antioxidants	mg/l
Anthocyanins	758.6
Flavonols	80.5
Derivates hydroxycinnamic	245.7
Stilbenes	1.0
Ellagic acid	167.7
Total	1253.5

(solvent (A) and methanol (solvent B) was used. The elution was carried out at flow speed of 1 ml/min, with increases at 30, 40 and 50 min, returning to initial conditions at 55 and allowing a settling time in the column. All the analyses were carried out in triplicate and the results are expressed as mean values $\pm$SEM. The different compounds were characterized by comparison chromatography following commercial models and by the visible spectrum registered with the diode net detector. The anthocyanins were quantified as cyanidin-3-rutinoside at 510 nm, the hydroxycinnamic derivatives at 320 nm as chlorogenic acid and the flavonols at 360 nm, as quercetin-3-glucoside and stilbenes as resveratrol at 320 nm (standards used).

Determination of maximal aerobic capacity

Maximal oxygen uptake was estimated by means of a continuous incremental exercise test on a electromagnetically braked cycloergometer Technogym Spin Trainer (Gambettoia, Italy), with a start load simulating a speed of 12 Km/h, increasing by 2 Km/h every minute, at a constant slope of 2%. Heart rate was continuously recorded on an electrocardiogram (ECG) G.E. Medical Systems Company; (Buc, France). Gas exchange data were obtained using an automated breath-by-breath system Sensormedics Vmax Series 29-C; (Yorba Linda, CA). Relationship from the $VO_{2\,max}$—work rate and the work rate equivalent to 70% $VO_{2\,max}$ was interpolated.

Exercise tests

Each subject performed two bouts of exercise on different occasions separated by one week: 1 of them testing the study drink (A), and another one with the placebo (P). The order of the trials was randomly assigned for each cyclist, but only once, before the first occasion (in this way, each cyclist play as his own control). Both beverages were identical in appearance and taste. The flavor of the two was indistinguishable. During the exercise no other beverages or food were ingested. The trials were double-blinded (to the subjects and to the researcher).

Two 90-min rectangular tests were performed on a bicycle ergometer at 70% of the $VO_{2\,max}$. Laboratory conditions were 25$\pm$0.2 °C and 70% relative humidity. The subjects drank 200 ml of beverage (A or P) every 15 min and initiated ingestion 15 minutes before the exercise. The sportsman drink 1600 ml of beverage for each test, this volume is considered usual in the habitual ingestion of liquid that the cyclists consume during the development of a sports test. Each cyclist was weighed immediately before and after exercise.

Following arrival at the laboratory at 9.00 a.m. each cyclist consumed a standard breakfast [500 ml of entire milk (Hero®), 200 ml of orange juice (Hero®), 25 g of jam of peach (Hero®), 2 toasts (Bimbo®), 1 snack of cereals (Hero Muesly®), sugar: 1 teaspoonful is equivalent to 3 g, soluble coffee (Nescafé®): 1 teaspoonful is equivalent to 1 g, soluble cocoa (Nesquik®): 1 teaspoonful is equivalent to 2 g] 45 min before the beginning of the experiment. Before initiating the test each survey subject was monitored for 24 h.

Biochemical blood analyses

For each volunteer, blood samples were collected immediately before exercise, immediately after exercise and 45 min post-exercise by venous puncture from the antecubital vein. Immediately an aliquot was transferred to chilled heparinized glass tubes. Samples were centrifuged (6000 rpm/10 min), and plasma was divided into aliquots and stored at −80 °C.

Measurements of hematocrit and hemoglobin

Hematocrit ratio (Htc) was measured in venous blood samples for triplicate with a microhematocrit centrifuge and corrected (0.96) for plasma trapped with the packed red cells. An additional correction (0.93) was made for the venous -to- total body Htc ratio. Hemoglobin was measured by the cyanmethemoglobin method. The methodological error for hematocrit and hemoglobin were $\pm$0.3% and $\pm$0.5%, respectively; and percentage changes in plasma volume were estimated using the method of Dill and Costill.[20]

Creatine kinase (CK) and lactate dehydrogenase (LDH) activity

Serum CK and LDH were used as an indirect index of exercise-induced muscle damage[21–23] and were measured using two diagnostic kits Sigma Chemical Co. (St. Louis, MO). We have measured serum CK activity, which is used as a marker of the muscular damage, and it is associated indirectly with the increment of the permeability in the muscle cell membrane[49,50] which it is produced in parallel way that the detection of oxidative stress induced by physical exercise. As a minimum triplicate analyses were made of each sample, and CK and LDH activities were calculated as the mean of three values that differed by no more than 10% of the lower value. The inter-assay variation coefficients (cv) were 3.5% for CK and 3.8% for LDH.

Lipid peroxidation measurement

Plasmatic levels of lipid peroxidation were measured as formation of a thiobarbituric acid (TBA) adduct of MDA, separated by HPLC.[51] The sample is added to lysis buffer (0.2% Triton X-100 in KH_2PO_4/KOH, pH 7.4) plus butylated hydroxytoluene (100 ml of a solution of 2 g/l butylated hydroxytoluene in ethanol) (Sigma). Two 250 ml samples were incubated at room temperature for 10 min and then 500 ml 1% (w/v) TBA was added. Reaction mixtures were incubated at 90 °C for 90 min, allowed to cool and centrifuged (3000*g*, 10 min). Twenty microliters of the supernatant were injected into a Spherisorb ODS_2 (C18) column (Waters, Milford, MA, USA) with a guard column (Hiber C18) (Waters). Elution was performed with 65% (v/v) 50 mm KH_2PO_4/KOH buffer (pH 7.0) and 35% (v/v) methanol at a flow rate of 1 ml/min. The absorbance of the sharp peak at retention time around 6 min was read at 532 nm as the level of the $(TBA)_2$-MDA adduct. Assays were performed in triplicate and results were expressed as μmol TBARS/ml plasma. This method is based in the addition of some reactives which produce cell lysis, and the products of the lipid peroxidation are poured off to the plasma and, therefore, the results are expressed as plasmatic measurements.

Plasmatic antioxidant capacity

The TAC of plasma is a quantitative indication of the state of balance of these various components in the course of time. Total antioxidant status (TAS) in plasma was measured by a chromogenic method Randox Laboratories Crumham's, North Ireland). In this assay metmyoglobin reacts with hydrogen peroxide to form ferrylmyoglobin free radical species. Ferrylmyoglobin was incubated with the substrate 2,2′-amino-di(3-ethylbenzthiazole sulphonate) and measured at 600 nm.[24]

Protein carbonyl measurement

Protein carbonyl content was measured by forming labelled protein hydrazone derivates, using 2,4-dinitrophenylhydrazide (DNPH), which were then quantified spectrophotometrically.[25–27] Briefly, after precipitation of protein with an equal volume of 1% trichloroacetic acid (TCA), the pellet was resuspended in 1 ml of DNPH 10 mM in 2 N HCl. Separate blanks were prepared by adding 1 ml of 2 N HCl without DNPH. Samples were left at room temperature for 1 h in the dark and vortexed every 15 min. An equal volume of 20% TCA was added and after centrifugation at 12,000*g* for 15 min at 4 °C, pellets were washed three times with 1 ml of ethanol–ethylacetate mixture (1:1) to remove the free DNPH and lipids contaminants. The final pellet was dissolved in 1 ml of 6 M urea and kept at 37 °C for 1 h in a water bath with mixer. The solution was centrifuged for 15 min at 12,000*g*. The carbonyl content was determined from the absorbance at 370 nm with the use of a molar absorption coefficient of 22,000 mol/l cm.

Statistical analyses

Statistical analysis was performed with the SPSS 12.0 program (Statistical Package for Social Science) for Windows. All data are presented as mean ± SEM. Comparisons were made by Student's *t*-test for normally distributed variables, and by Wilcoxon's test for those variables that did not follow a normal distribution. The normalization adjustment for each every variable was made by means of Kolmogorov–Smirnoff's test. The study of the differences between the averages at different moments was made by means of a one-way ANOVA with linear trend test. All contrasts were made considering $\alpha < 5\%$ to be an error. Correlation was evaluated using linear regression.

Results

The beverage studied showed an antioxidant capacity expressed as TEAC capacity antioxidant equivalents Trolox of 0.41 mM Trolox/ml, which does not change significantly (Fig. 1) for 6 months of storage in darkness at three different tempera-