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Research article

Grape extract improves antioxidant status and physical performance in elite male athletes

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Abstract

Excessive physical exercise overproduces reactive oxygen species. Even if elite sportsmen increase their antioxidant status by regular physical training, during the competition period, this improvement is not sufficient to limit free radical production which could be detrimental to the body. The aim of this randomized, double-blind, placebo controlled, and crossover study on 20 elite sportsmen (handball = 10, basketball = 5, sprint = 4, and volleyball = 1) during the competition period was to determine if the consumption of a grape extract (GE; *Vitis vinifera* L.) was able to improve the parameters related to (i) anti-oxidative status and oxidative stress and (ii) physical performance. Specific biomarkers of antioxidant capacity, oxidative stress, skeletal cell muscle damage, and other general biomarkers were determined in plasma and urine before (D0) and after one month (D30) of placebo or GE supplementation (400mg·d^{-1}). Effort tests were conducted using the Optojump® system, which allows determining the total physical performance (EnRJ45), explosive power (RJ110), and fatigue (RJL5). The plasma ORAC value was not modified in the placebo group; however, GE increased the ORAC value compared to the placebo at D30 (14 966+/-335 *vs* 14 242+/-339 µmol Teq·L^{-1}; $p < 0.05$). The plasma FRAP value was significantly reduced in the placebo group, but not in the GE group. Therefore, GE limited the reduction of FRAP compared to the placebo at D30 (1 053.7+/-31.5 *vs* 993.7+/-26.7 µmol Teq·L^{-1}; $p < 0.05$). Urinary isoprostane values were increased in the placebo group, but were not modified in the GE group. Consequently, GE limited the production of isoprostanes compared to the placebo at D30 (1.24+/-0.12 *vs* 1.26+/-0.13 ng·mg^{-1} creatinine; $p < 0.05$). GE administration, compared to the placebo at D30, reduced the plasmatic creatine phosphokinase concentration (CPK, 695.7+/-177.0 *vs* 480.0+/-81.1 IU·L^{-1}, $p = 0.1$) and increased hemoglobin levels (Hb, 14.5+/-0.2 *vs* 14.8+/-0.2 *vs* g·dL^{-1}, $p < 0.05$), suggesting that GE administration might protect cell damage during exercise. The high variability between sport disciplines did not permit to observe the differences in the effort test. Analyzing each individual group, handball players increased their physical performance by 24% ($p < 0.05$) and explosive power by 6.4% ($p = 0.1$) after GE supplementation compared to the placebo. Further analyses showed that CPK and Hb were the only biomarkers correlated with the increase in performance. In conclusion, GE ameliorates the oxidative stress/antioxidant status balance in elite athletes in the competition period, and enhances performance in one category of sportsmen (handball). Our results suggest that the enhancement in performance might be caused by the protective action of GE during physical exercise. These findings encourage conducting further studies to confirm the efficacy and mechanisms of action of GE on elite and occasional athletes.

Key words: Botanical extract, oxidative stress, exercise, physical performance, sportsmen, training, competition.

Introduction

Young athletes under a regular training program exhibit a substantial increase in antioxidant status when compared to healthy sedentary people (Brites et al., 1999). Such an effect results from an adaptative response to controlled physical activity, inasmuch as exercise represents a form of oxidative stress which may be defined as an increase in intracellular steady state concentration of oxidants over physiological values (Cazzola et al., 2003; Evelson et al., 2002; Radak et al., 2008). During a competition period and/or overload training, this adaptative response does not suffice to limit free radical production which could prove detrimental to the body (Klapcinska et al., 2005; Palazzetti et al., 2003; Pincemail et al., 2000). Antioxidants may be administrated before competition, when exercise is likely to be exhaustive and results in the generation of free radicals that overwhelm the defensive mechanisms, causing oxidative stress (Gomez-Cabrera et al., 2008). Different types of supplementation such as selenium, vitamin E, vitamin C or polyphenols revealed that it was possible to increase the already adapted antioxidant status (Margaritis et al., 2003; Morillas-Ruiz et al., 2005; 2006; Rokitzki et al., 1994a; 1994b). Some studies further evaluated the impact of an antioxidant effect on performance or endurance in athletes; the results, however, differed largely depending on the type of supplementation, subjects and protocol (Clarkson et al., 2000).

Oligomeric proanthocyanidins (OPCs) are polyphenols and more specifically, polymers of flavanols (Manach et al., 2004). Their main dietary sources are to be found in grapes, cocoa, and apples (Santos-Buelga and Scalbert, 2000). OPCs are famed for their potent antioxidant capacity *in vitro* (da Silva et al., 1991; Mazur et al., 1999; Shafiee et al., 2003; Teissedre et al., 1996; Vitseva et al., 2005; Yilmaz et al., 2004). As regards human consumption, there is substantial evidence that OPC intake from grapes or cocoa increase the antioxidant status among hypercholesterolemic, hyperlipidemic, hemodialysis patients, smokers or healthy volunteers (Castilla et al., 2008; Gorinstein et al., 2006; Preuss et al., 2000; Serafini et al., 2003; Simonetti et al., 2002; Vigna et al., 2003). Few studies have been released with respect to flavanols in athletes (Morillas-Ruiz et al., 2006; Pavlovic, 1999).

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To date, only a single study has been carried out on polyphenol consumption-related impact over performance (Pavlovic, 1999).

Thus, the aim of the randomized, double-blind, cross-over clinical study presented herein is to ascertain the effects of grape extract consumption (GE) titrated in flavanols on (i) anti-oxidative status and oxidative stress, and on (ii) the physical performance among elite athletes under regular training conditions and in competition.

Table 1. Subject (n =20) baseline physical, hematologic and lipemic characteristics. Values are means (± SEM).

Age (y)	21.6 (2.0)
Height (m)	1.87 (.02)
Weight (kg)	84.7 (3.2)
Body Mass Index ($kg \cdot m^{-2}$)	23.9 (.55)
Hb ($g.dL^{-1}$)	14.6 (.2)
Blood formula	Normal
Cholesterol ($g.L^{-1}$)	1.82 (.06)
Triglycerides ($g.L^{-1}$)	.56 (.06)

Methods

Subjects

Twenty elite male athletes were recruited after having submitted their written consent. All subjects practiced explosive sports in keeping with top elite standards (i.e. national leagues), namely: 10 handball players, 5 basketball players, 4 sprinters, and 1 volleyball player. The baseline characteristics of the latter are presented in Table 1. Participants proved to be in good health as assessed by way of a medical history questionnaire, physical examination, and clinical laboratory tests. All subjects fulfilled the following eligibility criteria: 1) Practice of high level national intensive sports under non-stop training conditions over a one-month period, including competition; 2) no metabolic disorders (type 1 or 2 diabetes, cardiovascular, hepatic, gastrointestinal or renal diseases); 3) no pharmacological treatment, antibiotic nor supplemental vitamin and mineral use over the 8 weeks prior to launching our study; 4) fewer than 2 cigarettes per day; 5) under 20g per day alcohol intake; 6) no vegetarian, vegetalian nor deviated diet behavior; 7) no recent surgery; 8) no blood transfusion over the three months prior to the study; and 9) no involvement in another clinical study. Moreover, as suggested by the Ethical Committee, they were asked not to consume any drugs which may enhance physical activity.

Study design

This study was a randomized, double-blind, placebo controlled, and crossover design. It was approved by the Ethical Committee from Salvator Hospital, Marseille, France (project # 206014). The promoter for the study was Naturex SA, Avignon, France. Dr Houvenaeghel, M.D., from Salvator Hospital, Marseille, France, ensured medical investigations. Avantage Nutrition, Luminy Technopole, Marseille, France, enforced scientific coordination for our study.

The subjects were randomly divided into 2 groups: each group was supplied with either 400mg GE supplementation, or 400mg of placebo (maltodextrin) under capsule conditioning (2 capsules) to be taken along with their breakfast over a one month period. After one month of supplementation and two weeks of a wash out period, the treatments were reversed (Figure 1). Placebo and GE capsules were indiscernible. Supplementation was given with breakfast over a one month period. The study was double-blind while neither the M.D. nor the coordinator, nor the participants knew the randomization codes. The promoter,--namely Dr Lafay--, was the sole member to have knowledge thereof. The subjects were allowed to maintain their standard eating patterns. They were asked to complete a questionnaire on food intake frequency. Moreover, the intensity of training had to be equivalent among all participants: hence, they were further requested to complete a questionnaire focusing on their training

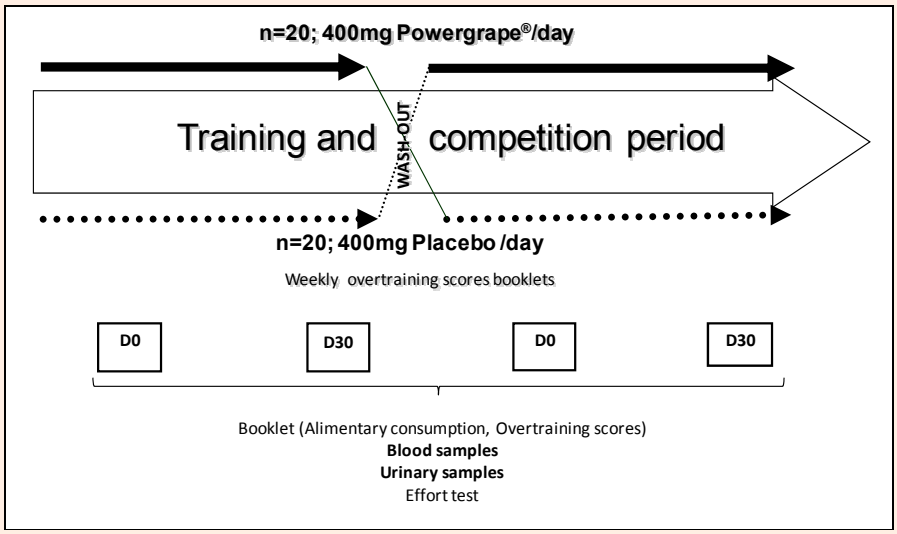

Figure 1. Study design. The subjects, randomly divided into 2 groups, were assigned to supplementation either 400mg of GE or 400mg of placebo (maltodextrin) under capsule forms (2 capsules) with their breakfast during one month. After one month of supplementation and two weeks of wash out period, the treatments were reversed. Effort test, blood and urine samples were done at day 0 (D0) and day 30 (D30) of each period.

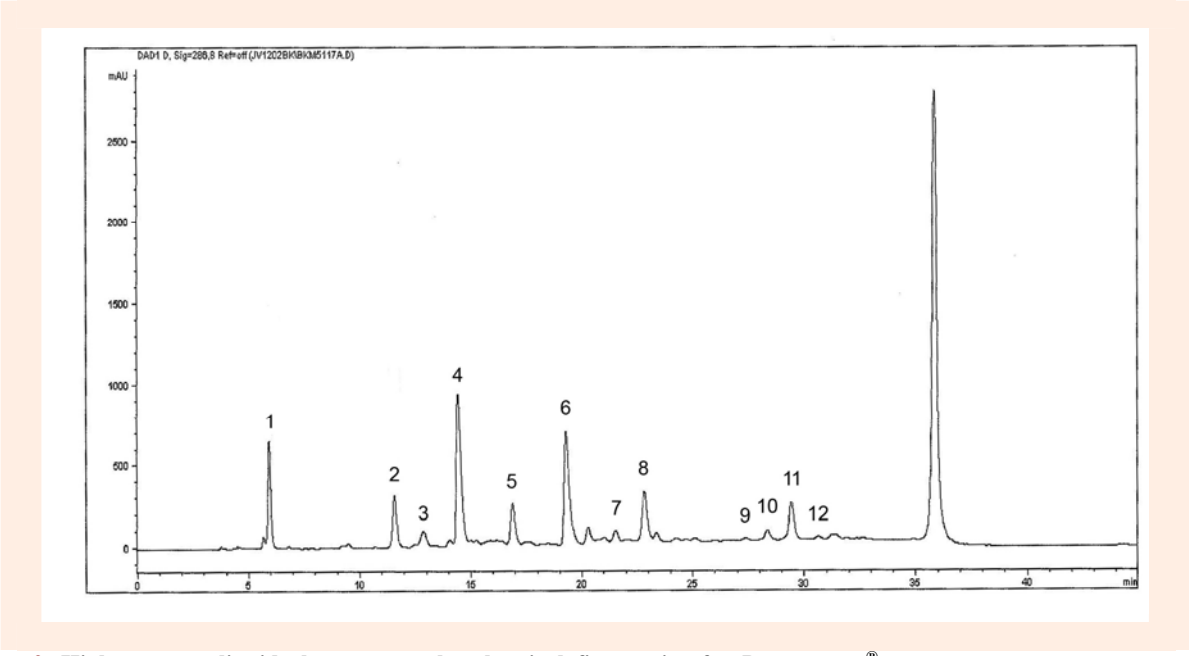

Figure 2. High-pressure liquid chromatography chemical fingerprint for Powergrape®: (1) gallic acid (5.9min); (2) B1 dimer (11.5min) ; (3) B3 dimer (12.9); (4) catechins (14.4min); (5) B2 dimer (18.9min); (6) epicatechin (19.3min); (7) C1 trimer (21.5min); (8) B2-3-*O*-gallate dimer (22.8min); (9) digallate trimer (27.3min), (10) digallate dimer (29.3min) ; (11) epicatechine-3-*O*-gallate (29.4min); (12) gallate trimer (30.6min).

levels (over-training scores). At the beginning of the study (D0), and after 30 days of supplementation of GE or placebo (D30), blood and urine were sampled from the subjects after they had fasted overnight for > 10h. Moreover, an effort test was conducted using a validated system Optojump® (Microgate SRL, Bolzano, Italy) (Lehance et al., 2005; Wilmot et al., 2004) associated with a cardiofrequence-meter. Strict compliance with terms and conditions was assessed as follows: a cross-analysis of information contained in a diet consumption frequency questionnaire together with the counting of the capsules brought back to the laboratory at D30 of each period.

Grape extract

The GE used in this trial was obtained from whole grapes, *Vitis vinifera* L. *(Vitaceae)*. This extract, registered as Powergrape® (batch 4990), was manufactured by Naturex SA, France. The extract is obtained using a hydro-alcoholic solvent (30% ethanol) and the plant:extract ratio is between 10:1 and 15:1, calculated on dry basis. This extract is spray dried with maltodextrin (0 to 8% w/w) and silica (0 to 2% w/w). It is standardized in total polyphenols (>90%) by the Folin method (Folin and Denis, 1915), in total flavanols (>50%) by the vanillin method (Broadhurst et al., 1978) and in flavanols monomers and gallic acid (>12%) by HPLC-DAD.

The high-pressure liquid chromatography chemical fingerprint for the whole grape extract is presented in Figure 2. The method for performing this analysis was as follows: HPLC was achieved using column SBC18, 250 X 4.6mm at 25°C. Mobile phase A involved the use of distilled water/trifluoroacetic acid 5.10^{-3}%, whereas mobile phase B consisted in acetonitrile 65%/trifluoroactic acid 5.10^{-3}%. The gradient used is presented in Table 2. The flow rate amounted to 0.7mL.min^{-1}, and the elution was monitored at 280 nm. Analyses were performed by Naturex SA.

Microbiology, heavy metals, pesticides and mycotoxins were controlled for compliance.

Table 2. Mobile phase gradient used to obtain high-pressure liquid chromatography chemical fingerprint of the grape extract.

Minutes	Phase A[1]	Phase B[1]
0	85	15
30	65	35
32	0	100
40	0	100
41	85	15
45	85	15

[1]Phase A: distilled water/trifluoroacetic acid 5.10^{-3}%; phase B: acetonitrile 65%/trifluoroactic acid 5.10^{-3}%. HPLC was achieved using column SBC18 (250 X 4.6mm) at 25°C.

Sample collection and analyses of endogenous parameters

Sample collection

Blood samples were collected in dry, EDTA/K3-coated and lithium/heparin-coated tubes. One EDTA/K3-coated tube was used for hemoglobin (Hb) analysis conducted less than two hours after sampling. Serum (blood in dry tubes) and plasma (blood in EDTA/K3-coated tubes) were separated by centrifugation at 4°C and 2000g for 10min. Serum was immediately treated with 10µL·mL^{-1} of inhibitor cocktail (EDTA, antibiotic and anti-protease). Suitable portions for the biochemical analysis described below were stored at -20°C.

One portion of plasma coming from lithium/heparin-coated tubes was treated with pyrogallol 12% (20µL·mL^{-1}) for vitamin E analysis. Another portion was treated with metaphosphoric acid 5% (4µL·mL^{-1}) for vitamin C analysis. The remaining plasma was directly stored at -80°C for antioxidant capacity determination. Plasma coming from EDTA/K3-coated tubes was stored

at -80°C for oxidized-LDL analysis. Red blood cells coming from EDTA/K3-coated tubes were washed with 5 mmol·L^{-1} phosphate-buffered saline, pH 7.4, hemolized at 4°C with bi-distilled water and stored at -80°C for endogen antioxidant enzyme analysis. Urine samples were collected in sterile tubes for creatinine and isoprostane analysis.

Analyses

Biomarkers of antioxidant status and oxidative stress

Oxygen radical absorbance capacity (ORAC): ORAC of plasma samples was determined. This fluorescence method is widely used for assessing antioxidant capacity in biological samples. It is based on the inhibition of a peroxy-radical-induced oxidation initiated by the thermal-based decomposition of azo compounds such as AAPH using fluorescein as a fluorescent probe and Trolox as a standard substrate (Huang et al., 2002; Ou et al., 2001).

Ferric reducing ability of plasma (FRAP): FRAP was also determined on 100µL plasma samples diluted 1:4 and the tripyridyltriazine complex formed with the reduced ferrous ions was measured with spectrofluorometry (LS 5, Perkin Elmer, Norwalk, CT) (Benzie et al., 1996).

LDL oxidation: Anti-oxLDL autoantibodies were determined using a commercially available ELISA kit (Biomedica, Vienna, Austria).

Antioxidant enzymes: Superoxide dismutase (SOD), gluthation peroxidase (GPx), and catalase: SOD (Peskin and Winterbourn, 2000), GPx (Ozdemir et al., 2005; Prasad et al., 2005) and catalase (Bai et al., 1999; Deisseroth and Dounce, 1970) were determined on red blood cells with three different commercial kits (FLUKA, Buchs, Switzerland; TREVIGEN Inc, Gaithersburg, MD, USA; SIGMA, Saint Louis, MO, USA, respectively).

Plasma vitamin E: To determine plasma vitamin E concentration, the used HPLC equipment included a photodiode array detector on-line with a Waters Millennium. The separation was carried out using a Nucleosil column (150mm × 4.6mm, 5 µm, Interchim, Montluçon, France). Elution was performed with an isocratic mobile phase of pure methanol at a flow rate of 2mL.min^{-1}. Alpha-tocopherol acetate (Sigma Aldrich) was added to plasma samples as an internal standard. Samples were extracted twice with hexane after precipitation of the proteins. The extract was evaporated to dryness under N_2, dissolved in ethanol-methylene chloride (65:35, v:v) and injected into the HPLC.

Ascorbic acid: Ascorbic acid was determined according to the method previously described by Tessier et al. (1996). Ascorbic acid from 500µL of supernatant was oxidized with 200µL of 2g·L^{-1} of potassium ferricyanide (Sigma) and 3.68 mol·L^{-1} of sodium acetate buffer (pH 6.9) and processed into the following derivative, i.e. quinoxaline complex with 200µL of 10 mg·mL^{-1} of o-phenylenediamine (Sigma). Dehydroascorbic acid was determined using a Waters HPLC/fluorescence detector. The sample (40µL) was injected into a Hypersil BDS-C18 column (5µm, 25cm) at 30ºC and eluted with a mobile phase consisting of 80mmol·L^{-1} Na_2HPO_4, 80mmol.L^{-1} NaH_2PO_4 and methanol (30:30:40 v/v/v) at an 0.8mL.min^{-1} flow rate. The excitation wavelength was 360nm and the emission wavelength was 440nm. The method has been previously validated, and we implemented quality control checks by spiking plasma samples.

Urinary isoprostanes: The level of isoprostanes in urine samples was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI and Oxford Biomedical Research, Oxford, MI). The amounts of isoprostanes were expressed in nanograms and corrected with creatinine values.

Biomarker of skeletal muscle damage

Plasma creatin phosphokinase (CPK) was assessed using specific enzymatic and colorimetric kit methods (Biomérieux, Craponne, France).

General plasmatic biomarkers: Triglycerides, cholesterol, serum ferritin, urea, and red blood cell hemoglobin

Plasma triglycerides and cholesterol levels were determined using commercial kits (Biotrol, Paris, France and Biomérieux, Charbonnière-les-bains, France, respectively). A polyvalent control serum (33-plus Biotrol, Paris, France) was treated in parallel to samples and served as control in order to appreciate result accuracy in the triglycerides and cholesterol analyses. Serum ferritin was assessed using a specific enzymatic kit (Roche Diagnosctics, Meylan, France). Serum urea was assessed using specific enzymatic and colorimetric kit methods (Biomérieux, Craponne, France). Red blood cell Hb was measured using a haematology blood cell counter (Sysmex XT2000i, Roche Diagnostics, Meylan, France).

Physical performance measurements (Effort tests)

For measuring physical performance, the Optojump® method was used (Microgate, Bolzano, Italy). This method calculated the energy in Joules (J) produced by each sportsman by measuring the flight time during several single vertical rebound jumps. The Optojump® system is able to measure with 1/1000th precision all flying times. This method consists of 2 bars (100 x 4 x 3 cm), one containing the reception and control unit, the other embedding the transmission electronics.

The Optojump® test was conducted at D0 and D30 of each period. Figure 3 summarizes the Optojump® test for each sportsman, which consisted of: (i) 3 successive single vertical Counter Movement Jumps (CMJ), starting from a resting standing position, from which the subjects were instructed to jump as high as possible, keeping their hands on their hips throughout the jump, starting with knees bent at 90°. For the CMJ test, they had to jump starting with a preliminary counter movement until a 90° angle. The flight time was recorded which allowed the determination of the energy (J) reached during the jump. Each single CMJ was separated from the other by a 1 minute rest. This first series of CMJ was considered as the warm-up period necessary for the adaptation to the test. (ii) A 2 minutes rest period. (iii) Sportsmen performed a series of vertical Rebound Jumps (RJ) during 45 seconds, from which they were instructed to jump as high and as fast as possible, keeping their hands on their hips throughout the jump, starting with knees bent at 90°. The Total Rebound Jump Power (EnRJ45) is the total

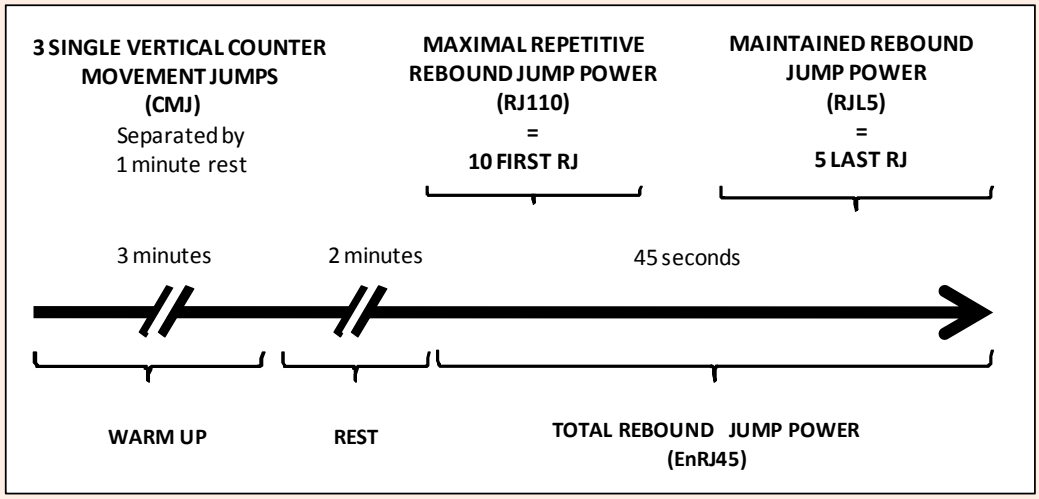

Figure 3. Optojump® methodology. Each sportsmen performed the test at day 0 (D0) and after 30 days (D30) of each period. The test included 3 steps: (i) a warm up phase of adaptation where they had to perform three single vertical Counter Movement Jumps (CMJ) separated by 1 minute rest, (ii) a 2 minutes of rest, and (iii) to perform a continuum Rebound Jumps (RJ) during 45 seconds, namely Total Rebound Jumps Power (EnRJ45), which represents the total physical performance. The Maximal Repetitive Rebound Jump Power (RJ110), which represents the explosive power, is the average energy (J) calculated from the 10 first RJ of this 45 seconds sequence; and the Maintained Rebound Jump Power (RJL5), which represents the fatigue, is the average energy (J) calculated from the last 5 RJ of this session.

accumulated energy (J), calculated from the successive RJ developed during 45 seconds. The Maximal Repetitive Rebound Jump Power (RJ110) is the average energy (J) calculated from the 10 first RJ of this 45 second sequence, and the Maintained Rebound Jump Power (RJL5) is the average energy (J) calculated from the last 5 RJ of this session.

EnRJ45 represents the total physical performance; RJ110 gives the average explosive power at the beginning of a repeated resistance exercise; and RJL5 represents the capacity to maintain a maximal explosive force in the ultimate phase of a resistance exercise, which is a measure of fatigue.

Statistical analyses

Data are presented as means +/- SEM. All observed results at the start of the second phase were compared with those at the start of the first phase by a paired *t* test. Because baseline data of the 2 phases did not differ, data from both groups were pooled by placebo or grape extract treatments (n = 20).

Comparisons of after-treatment values and within treatments were made by the paired *t* test. Comparisons of relative values were performed by using paired *z* tests. Pearson's correlation analysis was used to probe the correlation between different biomarkers determined in the blood, and the results obtained with the effort test. Values of $p < 0.05$ were considered significant. Statistical analyses were carried out using XLStat software (Version 2008.1.03, Addinsoft, France).

Results

Biomarkers of antioxidant status and oxidative stress

Regardless of the method used to determine the plasma antioxidant capacity, a significant increase of the plasma antioxidant capacity is showed when volunteers consumed whole grape extract capsules compared to the placebo capsules (Table 3). With the ORAC method, plasma antioxidant capacity increased from 13 885 ± 360 to 14 996 ± 335µmol Trolox eq·L^{-1} ($p < 0.05$) after one month of GE supplementation; whereas, this parameter remained unaltered after one month of placebo supplementation (14 488 ± 380 *vs* 14 242 ± 339µmol Trolox eq·L^{-1} at D0 and D30 respectively; $p = 0.7$).

With the FRAP method, a significant decrease in plasma antioxidant capacity is exhibited following placebo capsule consumption (1 084 ± 43 *vs* 994 ± 27 µmol Fe^{2+}·L^{-1}; $p < 0.05$) whereas no significant modification was observed after the GE period (1 071 ± 32 *vs* 1 054 ± 32 µmol Fe^{2+}·L^{-1}; $p = 0.3$).

No significant variation of oxidized LDL concentration, catalase, and SOD activities was disclosed; whereas, such was not the case as regards glutathione peroxidase (GPx) (Table 3). This enzyme activity decreased during the placebo period ($p < 0.05$) while remaining unaltered throughout the whole grape extract intake period.

Analyses of the vitamins C and E revealed that ascorbic acid concentration was not modified during the study, whereas plasma vitamin E concentration was significantly higher after GE supplementation as compared to placebo supplementation ($p < 0.05$; Table 3).

During placebo supplementation, urinary excretion of isoprostanes was markedly increased, disclosing thereby, an increase in oxidative injury and inflammation (1.18 ± 0.11 *vs* 1.64 ± 0.18ng·mg^{-1} creatinine at D0 and D30, respectively; $p < 0.05$). Such an increase did not appear during the GE supplementation (1.24 ± 0.12 *vs* 1.26 ± 0.13ng·mg^{-1} at D0 and D30, respectively).

Skeletal muscle damage

CPK activity reached 696 ± 177 and 480 ± 81 IU·L^{-1} after placebo and GE periods, respectively ($p = 0.1$) (Table 3).

General biomarkers: No significant difference on cholesterol and triglycerides was evidenced whatever

Table 3. Body weight, plasma and urinary parameters, and relative values of effort test, before (D0) and after (D30) supplementation of GE or placebo in elite sportsmen. Values are means (± SEM) of 20 determinations performed in duplicate on samples from different subjects; n = 20 elite sportsmen.

	Placebo n=20		Grape extract n=20	
	D0	D30	D0	D30
Weight (kg)	84.4 (3.1)	83.7 (2.9)	84.8 (3.1)	84.0 (3.1)
Biomarkers of antioxidant status and oxidative stress				
ORAC (µmol·L^{-1})	14 488 (380)	14 242 (339)	13 885 (360)	14 966 (335) *#
FRAP (µmol·L^{-1} Fe^{2+})	1 084 (43)	994 (27) *	1 071 (32)	1 054 (32) #
LDLox (mU·mL-1)	481 (98)	473 (106)	556 (119)	576 (128)
SOD (U·g^{-1} Hb)	2 368 (85)	2 438 (77)	2 523 (105)	2 541 (92)
GPx (µmol·min^{-1}·g $^{-1}$ Hb)	17.1 (1.0)	15.1 (.9) *	18.9 (1.1)	17.4 (.9) #
Catalase (mol·min^{-1}.g $^{-1}$ Hb)	.625 (.039)	.560 (.051)	.653 (.030)	.673 (.057)
Vitamin E (µg·mL^{-1})	12.49 (.36)	12.50 (.46)	12.79 (.49)	13.15 (.41) #
Vit E/cholesterol ratio (µg·mg^{-1})	6.74 (.20)	6.97 (.20)	6.99 (.21)	7.24 (.25) #
Vit C (µmol·L^{-1})	62.8 (4.9)	57.6 (4.6)	55.8 (3.2)	54.5 (4.3)
Isoprostanes (ng·mg^{-1} creatinine)	1.18 (.11)	1.64 (.18) *	1.24 (.12)	1.26 (.13) #
Biomarker of skeletal muscle damage				
Creatine phosphokinase (UI·L^{-1})	529 (122)	696 (177)	605 (158)	480 (81)
General plasmatic biomarkers				
Triglycerides (g·L^{-1})	.54 (.05)	.58 (.06)	.59 (.07)	.68 (.10)
Cholesterol (g·L^{-1})	1.87 (.05)	1.81 (.05)	1.84 (.06)	1.85 (.05)
Ferritin (µg·L^{-1})	76.9 (9.7)	69.2 (9.3) *	79.0 (10.9)	69.8 (10.8) *
Urea (g·L^{-1})	.287 (.012)	.279 (.013)	.301 (.013)	.291 (.019)
Hemoglobin (g·dL^{-1})	14.7 (.1)	14.5 (.2)	14.5 (.2)	14.8 (.2) *#
Effort test				
Performance (EnRJ45, %)	-.47 (3.77)		4.59 (5.99)	
Explosive power (RJ110, %)	-1.53 (1.92)		.73 (2.65)	
Fatigue (RJ5, %)	1.64 (3.22)		9.55 (5.34)	

* Significantly different from the pre-treatment (D0); $p < 0.05$ (paired t test). # Significantly different from the placebo post-treatment (D30); $p < 0.05$ (paired t test).

the period (placebo or GE) (Table 3). Regardless of the capsule content the athletes had, no modification of urea concentration was observed; whereas, ferritin concentration was significantly reduced in both cases (Table 3). Hb concentration was not modified in the placebo group; however, GE supplementation increased Hb concentration, as opposed to placebo intake, respectively (14.8 ± 0.2 *vs* 14.5 ± 0.2 g·dL^{-1}, $p < 0.05$; Table 3).

Effort tests

All sport categories

No significant effect was found regarding total physical performance (EnRJ45), explosive power (RJ110), and fatigue (RJL5) (Table 3). More pointedly, inter-subject variations observed in the present study reduced the likelihood of establishing a significant result. Even if the intensity of each volunteer's training program was controlled and remained consistent throughout the course of our study, subjects (i.e. selected athletes) practiced a variety of elite sports: hence, training protocols had to be adjusted accordingly. In order to reduce the variability component, we decided to analyze the effort test results as a function of the following criteria: the type of training, and the sportsmanship category involved therefore. We considered that only the handball category comprised a sufficient number of volunteers to allow for statistical analysis (n = 10).

Sub-group: the handball category (n = 10): Concerning the effort test for the handball sub-group (Table 4), performance (EnRJ45) is increased significantly during the grape extract supplementation phase compared to the placebo supplementation (19.5 ± 9.7% *vs* -4.4 ± 6.1% respectively; $p < 0.05$). There was also significant evidence of an effective trend towards explosive power (RJ110) (-3.6 ± 2.5% *vs* 2.8 ± 4.3% for placebo supplementation *vs* GE supplementation respectively; $p = 0.1$). First, it is noteworthy that results on general parameters for the handball category (Table 4) are equivalent to those found in the entire subject pool (Table 3). Different correlation tests were performed among handball players: results evidenced unequivocal cross-relation between GE-induced Hb concentration increase and performance ($r = 0.717$; $p = 0.02$) and CPK results ($r = -0.67$; $p = 0.034$).

Discussion

In the present study, we hypothesized that a grape extract (GE) supplementation at physiological doses would partially avoid antioxidant system down-regulation and consequently lower chronic and/or acute exercise-induced oxidative damage in elite athletes while in competition. Moreover, oxidative stress generated under such conditions is likely to trigger oxidative skeletal muscle fatigue and damage (Barclay et al., 1991; Powers et al., 1999) which can affect exercise performance. Thus, the second objective of this study was to cross-evaluate the effect of the GE supplementation on these parameters, along with oxidative stress and the antioxidant status.

We demonstrated that ingestion of 400mg of GE per day during one month resulted in a 9.48% increase in antioxidant capacity determined by ORAC method compared to placebo ingestion. *In vitro,* ORAC values of the

Table 4. Body weight, plasma and urinary parameters, and relative values of effort test, before (D0) and after (D30) supplementation of GE or placebo in handball players. Values are means (±SEM) of 10 determinations performed in duplicate on samples from different subjects; n = 10 handball players.

	Placebo n=10		Grape extract n=10	
	D0	D30	D0	D30
Weight (kg)	88.5 (5.7)	86.5 (5.4)	88.4 (5.7)	87.1 (5.7)
Biomarkers of antioxidant status and oxidative stress				
ORAC (µmol·L^{-1})	13 678 (487)	13 946 (482)	13 336 (384)	15 132 (536) ***
FRAP (µmol·L^{-1} Fe^{2+})	1 168 (57)	988 (39) ***	1 110 (45)	1 060 (55) #
LDLox (mU·mL-1)	537 (174)	562 (189)	622 (202)	693 (229)
SOD (U·g^{-1} Hb)	2 381 (116)	2 549 (122) *	2 710 (157) ‡	2 679 (113) #
GPx (µmol·min^{-1}·g -^{1} Hb)	17.0 (1.4)	14.2 (1.5) *	18.8 (1.7)	18.0 (1.6) #
Catalase (mol·min^{-1}.g -^{1} Hb)	.580 (.056)	.545 (.090)	.640 (.044)	.681 (.088)
Vitamin E (µg·mL^{-1})	12.0 (.5)	12.0 (.7)	12.2 (.6)	12.9 (.7) ##
Vit E/cholesterol ratio (µg·mg^{-1})	6.7 (.3)	6.9 (.3)	6.9 (.4)	7.5 (.5) *#
Vit C (µmol·L^{-1})	65.8 (9.1)	55.5 (9.1)	59.3 (5.1)	54.7 (5.8)
Isoprostanes (ng·mg^{-1} creatinine)	1.3 (.1)	1.7 (.2) *	1.3 (.2)	1.3 (.2) #
Biomarker of skeletal muscle damage				
Creatine phosphokinase (UI·L^{-1})	704 (223)	725 (326)	790 (300)	464 (120)
General plasmatic biomarkers				
Triglycerides (g·L^{-1})	.47 (.04)	.58 (.07)	.53 (.05)	.66 (.10)
Cholesterol (g·L^{-1})	1.81 (.1)	1.76 (.10)	1.78 (.10)	1.78 (.10)
Ferritin (µg·L^{-1})	69.1 (14.7)	57.8 (13.3) *	68.8 (15.5)	56.8 (13.0) *
Urea (g·L^{-1})	.29 (.02)	.28 (.01)	.30 (.05)	.27 (.06)
Triglycerides (g·L^{-1})	14.6 (.20)	14.56 (.19)	14.52 (.21)	14.87 (.30) *
Effort test				
Performance (EnRJ45, %)	-4.4 (6.1)		19.5 (9.7) †	
Explosive power (RJ110, %)	-3.57 (2.50)		2.82 (4.25)	
Fatigue (RJ5, %)	2.95 (3.47)		10.85 (6.88)	

*, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, from the pre-treatment (D0) by paired t test. # and ## denote $p < 0.05$ and $p < 0.01$, respectively, from the placebo post-treatment (D30) by paired t test. † significantly ($p < 0.05$) different from the placebo relative values by paired z test. ‡ significantly ($p < 0.05$) different from the placebo pre-treatment (D0).

GE used in the present study approximates 14 000µmol Trolox eq·g^{-1} (External analysis, Lareal, France); hence, ingestion of 400mg of GE enables to increase the plasma ORAC value, matching a consumption of 5 600µmol Trolox equivalent per day. This result is congruent with the work of Cao et al. (1998), to the effect that a diet involving around 3 300µmol Trolox per day increases significantly the plasma ORAC value of the participants. Additionally, Prior et al. (2007) recently demonstrated that consumption of mixed grape powder increases dramatically post-prandial antioxidant capacity. Interestingly, the variation of the plasma antioxidant capacity determined by the FRAP value differs substantially from our findings obtained through the ORAC method. While the ORAC method allows to highlight an increase of the antioxidant capacity with GE, the FRAP method, -- considered more so as an indirect method (Prior and Cao, 1999) --, revealed that GE limited the decrease of the antioxidant capacity compared to placebo. These results evidenced that GE consumption increases the athletes' antioxidant status; they further revealed, however, that the interpretation of the changes in plasma antioxidant capacity relies for the most part on the method used, a case in point formerly demonstrated by Prior and Cao (1999).

The increase of the plasma vitamin E concentration and the preservation of the GPx levels could be explained by the increase of the plasma ORAC value and the limitation of the FRAP value reduction induced by the consumption of GE polyphenols. The same result on vitamin E was shown in endurance athletes (Vasankari et al., 1997b) or in triathletes (Palazzetti et al., 2004) after absorption of different antioxidant blends. Palazzetti and coworkers (2004) also demonstrated the significant increase of GPx activity after antioxidant supplementation compared to placebo. As regards amateur male athletes while under competition conditions, the antioxidant supplementation maintained the glutathione reductase activity in the treated group after three months; whereas, it decreased significantly in the placebo group (Tauler et al., 2006). This decrease was also exhibited in soccer players having volunteered to participate in an incremental treadmill running exercise (Klapcinska et al., 2005). This enzyme is clearly linked with GPx because it regenerates the oxidized glutathione, cofactor of GPx; it is noteworthy therefore, that there was solid evidence of enzyme activity preservation, akin to our GPx related findings. Even if the type and the dose of antioxidant supplementation differ in these studies, results are congruent with the present study. The optimization of plasma ORAC and FRAP values could also be explained by the free scavenging activity of absorbed metabolites from grapes. Plasma flavanol metabolites were not determined in our study hereby; however, numerous studies to date (Baba et al., 2002; Donovan et al., 2000; Donovan et al., 2002; Gonthier et al., 2003; Holt et al., 2002; Sano et al., 2003) substantiated the bio-availability of grape or cocoa flavanols under different bio-active forms (Spencer et al., 2001; Tomas-Barberan et al., 2007).

Other blood antioxidant determined biomarkers, enzymatic such as superoxide dismutase, catalase or non enzymatic such as vitamin C, did not differ significantly whatever the period. These results are in agreement with

those of Palazzetti et al. (2004) and Margaritis et al. (2003); the latter revealed no variation in vitamin C and SOD in supplemented triathletes compared to placebo. Concerning catalase activity, Tauler et al. (2003) studied its variation on athletes supplemented with vitamin C, or with a blend of vitamin E, vitamin C and beta-carotene (Tauler et al., 2006). In both cases they exhibited an increase in catalase activity that was not disclosed in our study.

One of the biomarkers most commonly investigated within biological systems remains the degree of lipid peroxidation; to such an end, several methods may be implemented (Slater, 1985; Vasankari *et al.*, 1995). In the present study, lipid peroxidation was determined in blood, using specific autoantibodies against oxidized LDL and in urine using the ELISA method to determine isoprostanes. No effect was shown on oxidized LDL whatever the period, whereas significant results were obtained for isoprostane urinary excretion. Urinary isoprostane values were significantly increased in the placebo group, but were not modified in the GE group. Therefore, compared to the placebo, GE limited the production of isoprostanes significantly after the administration period. Oxidized LDL results are in agreement with results released by Vasankari et al. (1997b; 1997c); however, oxidized LDL results are not consistent with some studies showing an increase of this parameter in soccer and basket-ball players (Pincemail et al., 2000), in weekend warriors (Chang et al., 2002) or a decrease in sedentary volunteers (Vasankari et al., 1998a) after intense exercise. To our knowledge, no existing study has evidenced as yet, a decrease of oxidized lipoproteins following antioxidant supplementation in elite athletes.

Isoprostanes are specific end-products of non enzymatic free radical catalyzed oxidation of arachidonic acid. It is believed that isoprostanes are formed while still esterified to phospholipids in cellular membranes and are released through the action of phospholipase to circulate freely in the body (Mastaloudis et al., 2001; Morrow et al., 1992; Watson et al., 2005). Quantification of isoprostanes has been referred to as the ultimate criterion for *in vivo* lipid peroxidation and oxidative stress, notably in acute or chronic inflammatory conditions (Basu, 2008; Pratico et al., 1998). An increase in isoprostane concentration has been reported in athletes during exercise in several studies (Mastaloudis et al., 2001; Steensberg et al., 2002) showing induced-exercise oxidative stress. The effect of antioxidant supplementation on this biomarker has been probed and widely documented as regards smokers, menopausal women or patients with degenerative diseases (Abu-Amsha Caccetta et al., 2001; Devaraj et al., 2007; Wiseman et al., 2000; Zern et al., 2005). Effects on athletes, however, remains subject for debate (Mastaloudis et al., 2004; McAnulty et al., 2005; McAnulty et al., 2008). The present trial is the first one ever to substantiate evidence that a GE supplementation during one month optimizes significant limitation of exercise-induced urinary isoprostanes production. As for plasma FRAP and ORAC values, this result is probably explained by the significant increases of vitamin E concentration, of GPx activity and by the presence of flavanol metabolites in the body.

Numerous studies have reported that exogenous antioxidants protect against skeletal muscle fatigue (Barclay and Hansel, 1991; Diaz et al., 1994; Khawli and Reid, 1994; Novelli et al., 1990; Reid and Moody, 1994; Shindoh et al., 1990; Travaline et al., 1997). Most studies, however, have applied synthetic or enzymatic antioxidants and resorted to *in vitro* muscle preparations to quantify muscular contractions (Barclay and Hansel, 1991; Diaz et al., 1994; Khawli and Reid, 1994; Reid and Moody, 1994; Shindoh et al., 1990).

The determination of serum creatine phosphokinase (CPK) is used as an indirect index of exercise-induced muscle damage (Chevion et al., 2003) and is associated indirectly with the increment of the permeability in the muscle cell membrane (Janssen et al., 1989). This permeability is produced by the oxidative stress induced by physical exercise (Child et al., 1999; Palazzetti et al., 2003; Van der Meulen et al., 1991). Morillas-Ruiz et al. (2006) showed that consumption of a natural antioxidant beverage containing 1.2g per liter of polyphenols by cyclists increased the serum CPK concentration similarly to the placebo beverage suggesting that the antioxidant capacity of polyphenols has no repercussion on cellular muscle damage. The same result was obtained by Helgheim et al. (1979) with a vitamin E supplementation and by Margaritis and coworkers (2003), on triathletes supplemented with an antioxidant blend. Contrariwise, while using the same sportsmen and supplementation the latter revealed a significant reduction of the magnitude in duathlon-induced creatine kinase isoenzyme mass increase during normal and overload training (Palazzetti et al., 2004). Branched-chain amino acids (Coombes and McNaughton, 2000), vitamin C (Jakeman and Maxwell, 1993), allicin (Su et al., 2008) or CoQ10 (Kon et al., 2008) supplementations on different volunteers generated the same result: a lower CPK activity as compared to the control group. Because of the supplement antioxidant effects, muscle membrane integrity should be maintained, preventing the release of enzymes into blood circulation. All these results are in agreement with the findings presented here, hereby exhibiting a trend towards decreasing the serum CPK concentration among elite athletes involved in competition while absorbing the grape extract, as compared to placebo intake. Such a trend ($p = 0.09$) results from a very high variability already reported by Hartman and Mester (2000): authors observed that athletes with chronically low CPK exhibited mainly low variability; those with chronically higher values exhibited considerable variability. Nevertheless, this is the first time that this modification has been reported under GE supplementation.

Urea, ferritin and Hb markers are commonly used to determine overtraining signs (Aissa Benhaddad et al., 1999; Hartmann and Mester, 2000; Spodaryk, 2002). No modifications appeared in urea concentration, one nitric substance considered as a marker of protein catabolism. Differently, ferritin concentrations are significantly decreased whatever the period. This decrease of ferritin concentration is well-defined during training associated with the competition season or overtraining (Aguilo et al., 2004; Aissa Benhaddad et al., 1999; Diehl et al., 1986; Spodaryk, 2002). Whereas, we showed no difference in

ferritin concentration between the placebo and the GE supplementations. Aguilo et al. showed in 2004 that supplementation with vitamins E, C and beta-carotene prevented the decrease of serum iron and the iron saturation index. Such a difference in results may result from the type of supplementation, the dose and/or the used model: amateur trained male athletes supplemented with 1.530g per day of pure vitamins for Aguilo and co-workers or elite trained male athletes supplemented with 400mg per day of a complex botanical extract for the present study.

Several authors have described a significant increase in the destruction of the red blood cells after intensive exercise (Gilligan et al., 1943; O'Toole et al., 1988). One of the causes for this hemolysis is the fact that after strenuous exercise, the red blood cells are more responsive to stress, such as oxidative stress (Smith et al., 1995). Hb represents more than 97% of the dry content in the red blood cells, being the normal concentration in sedentary men between 13.5 and 16.5 $g \cdot dL^{-1}$ (Dominguez de Villota et al, 1981). In the present study, Hb was increased by 0.3 $g \cdot dL^{-1}$ among elite athletes after GE administration. Other studies on sportsmen and antioxidant supplementation yielded no variation of this parameter (Aguilo et al., 2004; Morillas-Ruiz et al., 2006). Our results might suggest that the increase of blood antioxidant capacity, induced by GE, protected red blood cells against free radicals generated under competition conditions, but further research is needed in order to confirm this hypothesis.

Recent publications demonstrated that total physical performance, average explosive power, capacity to maintain a maximal explosive force, or fatigue can be evaluated with confidence according to trial programs measuring flight times. These programs consist of series of individual Counter Movement Jumps (CMJ) or Rebound Jumps (RJ). These methods have been validated with different computerized systems measuring physical performance (García-López et al., 2005) and have been used as predictive tools to evaluate performance in sprint (Smirniotou et al., 2008), handball (Buchheit et al., 2009), volleyball (Newton et al., 2006), or soccer (le Gall et al., 2008) disciplines. Moreover, they were dedicated to the assessment of the response from the neuromuscular system after a treatment of 8 weeks of whole-body vibrations compared to the same period without treatment (Di Giminiani et al., 2009). They were also used to determine the force production capability after anterior cruciate ligament reconstruction (Flanagan et al., 2008). In our study, we did not find a significant improvement on any physical parameter when evaluating all disciplines together. The performance evaluation was not the prime criterion in this study, and consequently was not taken into account to determine the type of sportsmen to include and the sample size. These choices probably explain the variability obtained on the effort test and better results could be obtained with a bigger population comprising just one type of sport. When analyzing each category, except volleyball (n = 1), only the handball players group, including 10 sportsmen, showed a significant enhancement of overall physical performance (EnRJ45) and a trend to improve explosive power (RJ110) after one month of GE supplementation compared to the placebo.

In the handball players group, we found that there are two factors directly correlated with the enhancement of physical performance: plasmatic CPK concentration, and Hb levels. This evidence suggests for the first time that the protective effect of GE against oxidative stress might help to enhance physical performance. In fact, there is little evidence indicating that specific supplementation with dietary components such as vitamins and minerals improve performance (Maughan, 1999). Several studies have investigated the effect of vitamin E on exercise performance and have showed that such supplementation protects against exercise-induced muscle damage but has no effect on performance (Lawrence et al., 1975a; Lawrence et al., 1975b; Sharman et al., 1976; Sharman et al., 1971). The same conclusion can be reached with vitamin C (Keith and Driskell, 1982; Keith and Merrill, 1983; Keren and Epstein, 1980). Using a cysteine donor to boost endogenous glutathione synthesis, Lands et al. (1999) had reported an improvement in human exercise tolerance during short-duration exercise. Until now, only one study conducted with flavanol polymers collected from pine bark demonstrated that these polyphenols improved endurance among healthy regular runners (Pavlovic, 1999). Thus, the present trial ranks first insofar as it provides breakthrough evidence as regards performance enhancement under GE supplementation. Such evidence was obtained by using a subclass from our pool of subjects depending on sports category, so as to reduce the inter-variability index generated by the effort trial. Indeed, inter-subject variation observed in the present study with the effort test reduced the likelihood of establishing a significant result. Even if the intensity of the training of each volunteer was controlled and remained consistent throughout our study, subjects took part in different elite sports category and consequently, had different types of regular training. Thus, the type of the training associated with genetic predisposition is a contributory factor for the observed inter-subject variability during the effort test.

Conclusion

In summary, this trial is the first showing that consumption of GE standardized in flavanols permits to ameliorate the oxidative stress/antioxidant status balance in elite athletes during a competition period, and to enhance physical performance in one category of sportsmen (handball). The administration of GE seems to protect cells against oxidative stress damage, as suggested by the decrease in CPK concentration and the increase of Hb levels in plasma. In fact, these two biomarkers are correlated with the enhancement of performance in handball players. This evidence suggests that the enhancement in performance might be caused, at least in part, by the protective action of GE during physical exercise. Further studies have to be conducted in order to confirm the link between the oxidative stress/antioxidant status balance, cellular protective action, and performance enhancement effects caused by the consumption of GE in elite and occasional athletes.

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Competing interests

Naturex is involved in research/development and marketing/sales of Powergrape® as an antioxidant and tonic ingredient. The general goal of NAT'Life division of Naturex is to develop botanical extracts with scientifically proven well-being benefits. Powergrape® consists of a grape extract standardized and patented. Therefore Naturex has a commercial interest in this publication. Avantage Nutrition, the conducting laboratory, was paid by Naturex to perform and report the scientific wok which formed the basis of the publication. Avantage Nutrition and Naturex declare that the data presented in this publication represent a true and faithful representation of the work performed.

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Key points

- Grape extract consumption improves the oxidative stress/antioxidant status balance in sportsmen.
- Grape extract consumption enhances physical performance in one category of sportsmen (Handball).
- The performance enhancement might be caused by the protective action of grape extract during physical exercise.

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