

BLACK GRAPE EXTRACT SUPPLEMENTATION ATTENUATES BLOOD OXIDATIVE STRESS IN RESPONSE TO ACUTE EXERCISE

■ Accepted
for publication
10.08.2007

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ABSTRACT: The flavonoid-rich active compound in grapes is claimed to be one of the most important natural products. Hence, the objective of our research was to study parameters of the prooxidative-antioxidative balance in athletes supplied with Panace-Vid 2000® preparation consisting of black wine grape extract (*Vitis vinifera*). The study was carried out on 22 male rowers. The subjects from the supplemented group (n=10) were given one gelatin capsule containing Panace-Vid 2000® three times a day, for six weeks, while the control group (n=12) was given placebo. Before and after the supplementation period, the athletes performed a physical exercise test on the rowing ergometer; varying between 40 and 90% of maximal aerobic power. Each 3-min exercise session was followed by thirty seconds of rest. Blood was sampled from the rowers before the exercise test, one minute after its completion, and after a 24-h recovery period. The activity of antioxidative enzymes (superoxide dismutase, glutathione peroxidase) was determined and the concentration of thiobarbituric acid-reactive substances was measured in the hemolysate of red blood cells. The Total Antioxidant Capacity was determined in the blood plasma. The concentration of lactic acid was measured in the whole blood. An analysis of the results revealed that the supply of grape extract, in the form of Panace-Vid 2000® preparation, contributed to a significant increase in plasma antioxidative capacity and to an insignificant increase in superoxide dismutase, as well as a lower activity of glutathione peroxidase and reduced concentration of lipid peroxidation product levels.

KEY WORDS: antioxidant enzymes, TBA-reactive products, flavonoids, physical exercise

INTRODUCTION

Intensive physical exercise has been classified as one of the factors inducing disturbances in the prooxidative-antioxidative balance, which may lead to impairment in basic cellular structures [10,36,40,41]. Under physiological conditions, a balanced diet is sufficient to keep the prooxidative-antioxidative balance of the body, while an increase in the level of prooxidants during intensive physical exercise points to a failure of the antioxidative system. In order to counteract such unfavorable phenomena, antioxidants (e.g., vitamin C, E, β carotene) can be used to neutralize reactive oxygen species (ROS), and thus protect cellular structures against damage.

Recent epidemiological studies [16,17,18] have pointed to a significant relationship between the intake of polyphenols and a low risk of neoplasia and heart diseases. Evidence suggests that polyphenols contained in red wine, play an essential role in stabilization of the adequate oxidoreductive potential in cells by neutralizing metabolic ROS [9,12,23,24,26,29,37].

However, the presence of alcohol in red wine is a factor which limits its consumption. Therefore, many researchers [7,12,18,23,24] use wine grape extract in treatment and prevention of diseases of

free radical etiology. Natural extract from black grape peels and seeds is characterized by a high content of biologically active substances, such as anthocyanins, procyanidins, flavonoids, and resveratrol. These substances exert a protective effect by scavenging free radicals [7,25], chelation of metals, mainly copper and iron [3,24], and inhibiting oxidation of endogenous antioxidants (vitamins E and C) [26].

With this information in mind, we conducted a study to determine the influence of the six-week period of supplementation with Panace-Vid 2000®, containing black wine grape peel and seed extract (*Vitis vinifera*), on the prooxidative-antioxidative balance in rowers who performed a physical exercise test.

MATERIALS AND METHODS

The subjects were 22 male members of the Polish Rowing Team, who took part in a six-week training camp between the preparation and competition periods. The subjects' characteristics are presented in Table 1. The subjects under the study were randomly assigned to Panace-Vid 2000® preparation - black wine grape peel and seed

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extract (*Vitis vinifera*) (the supplemented group, $n=10$), or to the placebo (the control group, $n=12$). The rowers in the supplemented group were given one capsule of Panace-Vid 2000® (produced by Valefarma S.L., Spain), three times a day, for six weeks. One gelatin capsule of 367 mg contained 188 mg/g of polyphenols (catechin, gallic acid, quercetin, trans-resveratrol, cis-resveratrol) and 35 mg/g of anthocyanins (malvidin, peonidin, petunidin, delphinidin, cyanidin). The rest of the preparation was composed of auxiliary substances: magnesium stearate and calcium carbonate. At the same time and with the same dosage regime, the subjects from the control group received dyed gelatin capsules containing maltodextrin.

Every day over a week the subjects were to fill in their food intake questionnaires, which allowed us to calculate the energy equivalent of the food rations and the content of antioxidant vitamins. Data concerning the daily energy and antioxidant vitamin intake in the supplemented and control groups are given in Table 2, according to Tables of Composition and Nutritional Value of Foodstuffs [22].

On the first day (before supplementation) and at the end of the training camp (after supplementation), the athletes performed a controlled 2000-m rowing exercise test (Concept II, USA) in the fastest possible time. The maximal power achieved during the tests amounted to 450-500 W. The day after the 2000-m test the athletes were told to abstain from physical exercise, and on the following day they performed an incremental rowing ergometer exercise (Concept II, USA). The initial workload was 40% of maximal power achieved during the preceding 2000-m test (100% VO_{2max}). The workload was increased every three minutes to 50, 60, 70, 80 and 90% of the maximal power. Each 3-min exercise session was followed by thirty seconds of rest.

Blood samples for redox parameters were taken from an antecubital vein, with calcium disodium - before each incremental exercise test (in the morning, after an overnight fast), 1 minute after the test completion, and following the 24-h recovery period. Samples were centrifuged immediately to separate red blood cells from plasma. Packed erythrocytes were washed three times with saline and lysed with ice-cold, redistilled water. Plasma and lysed erythrocytes were frozen immediately and stored at $-28^{\circ}C$ until use (up to one week).

TABLE I. BASIC CHARACTERISTICS OF THE STUDIED GROUPS (MEANS \pm STANDARD DEVIATIONS)

| Parameters | Supplemented group (n=10) | Control group (n=12) |
|-----------------------------------|---------------------------|----------------------|
| Age (years) | 20.4 \pm 1.1 | 20.6 \pm 1.2 |
| Body mass (kg) | 82 \pm 8.7 | 83.3 \pm 8.2 |
| Body height (cm) | 187.9 \pm 5.0 | 191.1 \pm 5.4 |
| Years of training (years) | 6.7 \pm 1.1 | 6.2 \pm 1.6 |
| Sport class (number of subjects): | | |
| Country Master Class | 7 | 8 |
| Class I | 3 | 4 |

Additionally, capillary blood samples were taken by fingerprick before and after each exercise test to assess the lactate levels (LA).

The Total Antioxidant Capacity (TAC), according to Miller et al. [31], used as the overall measure of plasma antioxidant capacity, was assessed using commercial kits (Randox-TAS, Cat No. NX 2332, UK).

The superoxide dismutase (SOD) activity was measured in washed erythrocytes after their lysis, by means of commercial kits (Randox-Ransod, Cat No. SD 125, UK). The superoxide dismutase activity was expressed in U/gHb.

The glutathione peroxidase (GPx) activity in the hemolysate samples was measured using commercial kits (Randox-Ransel, Cat No. RS 506, UK). According to the method of Paglia [34] and glutathione peroxidase activity was expressed in U/gHb.

The concentrations of the thiobarbituric acid reactive substances (TBARS) in the hemolysate samples were assessed as a measure of oxidative damage to red blood cells. TBARS concentrations were evaluated with the method described by Buege [4] involving the acidic breakdown of lipid peroxides into malonaldehyde molecules. The concentrations of TBARS (malondialdehyde equivalents) were expressed as $\mu\text{mol/gHb}$.

The concentration of hemoglobin was assessed using the cyanmethemoglobin method with the Drabkin's reagent. The results were expressed as g/100 mL.

The lactate levels in capillary blood were determined immediately after the collection of the samples using a diagnostic kit (Dr Lange, Cat No. LKM 140, Germany). The lactate concentration was expressed in mmol/L.

Statistical analyses were performed with STATISTICA v. 6.0 software package. The normally distributed data (TAC, TBARS) were compared using 2 (supplemented and placebo groups) \times 3 (times of measurement) repeated measures analysis of variance (ANOVA). When significant changes were observed in ANOVA tests, these data were also analyzed by paired and unpaired Student's t-tests, with Scheffe's post-hoc test for multiple comparisons. The data without normal distribution (SOD, GPx, LA) were analysed with nonparametric tests. The Mann-Whitney test was used to compare mean values between the two groups, and the data within each group were analyzed with the Wilcoxon test. All values were reported as mean \pm SD. Statistical significance was set at $p<0.05$.

RESULTS

The results of the study are summarized in Tables 1-3. Table 1 presents basic characteristics of all the studied athletes: age, body mass, body height, years of training. The anthropometric profile of the rowers was similar in both groups.

The average daily intake of energy and antioxidants is shown in Table 2. No significant dietary differences were observed between the supplemented and control groups. The diet for both studied groups was composed in accordance with the dietary recommendations for subjects with high-energy expenditure [46].

TABLE 2. DAILY ENERGY AND ANTIOXIDANT VITAMIN INTAKE IN THE SUPPLEMENTED AND THE CONTROL GROUPS (MEANS ± STANDARD DEVIATIONS)

| | Supplemented group | Control group | Student's t-test |
|--------------------|--------------------|---------------|------------------|
| Kcal | 4276±780 | 4079±941 | n.s. |
| Beta-carotene (IU) | 4242±1494 | 4570±1230 | n.s. |
| Vitamin C (mg) | 113±45 | 105±32 | n.s. |
| Vitamin E (mg) | 9±2 | 10±2 | n.s. |

n.s. - difference non-significant (p<0.05)

Athletes participating in the study during the 2003/2004 season had the following sports achievements:

- lightweight quadruple sculls, gold medal at Junior World Championships,
- quadruple sculls, silver medal at Junior World Championships,
- pairs without cox, bronze medal at Junior World Championships

A comparable analysis of the results obtained before and after supplementation within each group is presented in Table 3. The following parameters were significantly changed in the second phase of the study (in comparison with the first phase) in the supplemented group: TAC of the plasma increased at rest (p<0.05); lactic acid concentration increased after the exercise test (p<0.05); the GPx activity decreased after the 24 h recovery period (p<0.05); the TBARS concentration decreased (p<0.05), and the TAC increased

(p<0.05). Table 3 also shows post exercise changes in the studied parameters with reference to the values obtained at rest (p<0.05).

Table 3 contains a comparable analysis of mean values of the oxidative stress parameters investigated in the athletes from both groups during the two phases of the study. In the first phase (before the supplementation), an ergometric physical exercise test at an intensity increasing up to 90% of the maximal power induced changes in the concentrations of the investigated parameters which were similar in both groups. In the second phase (after the supplementation), significantly higher values of the total antioxidative status of plasma (TAC) were found in the group supplemented with Panace-Vid 2000®.

In the control group, no statistically significant differences were found between the two phases of the study as regards mean values of all the parameters investigated.

DISCUSSION

Physical exercise, especially of high intensity, is generally recognized as a factor inducing an oxidative stress, while the degree of prooxidative changes is determined by the exercise intensity and time course [2,28]. This was documented by Goto et al. [10], who applied a cycloergometer physical exercise test in a study of three groups of young men, with the exercise intensity of 25, 50 and 75% of VO2max, respectively, for a 12-week period. The increase in the prooxidative

TABLE 2. THE BALANCE BETWEEN OXIDANTS AND ANTIOXIDANTS BEFORE AND AFTER SUPPLEMENTATION WITH THE VITIS VINIFERA EXTRACT - COMPARISONS WITHIN THE SUPPLEMENTED AND THE CONTROL GROUPS (MEANS ± STANDARD DEVIATIONS)

| Parameter | Supplemented group | | Statistical significance | Control group | | Statistical significance |
|-------------------------|--------------------|--------------|--------------------------|---------------|--------------|--------------------------|
| | Before suppl. | After suppl. | | Before suppl. | After suppl. | |
| GPx (U/gHb) | | | | | | |
| At rest | 45±10.6 | 42±9.6 | n.s. | 48±11.4 | 41±15.2 | n.s. |
| After ET | 49±10.4 | 48±8.1 | n.s. | 50±12.4 | 44±9.8 | n.s. |
| 24 h after ET | 48±7.3 | 39±9.1 | * | 44±7.7 | 41±6.2 | n.s. |
| SOD (U/gHb) | | | | | | |
| At rest | 1269±136.4 | 1283±75.2 | n.s. | 1234±114.8 | 1244±75.2 | n.s. |
| After ET | 1288±107.1 | 1309±165.9 | n.s. | 1447±197.5† | 1334±119.8† | n.s. |
| 24 h after ET | 1426±100.8† | 1398±126.5 | n.s. | 1487±136.9† | 1465±119.8† | n.s. |
| TAC (mmol/L) | | | | | | |
| At rest | 0.95±0.11 | 1.16±0.13 | * | 0.95±0.16 | 0.86±0.12 # | n.s. |
| After ET | 0.95±0.16 | 0.98±0.06† | n.s. | 0.93±0.14 | 0.82±0.07 # | n.s. |
| 24 h after ET | 0.91±0.08 | 1.18±0.10 | * | 0.89±0.15 | 0.85±0.08 # | n.s. |
| TBARS (µmol/gHb) | | | | | | |
| At rest | 1.2±0.06 | 1.3±0.09 | n.s. | 1.2±0.13 | 1.2±0.08 | n.s. |
| After ET | 1.5±0.32† | 1.6±0.31† | n.s. | 1.4±0.26† | 1.6±0.30† | n.s. |
| 24 h after ET | 2.1±0.44† | 1.7±0.34† | * | 1.9±0.17† | 1.8±0.42† | n.s. |
| LA (mmol/L) | | | | | | |
| At rest | 1.5±0.25 | 1.4±0.26 | n.s. | 1.6±0.25 | 1.6±0.56 | n.s. |
| After ET | 7.5±1.93† | 10.6±2.76† | * | 10.5±3.49† | 9.6±2.42† | n.s. |

Legend: The compared values were collected at rest (At rest), 1 min after the exercise test (After ET), and 24 h after the exercise test (24 hrs after ET). GPx - glutathione peroxidase; SOD - superoxide dismutase; TAC - Total Antioxidant Capacity; TBARS - thiobarbituric acid reactive substances; LA - lactic acid. n.s. - difference non-significant; * - difference significant at p<0.05. † - significantly different from rest value (p<0.05), # - difference between the supplemented and the control groups significant (p<0.05)

processes was only noted in the group loaded with a high intensity exercise.

In our study, performed on rowers of the Polish National Rowing Team during a training camp between the preparation and competition periods, the ergometric exercise test was applied, and its intensity increased from 40 to 90% of maximal power. This test appeared to have a considerable anaerobic component of the metabolism covering the energetic load of exercise. This was indicated by high concentrations of lactate in blood, between 7.5 to 10.6 mmol/L. In the supplemented group at the 2nd stage of study compared with the 1st stage of study exercise induced lactate concentration increased by about 3 mmol/L. According to Weltman [42] lactate concentration in blood during exercise with increasing intensity is not an indicator of oxidative efficiency; nevertheless decreased concentration of this metabolite in blood is observed at low muscular glycogen (Table 3). It should be stressed that the described supplementation in parallel with the realized resistance training resulted in an increase of both physical performance and tolerance to acidifying in the supplemented group; whereas no changes of these parameters were noted in the control group. Such high anaerobic metabolism suggested that the oxygen requirements during physical exercise in athletes under study were covered insufficiently, which eventually caused hypoxia. During hypoxia, the catabolism of ATP exceeds its resynthesis, which leads to accumulation of AMP, to be later deaminated in muscles into IMP, and then, after subsequent reactions, inosine and hypoxanthine are synthesized. Moreover, the increase in the free calcium concentration observed during hypoxia [8] brings about an intensified activity of the protease stimulating conversion of xanthine dehydrogenase into the oxidase form that oxidizes hypoxanthine to xanthine and subsequently to uric acid [11,13,14,15,20,38]. In reactions of oxidation of hypoxanthine to xanthine and uric acid, under hypoxia, the molecular oxygen becomes the acceptor of electrons, and, as a result of univalent or bivalent reduction, it is converted into the superoxide anion (O_2^-) or the hydrogen peroxide, respectively [19,30].

In the present paper, the intensity of the generation of free radicals during the exercise was indicated by the increase (by 25 and 16% after exercise, and a further increase by 75 and 58% during the recovery period) in lipid peroxidation products reacting with the thiobarbituric acid (TBARS) in both groups, in the first phase of the study (Table 3). In the second phase, after 24-h of recovery, the TBARS concentration was significantly lower in comparison to the first phase, in the group supplemented with *Vitis vinifera* extract, while changes in the control group were non-significant (Table 3). These favorable changes may be attributed to polyphenols contained in the preparation. The Panace-Vit 2000 preparation containing 188 mg/g of polyphenols and 35 mg/g of anthocyanins, taken by the studied rowers, corresponds to a daily dose of 200 ml of red wine. For people under high oxidative stress similar doses of red wine were proposed by Abu-Amsha Caccetta et al. [1], Serafini et al. [39], and

Young et al. [45]. Moreover, Yamakoshi et al. [44] studied the influence of this preparation and provided laboratory animals with doses of 4 g of the grapes extract per 1kg of body mass and observed no toxic effects. The study by Maffei Facino et al. [24] supports also the above conclusions. The cited authors have shown that procyanidins contained in the seeds of *Vitis vinifera* grapes influence not only the limitation of the xanthine oxidase activity, but also inhibit (depending on the amount) iron-catalysed peroxidation of lipids in phosphatidylcholine liposomes. In the latter function, these substances demonstrate a higher activity than catechins, which were used as the reference substance in the cited study.

During the post-exercise recovery period, the prooxidative-antioxidative balance was less impaired. This was indicated by a significantly lower activity of the glutathione peroxidase in the supplemented group, in the second phase of the study (Table 3).

The glutathione peroxidase degrades the hydrogen peroxide, which is generated in the reaction of superoxide anion dismutation, catalyzed by SOD. The major source of the superoxide anion during physical exercise is the xanthine oxidase [13,15]. The picture of changes established in our study showed a favorable influence of the preparation tested on the activity of the cellular antioxidative system. This conclusion may be supported by the experimental research of Cui et al. [7]. These authors demonstrated that standardized grape extract could directly scavenge both superoxide and hydroxyl radicals.

The extract from grapes administered in this study to the supplemented group during six weeks also improved the antioxidative capacity of plasma, expressed as a higher TAC level. It should be emphasized that after the exercise, lower levels of this parameter were noted in both groups under study (Table 3). It may suggest a reduction in the antioxidative defense system in the plasma. However, in comparison to the control group receiving placebo, the TAC levels in the group supplemented with the preparation were significantly higher at rest, after the physical exercise test as well as after the 24-h recovery period (Table 3).

The decrease in the total level of antioxidants in the plasma after intensive physical exercise has been reported in previous studies [27,35]. This points to the essential role of plasma antioxidants in maintaining the prooxidative-antioxidative balance. Therefore, addition of antioxidants contained in black grapes to the diet seems to be favorable in strengthening the antioxidative defense. Other authors, such as Lopez-Velez et al. [20], Maxwell et al. [29], or Whitehead et al. [43], suggested that moderate quantities of red wine be introduced in the diet to increase the total antioxidant status of the plasma. Such recommendations are supported by findings by Noroozi et al. [33], who presented direct evidence of the contribution of flavonoids in inhibiting the oxidation of ascorbate into dehydroascorbate. Moreover, it has been established that ascorbic acid may reduce tocopheryl radical to tocopherol [32] and also inhibit oxidative metabolism of flavonoids [33]. Buettner [5] proved that the sequence of "consumption" of individual antioxidants in the pla-

sma under oxidative stress was not equal. First ascorbate and thiol groups of proteins are used, followed by bilirubin, uric acid, and alpha-tocopherol. This observation may be used to explain an increase in ascorbic acid and alpha-tocopherol concentrations in rats supplemented with red wine, reported by Cestaro et al. [6]. On the other hand, studies by Eder et al. [9] showed a decrease in the activity of antioxidative enzymes administration followed by vitamin E. These authors suggest that the raised level of vitamin E have a considerable impact on the endogenous antioxidative system, which may be defined as a lower level of antioxidative enzymes in the blood. Finally, Eder et al. [9] suggest that enhancing a properly composed diet (Table 2) for high-energy expenditure subjects with

black grape peel and seed extract (in daily doses amounting up to 564 mg of polyphenols and 105 mg of anthocyanins), contributed to an increased TAC level in the plasma and a decreased frequency of damages to erythrocyte lipids produced during intensive physical exercises.

CONCLUSIONS

The results of this study support previous reports that polyphenols contained in black grape peel and seed extract enhance the endogenous antioxidative system by reducing proportions between amounts of prooxidants generated during intensive physical exercise and the level of endogenous antioxidants.

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