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# Effect of a special carbohydrate–protein bar and tomato juice supplementation on oxidative stress markers and vascular endothelial dynamics in ultra-marathon runners

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ABSTRACT

It is well established that exercise induces excessive production of reactive species leading to oxidative stress, which has been implicated in oxidative damage of macromolecules, immune dysfunction, muscle damage and fatigue. The present study examined the effect of supplementation of ultra-marathon runners for a two-months-period with a special whey protein bar containing carbohydrates and protein in a specific ratio (1:1) ($N = 16$), prepared using as starting material the by-products of cheese manufacturing, and supplementation with commercially available tomato juice ($N = 15$). Thiobarbituric-acid reactive substances and protein carbonyls were significantly decreased in both supplementation groups, while a pronounced increased in reduced glutathione was observed in the protein bar group. Total anti-oxidant activity remained unchanged in both groups. Flow-mediated dilatation, used as an estimate of endothelial function, was increased in both groups, with a significant rise observed only in the tomato juice administration group. In conclusion, supplementation of ultra marathon runners for a two-months-period with a special protein bar and tomato juice significantly improved the oxidative status of the subjects, while tomato juice also improved vascular endothelial function in these athletes.



## 1. Introduction

It is well established that exercise induces excessive production of reactive species leading to oxidative stress, which has been implicated in oxidative damage of macromolecules (Galhardi et al., 2009; Veskoukis et al., 2008), immune dysfunction (Schneider and Tiidus, 2007), muscle damage (Nikolaidis et al., 2007) and fatigue (Betters et al., 2004). During prolonged and high-intensity exercise, the disturbance of intracellular milieu, the mechanical stress and the potential risk of free radical formation are more pronounced (Dantas de Lucas et al., 2013). Marathon and ultra-marathon runners are particularly susceptible to oxidative stress and muscle damage (Gomez-Cabrera et al., 2006; Kaikkonen et al., 1998) generating the need for methods to counteract these adverse effects. At the same time, despite the favorable effects of exercise on endothelial function, vascular endothelial dysfunction is currently being recognized as a growing entity in prolonged endurance exercise (Jee et al., 2013).

Improving athletic performance via supplementation of beverages, most often, rich in carbohydrates, creatine and proteins/aminoacids has been used with non-consistent effects on performance (Kerasioti et al., 2012; Baty et al., 2007; Betts et al., 2007; Haff et al.,

*Abbreviations:* CK, creatinine kinase; DNPH, 2,4-dinitrophenylhydrazine; DPPH, 2,2′-diphenylpicrylhydrazyl; DTNB, 5,5′-dithiobis(2-nitro-benzoic acid); FMD, flow-mediated dilatation; GSH, redused glutathione; HCl, hydrochloric acid; HDL, high density lipoprotein cholesterol; HR, heart rate; LDL, low density lipoprotein cholesterol; $Na_2SO_4$, sodium sulfate; NO, nitric oxide; PBS, phosphate buffered saline; TAC, total antioxidant capacity; TBARS, thiobarbituric-acid reactive substances; TCA, tricholoroacetic acid; Tris, (hydroxymethyl)-aminomethane.

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2000). Recently supplementation of athletes with whey products and/or tomato juice, a well-known source of lycopene, has started to become popular. Little is known whether whey-derived bioactive peptides regulate vascular endothelial function (Ballard et al., 2013), while tomato juice consumption has proven effective in ameliorating lactate dehydrogenase and creatinine kinase responses to anaerobic training in anaerobically trained athletes (Tsitsimpikou et al., 2013).

The present study examined both the effect of a special bar containing carbohydrates and whey protein in a specific ratio (1:1), prepared using as starting material the by-products of cheese manufacturing, and the results of supplementation with commercially available tomato juice on oxidative stress and endothelial function of ultra-marathon runners by monitoring a constellation of oxidative stress and endothelial dysfunction parameters.

## 2. Materials and methods

### 2.1. Subjects

The demographic and other characteristics of the population study are presented in Table 1.

All subjects were non-smokers, had no medical history of hypertension and were not receiving anti-hypertensive or anti-inflammatory medication. A written informed consent to participate in the study was provided by all participants involved in the study. The procedures were in accordance with the Helsinki declaration of 1975 and approval was received by the human subjects committee of the University of Thessaly and the General Hospital of Giannitsa, where all medical practices were conducted.

### 2.2. Diet and activity before the experiment

The subjects underwent full echocardiographic assessment and stress test at baseline. They were instructed to follow their usual eating habits during the days before the experiment. The subjects enrolled in the study groups were instructed to replace their usual carbohydrate supplementation beverage, regularly consumed during and post-exercise sessions, with the special bar studied (protein bar $N = 16$) or with a tomato juice (tomato juice $N = 15$) commercially available with an equivalent nutritional value (see Table 2) for 2 months. The subjects were left free to consume equal to their usual carbohydrate supplementation amount of tomato juice or were eating 2 special bars per day. The control group continued any supplementation consumption they had. They were interviewed by the attending physician/cardiologist on their diet habits (Table 3) and a full medical history was recorded.

### 2.3. Measurement of endothelial function of the brachial artery

Flow-mediated dilatation (FMD) is used as an estimate of endothelial function. FMD was measured in all participants, in the morning at least 15 h post any training session, both during pre- and post-supplementation period, by high-resolution vascular ultrasound (Agilent Sonos 5500, Hewlett–Packard, Andover, Mass) according to guidelines (Corretti et al., 2002). Briefly, endothelium dependent FMD was assessed by measuring the changes in the diameter of the brachial artery for 2 min after reactive hyperemia for 5 min. Flow-mediated dilatation was defined as the maximum percentage change in brachial artery diameter compared with baseline; that is, FMD = [(postocclusion diameter − resting diameter)/resting diameter] × 100. Analyses were conducted offline by 2 different investigators blinded to treatment. The repeatability coefficient for FMD is 3% in our laboratory in accordance with the definition of the British Standard Institution.

### 2.4. Blood collection and handling

#### 2.4.1. Data collection and analyses

In the morning and after an at-least-30-min supine rest, fasting venous blood samples were drawn from all participants, pre- and post-supplementation period, centrifugated within 30 min and stored at −20 °C. Glucose was measured enzymatically with the glucose-oxidase/peroxidase method. Total cholesterol, triglycerides and high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL) were measured enzymatically with final measurements at 520 nm and 583 nm, respectively. For all the above, the Cobas Integra 800 automated system by Roche and all relevant diagnostic reagents were used.

Total antioxidant capacity (TAC), expressed in mmol DPPH/L was determined by the DPPH spectrophotometric assay which uses stable 2,2′-diphenylpicrylhydrazyl (DPPH) radical as reagent. The plasma was mixed with PBS and DPPH, it was then incubated and centrifuged and the absorbance was read at 520 nm.

Thiobarbituric-acid reactive substances (TBARS), expressed in μmol/L were measured in blood plasma using a previously described method (Paschalis et al., 2007). Briefly, plasma was mixed with TCA, Tris–HCl, $Na_2SO_4$ and thiobarbituric acid and incubated at 95 °C. TCA was added again, centrifuged and the absorbance was read at 530 nm.

GSH in plasma, expressed in μmol/L was measured as follows: 100 μl of plasma was pipetted into Eppendorf tubes containing 200 μl of a 10% solution of TCA (tricholoroacetic acid), vortexed and centrifuged at 4000*g* for 10 min at 10 °C. To 200 μl of the supernatant 700 μl of 400 mM Tris–HCl buffer, pH 8.9, was added followed by the addition of 100 μl of 2.5 mM DTNB dissolved in 40 mM Tris–HCl buffer pH 8.9. After 60 min at room temperature the extinction of the samples was measured at 412 nm spectrophotometer. Blank consisted of DTNB instead of plasma (Look et al., 1997).

Protein carbonyls were determined based on the method of Patsoukis et al. (2004). In this assay, 50 μL of 20% TCA was added to 50 μL of plasma and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000*g* for 5 min at 4 °C. The supernatant was discarded and 500 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) [in 2.5 N hydrochloride (HCl)] for the sample, or 500 μL of 2.5 N HCl for the blank, was added in the pellet. The samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min and were centrifuged at 15,000*g* for 5 min at 4 °C. The supernatant was discarded and 1 mL of 10% TCA was added, vortexed and centrifuged at 15,000*g* for 5 min at 4 °C. The supernatant was discarded and 1 mL of ethanol–ethyl acetate (1:1 v/v) was added, vortexed and centrifuged at 15,000*g* for 5 min at 4 °C. This washing step was repeated twice. The supernatant was discarded and 1 mL of 5 M urea (pH 2.3) was added, vortexed and incubated at 37 °C for 15 min. The samples were centrifuged at 15,000*g* for 3 min at 4 °C and the absorbance was read at 375 nm. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH. The intra- and inter-assay CV for protein carbonyls were 4.3% and 7.0%, respectively. Total plasma protein was assayed using a Bradford reagent from Sigma–Aldrich.

### 2.5. Statistical analysis

All results are presented as mean value ± SD (standard deviation). Statistical analyses were performed with SPSS version 14 (SPSS INC., Chicago, Illinois). Significant differences between means for the same parameters were investigated with repeated measures Anova and paired *t*-test analyses. Independent *t*-tests were used to compare mean values between groups. Pearson and Spearman correlations and linear regression analysis was conducted to investigate correlations between various variables. Differences between categorical variables were assessed by the chi-square test. A $P$ value $\leqslant 0.05$ was considered statistically significant.

## 3. Results

The results of the protein bar supplementation in the endothelial function (as depicted by the FMD), in common biochemical parameters (i.e. glucose, cholesterol, triglycerides, LDL and HDL) and in the oxidative status (TAC, GSH, TBARS and protein carbonyls) of 16 ultra-marathon runners are presented in Table 4, while the effects of the tomato juice supplementation on the 15 ultra-marathon runners are summarised in Table 5. No statistically significant differences were observed in the control group after the 2 months-monitoring period.

Endothelial function seems to ameliorate after the special bar consumption, although not statistically significantly. In 4 subjects the increase in FMD exceeded 100%, while in 7 subjects a decrease was observed (5–50%). The change in FMD was independent of all baseline characteristics of the subjects, except EF, which was negatively correlated ($r = -0.569$, $p = 0.034$) with the increase in FMD. Positive correlations were also found with the decrease in protein carbonyls ($r = 0.704$, $p = 0.007$) and negative correlations with the baseline FMD levels and the cholesterol levels ($r = -0.632$, $p = 0.012$).

On the other hand, FMD significantly increased in subjects consuming tomato juice for 2 months, reaching 186% for one subject (average increase 66.9% in 10 ultra-marathon runners). The change in FMD was independent of all baseline characteristics of the subjects and not related with any other change in the parameters monitored.

In general, the diet habits of the study population did not influence any of the changes observed during this study either in the protein bar or in the tomato juice supplementation groups. The usual biochemical parameters monitored that are mainly diet-