Accepted Manuscript

Effects of Pomegranate Juice in Circulating Parameters, Cytokines and Oxidative Stress Markers in Endurance-Based Athletes: A Randomised Controlled Trial

E. Fuster-Muñoz, E. Roche, L. Funes, P. Martínez-Peinado, J.M. Sempere, N. Vicente-Salar

PII: S0899-9007(15)00463-3

DOI: 10.1016/j.nut.2015.11.002

Reference: NUT 9649

To appear in: *Nutrition*

Received Date: 23 May 2015

Revised Date: 4 November 2015

Accepted Date: 5 November 2015

Please cite this article as: Fuster-Muñoz E, Roche E, Funes L, Martínez-Peinado P, Sempere JM, Vicente-Salar N, Effects of Pomegranate Juice in Circulating Parameters, Cytokines and Oxidative Stress Markers in Endurance-Based Athletes: A Randomised Controlled Trial, *Nutrition* (2016), doi: 10.1016/j.nut.2015.11.002.

EFFECTS OF POMEGRANATE JUICE IN CIRCULATING PARAMETERS, CYTOKINES AND OXIDATIVE STRESS MARKERS IN ENDURANCE-BASED ATHLETES: A RANDOMISED CONTROLLED TRIAL

Authors´ names:

E. Fuster-Muñoz[1], E. Roche[2], L. Funes[3], P. Martínez-Peinado[4], J.M.Sempere[4], N. Vicente-Salar[2]

Name and address of department(s) and institution(s):

1- Toxicology Unit. Institute of Bioengineering, University Miguel Hernandez. Av. De la Universidad s/n, 03202, Elche (Alicante) SPAIN.

2- Biochemistry and Cell Therapy Unit. Institute of Bioengineering, University Miguel Hernandez. Av. De la Universidad s/n, 03202, Elche (Alicante) SPAIN. CIBEROBN CB 12/03/30038.

3- Quality and Innovation Department, Vitalgrana® Pomegranate SL. Polígono Industrial de Poniente C/ 1-2, 03150, Catral, (Alicante) SPAIN.

4- Immunology Division, Biotechnology Department, University of Alicante. Carretera de San Vicente del Raspeig s/n, 03690, San Vicente del Raspeig (Alicant), SPAIN.

Corresponding Author:

Néstor Vicente-Salar

Biochemistry and Cell Therapy Unit. Institute of Bioengineering, University Miguel Hernandez. Av. De la Universidad s/n, 03202, Elche (Alicante) SPAIN.

e-mail: nvicente@umh.es

Telephone number: +34 965222041

Fax number: +34 965919546

Shortened version of the title: Pomegranate reduces oxidative stress in sport

Key words: pomegranate juice, antioxidants, sport, carbonyls, malondialdehyde.

ABSTRACT

The aim of the present study was to assess the effects of pomegranate juice on the level of oxidative stress in blood of endurance-based athletes. Pomegranate juice is rich in polyphenols, conferring it a higher antioxidant capacity than other beverages with polyphenolic antioxidants. A randomized, double-blind, multicenter trial was performed in athletes from 3 different sport clubs located in the southern region of Spain. Plasma oxidative stress markers (protein carbonyls and malondialdehyde (MDA)) as well as C-reactive protein and sE-selectin were measured. A total of 31 athletes participated in the study, supplemented during 21 days with 200 ml/day pomegranate juice (PJ) (n=10), 200 ml/day pomegranate juice diluted 1:1 with water (PJD) (n=11) and a control group not consuming pomegranate juice (C) (n=10). Nine volunteers were excluded due to protocol violations (n=4 in the PJ group and n=5 in the PJD group) since they did not respect the 24 h of rest before the last blood test. The control group increased levels of carbonyls (+0.7 ± 0.3 nmols/mg protein) and MDA (+3.2 ± 1.0 nmols/g protein), while PJ and PJD groups maintained or decreased their levels, respectively. On the other hand, lactate levels increased in the PJ group (from 10.3 at day 0 to 21.2 mg/dL at day 22). A non-significant decrease was detected in sE-selectin and C-reactive protein in the groups consuming pomegranate juice. Consumption of pomegranate juice during 21 days improves MDA levels and carbonyls, decreasing the oxidative damage caused by the exercise.

INTRODUCTION

Reactive oxygen and nitrogen species (RONS) play various roles in the cells, being both beneficial and deleterious. The beneficial effects of RONS include defense against infectious agents as well as intracellular signaling molecules in many processes [1]. On the other hand, persistently high RONS levels can produce harmful effects if the antioxidant defenses are overwhelmed, resulting in structural damage, including membrane lipids, proteins and nucleic acids. This phenomenon is called oxidative stress, and can be detected by analyzing in blood end-metabolites of the oxidation process such as malondialdehyde (MDA) from lipid peroxidation, or by measuring oxidatively-altered macromolecules such as the presence of carbonyl adducts in affected proteins [2,3,4].

Exercise could be considered as an exogenous source of oxidative stress due to an increase in oxygen consumption at the level of mitochondrial respiration, leading to punctual increases in RONS production [5]. However, this seems to be a controversial topic since the benefits of exercise are well documented in the prevention and/or treatment of chronic disorders such as diabetes mellitus, dyslipidemia, hypertension, obesity, cardiovascular and pulmonary diseases, muscle, bone and joint diseases, cancer and depression [6]. After moderately intense exercise, the muscle tissues produce RONS, which have been shown to act as intracellular secondary messengers [7]. However, when strenuous exercise or overloaded training is performed, an imbalance occurs between production of free radicals and the endogenous antioxidant systems [8,9,10]. Moreover, high levels of markers of oxidative stress and inflammation, such as E-selectin and CRP, could lead to endothelial dysfunction [11,12].

Nevertheless, diet is the main source of antioxidants and in this context, pomegranate juice, which is extracted from the fruit of the *Punica granatum* plant, is rich in polyphenols such as anthocyanins, flavonols and certain ellagitannins such as punicalagin [13]. Several studies have documented the benefits of pomegranate juice consumption in individuals affected with different disorders [14,15,16,17,18,19,20]. Regarding the field of physical activity, only a few studies have analyzed how pomegranate consumption can modulate performance during exercise [21,22]. However, there are no studies regarding the possible role of pomegranate juice consumption in oxidative stress modulation in athletes. Thus, the aim of the present study was to assess the effects of pomegranate juice on oxidative stress markers in a group of well-trained endurance-based athletes.

MATERIAL AND METHODS

Trial design

Participants were randomly assigned to one of the three groups that consumed the juice both training and non-training days. In the training days, juice intake was done just after the training session as a post-exercise meal: those that consumed a bottle of 200 ml/day of pomegranate juice (PJ group) (n=10), another group consumed a bottle of 200 ml/day pomegranate juice diluted 1:1 with water (PJD group) (n=11) and a control group that consumed fresh fruit (1 piece (200 g approx.) of seasonal fruit except pomegranate, which contains the same energy that 1 bottle (200 ml) of pomegranate juice) instead of pomegranate juice (C group) (n=8) for maintaining the same daily energetic intake (Table 1). The aim of a group taking a diluted form of PJ was to assess if there was a dose response in any of parameters studied. All the groups consumed fluids as water after exercise ad libitum. The design was a double-blind, parallel-group, randomized controlled trial conducted at Miguel Hernandez University of Elche (Spain). This work has been registered in: https://clinicaltrials.gov/ct2/show/NCT02293486, Protocol ID: GRA 01/ ClinicalTrials.gov ID: NCT02293486.

Table 1: Anthropometric values of each group at day 0 (d0) and day 22 (d22).

Group	**C (n=8)**		**PJ (n=6)**		**PJD (n=6)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Age (years)	33.3	9.0	35.2	8.5	37.5	11.4
Height (m)	1.7	0.1	1.7	0.1	1.7	0.1
Weight (kg) (d0)	71.3	11.8	67.2	3.4	70.0	12.2
Weight (kg) (d22)	70.3	11.7	66.8	3.8	70.1	12.1
% Fat mass (d0)	14.2	4.4	15.7	6.0	16.3	5.4
% Fat mass (d22)	13.1	4.2	14.5	5.2	15.7	4.8
% Muscle mass (d0)	46.1	4.7	46.4	4.0	43.9	5.3
% Muscle mass (d22)	46.3	4.6	46.7	4.2	43.3	4.8
Exercise caloric expenditure (Kcal/day)	514.5	193.3	544.4	207.3	708.5	156.4

Abbreviations used: C, control group not consuming pomegranate juice; PJ, group consuming pomegranate juice; PJD, group consuming pomegranate juice diluted 50% with water; d, day. There are not significant differences between groups in the parameters analysed.

Participants

Volunteers participating in the study (Table 1) were selected from sport clubs of different locations in southeastern Spain. The parameters for inclusion were to be adult males, perform

training sessions, and to have participated recently in a half marathon or similar event, which are held throughout the competitive season. To this end, the participants performed endurance-based training for more than 1h per session and more than 3 sessions per week. The exclusion criteria were: intake of antioxidant or anti-inflammatory supplementation, present a chronic disorder, smoker and alcoholic beverage consumption.

Interventions

Thirty-one volunteers were selected (Fig. 1), informed about the objective and demands of the study and gave their written consent to participate. The protocol was in accordance with local legal requirements and the Helsinki Declaration for research on human beings, and approved by the Ethics Committee of University Miguel Hernandez.

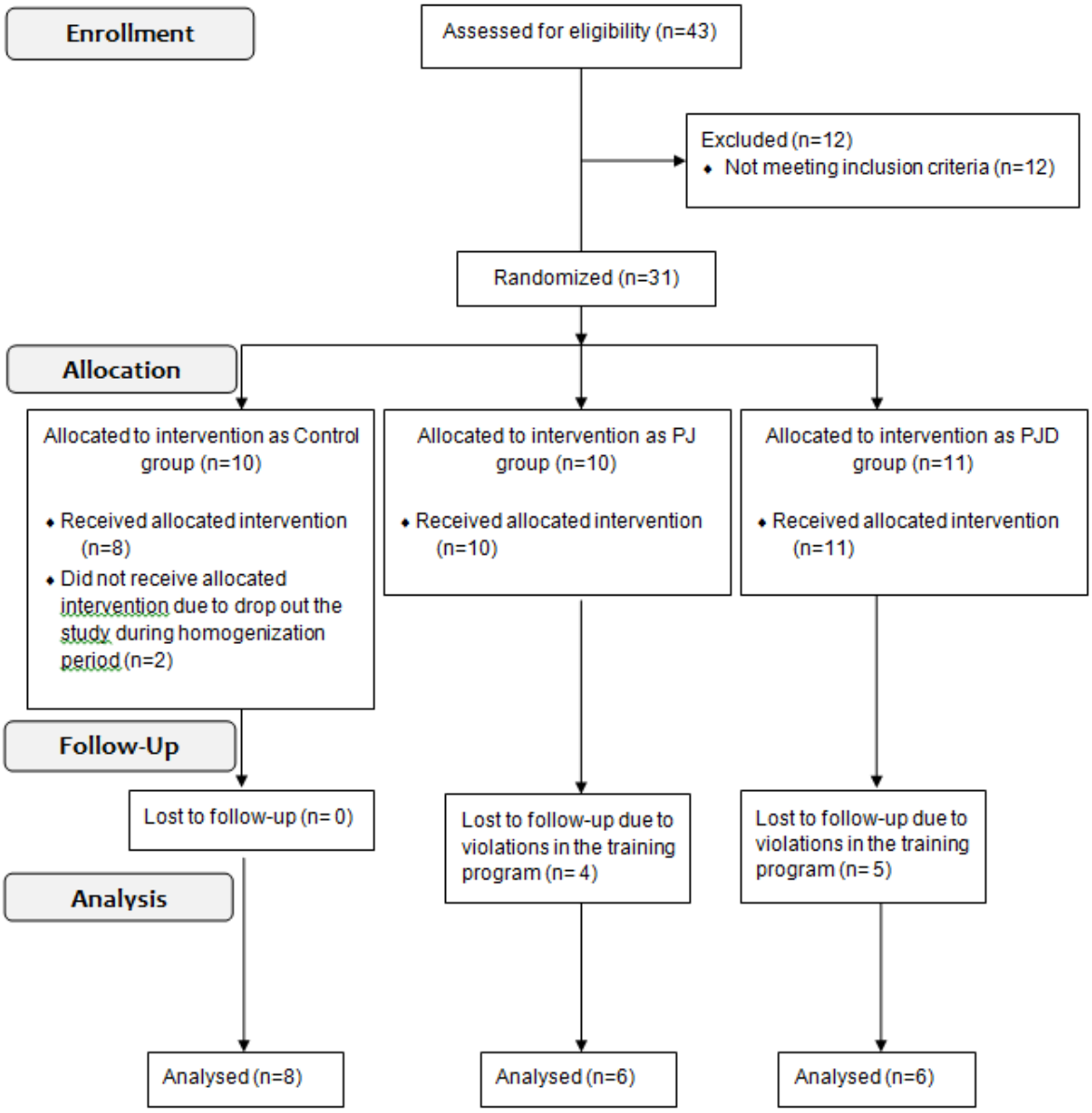


Fig. 1. Participant's flow diagram.

Pomegranate juice was provided by Vitalgrana SL®, elaborated by crushing the fruit and the seed. This produces the transfer of an oily phase to the final juice that is rich in unsaturated fatty acids, being punicic acid one of the most abundant. The complete composition of the juice is provided in (http://www.vitalgrana.com/es/productos-zumo-granada). The study lasted a total of 5 weeks (Fig. 1). The first two weeks of the experiment (homogenization period) were used to volunteers started their training sessions and verify accomplishment of the duration and frequency, to accustom volunteers to the individual diet plan and to solve doubts about the procedure. During the homogenization period, 9 volunteers were excluded due to protocol violations in the training program (Fig. 1). The recruitment process began in August 2012, and the intervention was carried out in February 2013.

Table 2: Energy and macronutrient composition of diets used in the study.

Abbreviations used: CHO, carbohydrate; P, protein; F, fats.

Weight range (kg)	kJ/day	% CHO	g/kg·day CHO	% P	g/kg·day P	% F	g/kg·day F
55 – 59	9,630		6.3 – 6.7		1.4 – 1.5		1.2 – 1.3
60 – 65	10,467	65	6.3 – 6.8	15	1.4 – 1.5	20	1.1 – 1.2
66 – 70	10,886		6.0 – 6.4		1.5		1.1 – 1.2
71 - 75	11,723		6.0 – 6.3		1.4-1.5		1.0 – 1.1

Diet plans were customized adjusting energy expenditures and macronutrients to the training activity and body weight (Table 2) in order of the diet composition or energy intake did not affect the study. Total energy expenditure (TEE) was estimated as an average of the rest energy expenditure (REE) for each weight range according to the FAO equation ([11.3·weight] + [16· [height/100]] +901)[21] and multiplied by 1.55 as a factor for activity. Therefore, all groups had the same diet plan according to the individual weight, with the only exception of the change of one portion of fruit in group C by the juice in groups PJ and PJD in order to maintain the energy intake. The subjects were instructed to manage their own diet plan by making proper equivalent food changes (maintaining the daily energy intake and macronutrient composition approximately) during the 3 weeks of intervention. Periodic meetings or email contact was maintained to solve any doubts the volunteers had during the

study. The next three weeks were considered as the intervention period when data was collected, coinciding with other studies that used similar periods of time [14,24]. At day 0, before starting the intervention and 48h without exercise, whole blood samples were collected and anthropometric measures performed according to recommendations of the International Society for Advancement of Kinanthropometry (ISAK). At the end of the study (day 22), volunteers repeated the above mentioned procedures. The subjects scored during the 22 days of intervention their physical activity and its duration. The energy expenditure by exercise of each subject was calculated through MET values of each activity and shown as the main of energy consumed per day (Table 1).

Blood samples were obtained from the antecubital vein in EDTA vacutainers at days 0 and 22 after overnight fasting. The plasma was obtained as a supernatant of the whole blood after centrifugation at 1000xg for 15 min at 4ºC and then stored at -80º C.

Circulating glucose was determined by the glucose oxidase method coupled to the peroxidase reaction [25]. HDL-cholesterol was determined by a direct enzymatic colorimetric method. HDL was dissolved with a detergent, while HDL-cholesterol was released to react with cholesterol esterase. Afterwards, free cholesterol was oxidized with cholesterol oxidase to cholest-4-ene-3-one and hydrogen peroxide, which was determined using the peroxidase reaction. The non-HDL lipoproteins were inhibited from reacting with the enzymes due to the absorption to the detergent [26]. Circulating triglycerides were determined from coupled reactions of lipoprotein-lipase, glycerol-kinase, glycerol phosphate oxidase and peroxidase, giving a color end-adduct as reported previously [27]. Ferritin was determined using an enzyme-linked fluorescent assay (BioMerieux, Madrid, Spain) according to the manufacturer´s instructions. Lactate was determined by lactate oxidase/peroxidase-coupled colorimetric reaction [28]. Plasma Na^+ and K^+ were determined by potentiometry using selective electrodes Spotlyte (Menarini, Badalona, Spain). Creatine kinase was determined photometrically (Spinreact, Girona, Spain) from coupled reactions of hexokinase and glucose-6-phosphate dehydrogenase, giving rise to NADPH. Aspartate aminotransferase (AST) was determined photometrically (Spinreact, Girona, Spain) by analyzing the decreased NADH concentration from the coupled reaction with malate dehydrogenase. Alanine aminotransferase (ALT) was determined in a similar manner as AST, only the coupled reaction was performed on lactate dehydrogenase.

Oxidative stress markers were determined in plasma. Protein carbonyl derivatives were determined using an adaptation of the method published in Levine et al [29]. MDA was determined by HPLC with fluorescence detection according to the method described by

Laporta et al [30]. Briefly, 100 µl of plasma were mixed with 100 µl of 0.05% butylated hydroxytoluene in ethanol and 100 µl of 20% trichloroacetic acid in 0.6M HCl. The samples were incubated 15 min on ice and then centrifuged at 5000xg during 15 min at 4ºC. The supernatant was collected and 100 µl of 0.6% thiobarbituric acid (TBA) in water was added. Then, the mixture was incubated at 97ºC for 30 min, allowed to cool down and extracted with 200 µl of n-butanol through vigorous shaking. Finally, the samples were centrifuged at 10000xg for 3 min at 4ºC. The TBA–MDA chromogen was determined using a HPLC and fluorescence detection system.

Cytokine concentrations in plasma, specifically C-reactive protein (CRP) and sE-selectin, were determined in 25 µl of plasma by an immunoassay analyzed on a flow cytometer (BD FacsCanto II, San Jose, CA, USA) according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). The lower limits of detection were: 67.0 ng/l for CRP and 1.2 ng/ml for sE-selectin.

Outcomes

The primary endpoint was to assess whether pomegranate juice consumption can modulate changes in plasma protein carbonyls and MDA levels in volunteers. The secondary endpoint was to assess whether the same supplements can modulate changes in plasma markers related to the health status of each individual during the study.

Blinding

During the intervention, the participants, investigator and outcome assessor were blinded. Neither the group nor the volunteers knew who and what supplement were the other participants taking during the 22 days of the study. Participating groups were also unaware of the type of drink they were consuming, or of the existence of other volunteers in different locations. In this manner, it was not necessary for the flavor to be blinded. The groups were blinded by letters and each participant by numbers, indicating the first samples as d0 and the last samples as d22 to blind the investigator, which was given by the outcomes assessor. Finally, when the results were obtained, the investigator changed the codes before giving them to the outcomes assessor.

Statistical methods and sample size

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, v. 20.0 for Windows) to process the data obtained from the volunteers. Standard descriptive statistics were presented as mean ± standard deviation (SD) (mean ± SD). Since the volunteers presented different values in the circulating parameters analyzed at the beginning

of the study, the differences in values between day 0 and 22 were analyzed in each group using a transversal analysis. The difference between both values (Δparameter = $parameter_{day22}$ – $parameter_{day0}$) indicated the variation underwent by each parameter, positive values reported an increase in the corresponding parameter, while negative values indicated a decrease. One-sample K-S test (Kolmogorov-Smirnov test) and Homoscedasticity Levene test were performed in order to assess if the variables fit a normal distribution. Due to the volunteer exclusions during the study, the n of all groups was between 6 and 8 athletes. Therefore, non-parametric tests for dependent samples (Wilcoxon test) were used when comparing the intra-group variation between day 0 and day 22. A non-parametric two-way analysis of variance (Kruskal-Wallis) was used to test the inter-group effect of juice consumption and aerobic training after 22 days. Values with a $p<0.05$ were considered significant.

RESULTS

Effect of pomegranate juice consumption in plasma circulating parameters

No variation in body composition or weight was detected in any of the volunteers during the experimental protocol. In a comparative analysis between days 0 and 22, glucose tended to increase in all groups, although always in the healthy range, being most significant in the C and PJD group (Table 3).

Table 3: Significant changes in plasma parameters of each group comparing day 0 and 22

Group	**C (n=8)**				**PJ (n=6)**				**PJD (n=6)**			
Day	**0**		**22**		**0**		**22**		**0**		**22**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Glucose (mg/dL)	74.4	9.5	80.5	13.0*	79.0	8.0	80.8	9.7	73.3	8.0	83.3	5.3*
HDL (mg/dL)	41.9	4.6	42.9	6.5	53.3	10.2	54.3	11.1	48.8	7.3	51.0	7.0*
AST (U/L)	29.5	5.8	23.5	4.3*	22.5	2.7	24.5	3.4	24.2	4.0	29.2	15.7
ALT (U/L)	21.2	5.9	18.4	5.2	17.0	3.0	22.0	11.4	17.0	3.4	25.2	13.6
K^+ (mEq/L)	4.2	0.2	4.2	0.3	4.1	0.1	4.4	0.2*	4.3	0.3	4.3	0.4
Na^+ (mEq/L)	139.1	1.5	139.1	1.0	139.5	0.7	139.5	0.5	139.2	1.2	139.4	1.0
Lactate (mg/dL)	8.9	3.4	10.1	5.8	10.3	2.0	21.2	4.5†	7.9	4.9	13.7	9.2
Ferritine (ng/mL)	71.7	39.6	65.1	38.4	69.2	37.4	55.0	36.7*	58.7	27.9	57.6	12.7

Abbreviations used: C, control group not consuming pomegranate juice; PJ, group consuming pomegranate juice; PJD, group consuming pomegranate juice diluted 50% with water. *$p<0.05$ versus day 0; † $p<0.01$ versus day 0.

Similarly, lactate also tended to increase and ferritine to decrease in all groups, being only significant in the PJ group. As for the change score of lactate (Δlactate), all groups tended to increase, being significantly higher in PJ than C (Table 4).

Table 4: Significant change scores in biochemical plasma parameters between groups at the end of the study.

Group[a]	**C (n=8)**		**PJ (n=6)**		**PJD (n=6)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
ΔGlucose (mg/dL)	6.1	4.9	1.8	10.0	10.0	6.2
ΔHDL (mg/dL)	1.0	4.0	1.0	2.8	2.2	1.5
ΔAST (U/L)	-6.0	6.1	2.0	3.0	5.0	14.5
ΔALT (U/L)	-2.9	5.6	5.0	10.1	8.2	14.0
ΔK^+ (mEq/L)	0.1	0.2	0.3	0.3	0.0	0.3
ΔNa^+ (mEq/L)	0.0	1.5	0.0	0.9	0.2	0.8
ΔLactate (mg/dL)	1.2	3.9	10.9	6.0*	5.9	8.7
ΔFerritine (ng/mL)	-6.6	20.8	-14.2	3.9	-1.1	18.2

Abbreviations used: C, control group not consuming pomegranate juice; PJ, group consuming pomegranate juice; PJD, group consuming pomegranate juice diluted 50% with water. [a]Δ = (value obtained at day 21) - (value obtained at day 1). *$p<0.05$ C versus PJ.

On the other hand, the study of damage tissue markers showed that transaminases (AST and ALT) tended to increase no significantly during the 3 weeks of intervention only in PJ and PJD groups, but not in C group, where AST decreased significantly at day 22 (Table 3). No significant differences were observed in the change scores of transaminases between groups (Table 4) possibly due to the need of more intervention days for these changes to be significant. Parameters related with lipid metabolism showed an increase in HDL cholesterol in all groups at the end of the study, being significant only in the PJD group (table 3). As in the case of the transaminases, no differences were observed in the changes of HDL levels when compared between the groups (table 4). Finally, the electrolyte status reflected by the determination in plasma of K^+ and Na^+ presented only significant changes for K^+ at the end of the study in the PJ group (Table 3), albeit in the healthy range in all cases. Na^+ levels were

stable in all groups. No differences in K^+ and Na^+ were detected between groups when analyzing their change scores (ΔK^+ and ΔNa^+).

Effect of pomegranate juice consumption in plasma protein carbonyls and MDA

Significant differences were observed in oxidative stress markers MDA and protein carbonyls both between groups as well as in the change scores (Δcarbonyls and ΔMDA) (Fig. 2). At day 22, protein carbonyl levels significantly increased in C group (passing from 1.1 at day 0 to 1.8 nmols/mg protein at day 22), while PJ and PJD groups presented no significant changes (Fig. 2). In the case of MDA, C group also presented a significant increase at the end of the study (14.1 nmols/g protein) compared to day 0 (10.9 nmols/g protein). On the other hand, decreased MDA levels were detected in PJ and PJD groups in the same time period (Fig. 2).

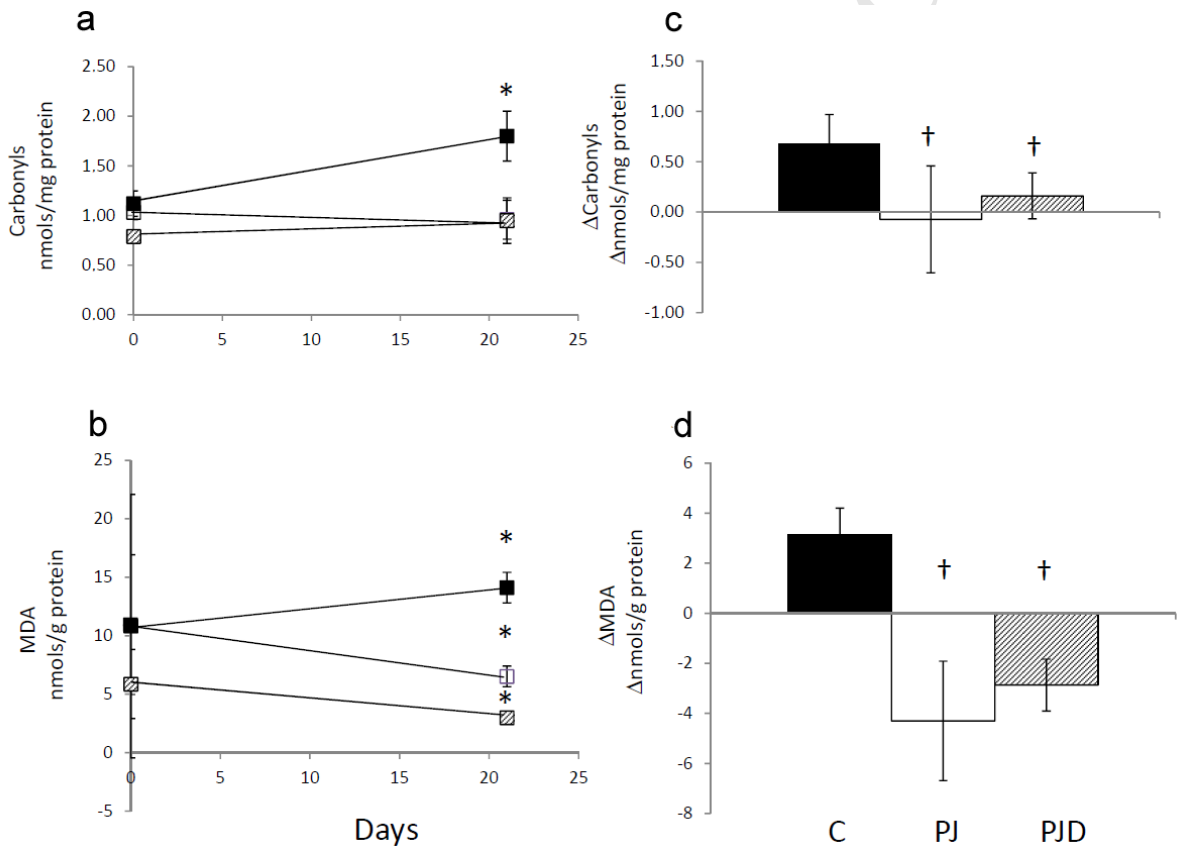


Fig. 2. Changes in carbonyls and MDA levels (a and b respectively) between days 0 and 22 during the consumption of PJ (dashed squares), PJD (empty squares) and C (black squares). *P< 0.01 versus day 0. Change scores and comparison between PJ, PJD and C groups (c and d). † P< 0.01 versus C.

Effect of pomegranate juice consumption in CRP and sE-selectin

None of the groups presented changes in CRP and sE-selectin levels throughout the experiment (not shown). However, a slight but not significant decrease in sE-selectin levels

was detected in PJ and PJD groups (-4.2 ± 23.3 ng/ml and -1.5 ± 9.5 ng/ml respectively), while the contrary was detected in the C group. On the other hand, a no significantly decrease was detected in the change scores for CRP (ΔCRP) of the PJD group (-0.7 ± 1.3 mg/l).

DISCUSSION

The present study assess the impact of pomegranate juice in the oxidative status of endurance-based athletes after consuming the beverage for over three weeks. All hematological and biochemical blood parameters were in the healthy range, and the variations detected were not related to any pathological process.

Trial limitations

The limitations that the study presents stem from the low number of volunteers due to the strict selection criteria used during the randomization trial. Despite the low number, there was a high rate of homogeneity in terms of gender, age, body composition and training routine.

On the other hand, for more exhaustive control of the diet adherence, a 24h recall questionnaires would be made every week.

External validity and applicability of the trial findings

The volunteers of this study were chosen from different sport clubs in Southeastern Spain. Nevertheless, we can state that the results could be applicable to adult males that frequently practice endurance-based sports, but further investigation of the use of pomegranate juice in endurance sport athletes need to be replicated in larger clinical trials.

Blood biochemical parameters.

Interestingly, lactate levels were higher in the PJ group at the end of the intervention period (10.3 at day 0 vs 21.2 mg/dl at day 22). Significant differences were also detected when comparing lactate levels between PJ vs C group. It is well known that lactate increase occurs during high-intensity exercise, as pyruvate conversion to lactate is a rapid method to obtain energy. In this context, the PJ group presented increased K^+ levels at the end of the study, although the differences compared to the other groups were not significant. A likely explanation to these observations is that high intensity exercise tends to increase extracellular K^+ levels in order to maintain an optimal muscle contractibility [31], playing a parallel role with lactate against muscular fatigue [32]. It must take in account that a bottle of PJ contains roughly 600mg of potassium and PJD and 200g of seasonal fresh fruit have 300 and 200-500mg respectively, thus it will also validate the role of the daily potassium intake to test his role in these blood changes. The results of this experiment, together with a significant decrease in AST (an aminotransferase that is released from the muscle after a high intensity exercise) [33] in the C group compared to the other groups, give rise to the hypothesis that

pomegranate juice intake could have optimized the training intensity during the period of study, improving the wellness or maybe the capacity of fatigue perception during the training sessions through an unknown mechanism. Differences in AST clearance from circulation between different groups should be taken into account as well. Further studies taking in account the percentage of VO_{2max} during the training sessions are necessary for a proper validation.

Only PJD group improve significantly HDL levels at day 22 although PJ group maintained a higher level than PJD even at day 0. Recent studies in animal models have demonstrated that consumption of pomegranate juice reduce the risk of atherogenicity [34,35].

Oxidative stress markers

Recent studies indicate that 15 days of pomegranate juice consumption reduces MDA, carbonyls and matrix metalloproteinases 2 and 9 levels and increases erythrocyte glutathione contents, serum superoxide dismutase and glutathione peroxidase levels in healthy non-active volunteers [24,36]. The most interesting observation of that study was that the antioxidant beneficial effects of the juice persisted 3 weeks after consumption was halted [24]. In our study, and despite the direct implication of pomegranate juice consumption in the changes observed in circulating lipids, neither PJ nor PJD groups presented significant changes in the lipid parameters. In this respect, it must be mentioned that all subjects participating in the study (C, PJ and PJD) presented healthy values in all parameters related to the circulating lipid profile, making it difficult to observe positive changes. This is in agreement with other previously published reports using either sedentary volunteers or hemodialysis patients [23,20]. In any case, the next question would be to identify the candidate components in pomegranate juice that may be responsible for the changes in circulating lipid parameters. For example, the polyphenols may be an appropriate candidate, due to their affinity to plasma lipid molecules [37]. In the present study, pomegranate juice consumption is capable of decreasing the initial MDA levels in a greater extent than plasma carbonyls. However, this seems to be dependent on the body compartment analyzed. For instance, mouse liver homogenates presented significantly decreased carbonyl content and 8-OH-guanosine levels, while MDA levels were not affected after four weeks of pomegranate juice consumption [19]. Several mechanisms have been proposed to explain the antioxidant effect of polyphenols. These include free radical scavenging, antioxidant recycling, antioxidant enzyme activity modulation and preservation of mitochondrial function [38]. In this context, the studies concerning polyphenol concentrations reveal that the inner and outer peels possess higher levels than the seeds [18]. Altogether, these observations strongly indicate that the method of

juice manufacturing is an instrumental factor in the final composition. The juice used in this study contains a mixture of inner and outer peels as well as from the seed. Therefore, the antioxidant capacity of the product tested in this study could be considered higher than that from other juices that do not use these subproducts. In addition, it has been well documented that pomegranate juice possesses higher levels of antioxidants than other beverages, including red wine, green tea or wine vinegars[13, 39].

Plasma citokynes

The levels of sE-selectin, a specific marker of endothelial dysfunction and associated with diabetes, tends to decrease in a non-significant manner in PJ and PJD groups. These results are in agreement with studies where adolescents with metabolic syndrome consumed pomegranate juice [16], but not in cases where hypertensive volunteers were analyzed [15]. CRP is also a marker for endothelial dysfunction and vascular inflammation. In the present study, pomegranate consumption did not significantly affect this parameter, whose values were inside healthy ranges (0.8-1.7 mg/l) before and after intervention, which corroborates the results observed in other studies with subjects with hypertension or metabolic disorders [15,16]. On the other hand, the levels of CRP tend to decrease significantly in healthy subjects with the consumption of pomegranate juice, but the baseline of their CRP values was unusually very high (6.4-6.8 mg/l) in that study [36]. So those changes could be due to the wide range of improvement that the subjects had. In this sense, it could be interesting to analyze the profile of cytokines that are related to exercise performance, such as tumor necrosis factor–α (TNF-α) [40] or interleukin-6 (IL-6), which act over the expression of endothelial adhesion molecules such as sE-selectin [41]. Further studies are necessary to confirm this point in physically-active volunteers.

CONCLUSION

In conclusion, and taking in account the data presented in this study, consumption of pomegranate juice during 22 days is capable of modulating fat and protein damage, as the changes in MDA and carbonyl levels indicated. The high presence of antioxidant polyphenols also supports this recommendation. Finally, the evaluation of pomegranate juice consumption regarding training intensity, as well as the study of circulating parameters such as blood lactate, K^+ and AST, needs to be analyzed in further studies.

AKNOWLEDGEMENTS

We greatly appreciate the technical support of Dr. José María Adsuar and the participants of this study. The following institutions are acknowledged: PROMETEO/2012/007 from

Generalitat Valenciana and CIBEROBN (Fisiopatología de la Obesidad y la Nutrición CB12/03/30038) Instituto de Salud Carlos III, Spain to E Roche.

FINANCIAL SUPPORT

This research received specific grant and provision of supplements from Vitalgrana® SL. Vitalgrana® SL had no role in the design, analysis or writing of this article.

CONFLICT OF INTEREST

E. Roche has received honoraria from Vitalgrana® SL and L. Funes is on the speaker's bureau for Vitalgrana® SL. For the remaining authors none were declared.

AUTHORSHIP

- E. Fuster-Muñoz: Anthropometric evaluation, biochemical determinations and analysis of results.
- E. Roche: Study design and writing the article.
- L. Funes: Supplement production.
- P. Martínez-Peinado: Cytokine determinations.
- J.M.Sempere: Cytokine determinations and writing the article.
- N. Vicente-Salar: Diet control and design, contact with participants, study design, analysis of results and writing the article.

REFERENCES

[1]. Poli G, Leonarduzzi G, Biasi F, et al. (2004) Oxidative stress and cell signalling. Curr Med Chem 11, 1163–1182. DOI: 10.2174/0929867043365323

[2]. Halliwell B (1996) Antioxidants in human health and disease. Ann Rev Nutr 16, 33–50. DOI: 10.1146/annurev.nu.16.070196.000341

[3]. Marnett LJ (1999) Lipid peroxidation—DNA damage by malondialdehyde. Mutat Res Mech Mutagen 424, 83–95. DOI:10.1016/S0027-5107(99)00010-X

[4]. Dalle-Donne I, Giustarini D, Colombo R, et al. (2003) Protein carbonylation in human diseases. Trends Mol Med 9, 169–176. DOI:10.1016/S1471-4914(03)00031-5

[5]. Scheele C, Nielsen S, Pedersen BK (2009) ROS and myokines promote muscle adaptation to exercise. Trends Endocrinol Metab 20, 95–99. DOI:10.1016/j.tem.2008.12.002

[6]. Petersen AM, Pedersen BK (2005) The anti-inflammatory effect of exercise. J Appl Physiol 98, 1154–1162. DOI: 10.1152/japplphysiol.00164.2004

[7]. Gomez-Cabrera MC, Domenech E, Viña J (2008) Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. Free Radic Biol Med 44, 126–131. DOI:10.1016/j.freeradbiomed.2007.02.001

[8]. Palazzetti S, Richard M-J, Favier A, et al. (2003) Overloaded training increases exercise-induced oxidative stress and damage. Can J Appl Physiol 28, 588–604.

[9]. Knez WL, Jenkins DG, Coombes JS (2007) Oxidative stress in half and full Ironman triathletes. Med Sci Sports Exerc 39, 283–288. DOI: 10.1249/01.mss.0000246999.09718.0c

[10]. Margaritelis NV, Kyparos A, Paschalis V, et al. (2014) Reductive stress after exercise: The issue of redox individuality. Redox Biol 2, 520–528. DOI: 10.1016/j.redox.2014.02.003

[11]. Blankenberg S, Barbaux S, Tiret L. (2003) Adhesion molecules and atherosclerosis. Atherosclerosis 170, 191-203.

[12] Libby P. (2006) Inflammation and cardiovascular disease mechanisms. Am J Clin Nutr 83, 456S-460S.

[13]. Gil M, Tomás-Barberán F, Hess-Pierce B, et al. (2000) Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agric Food Chem 48, 4581–4589. DOI: 10.1021/jf000404a

[14]. Aviram M, Dornfeld L, Rosenblat M, et al. (2000) Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. Am J Clin Nutr 71, 1062–1076.

[15]. Asgary S, Sahebkar A, Afshani M, et al. (2014) Clinical Evaluation of Blood Pressure Lowering, Endothelial Function Improving, Hypolipidemic and Anti-Inflammatory Effects of Pomegranate Juice in Hypertensive Subjects. Phytother Res 28, 193–199. DOI: 10.1002/ptr.4977

[16]. Kelishadi R, Gidding SS, Hashemi M, et al. (2011) Acute and long term effects of grape and pomegranate juice consumption on endothelial dysfunction in pediatric metabolic syndrome. J Res Med Sci 16, 245–253.

[17]. Vicinanza R, Zhang Y, Henning SM, et al. (2013) Pomegranate juice metabolites, ellagic acid and urolithin a, synergistically inhibit androgen-independent prostate cancer cell growth via distinct effects on cell cycle control and apoptosis. Evidence-based Complement Altern Med 2013, 247504. DOI: 10.1155/2013/247504

[18]. Aviram M & Dornfeld L (2001) Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. Atherosclerosis 158, 195–198. DOI:10.1016/S0021-9150(01)00412-9

[19]. Faria A, Monteiro R, Mateus N, et al. (2007) Effect of pomegranate (Punica granatum) juice intake on hepatic oxidative stress. Eur J Nutr 46, 271–278. DOI: 10.1007/s00394-007-0661-z

441 [20]. Shema-Didi L, Sela S, Ore L, et al. (2012) One year of pomegranate juice intake
442 decreases oxidative stress, inflammation, and incidence of infections in hemodialysis patients:
443 A randomized placebo-controlled trial. Free Radic Biol Med 53, 297–304. DOI:
444 10.1016/j.freeradbiomed.2012.05.013
445 [21]. Trombold JR, Barnes JN, Critchley L, et al. (2010) Ellagitannin consumption improves
446 strength recovery 2-3 d after eccentric exercise. Med Sci Sports Exerc 42, 493–498. DOI:
447 10.1249/MSS.0b013e3181b64edd.
448 [22]. Trombold JR, Reinfeld AS, Casler JR, et al. (2011) The effect of pomegranate juice
449 supplementation on strength and soreness after eccentric exercise. J Strength Cond Res 25,
450 1782–1788. DOI: 10.1519/JSC.0b013e318220d992.
451 [23]. Worl Health Organization (1985). Energy and protein requirements. Joint
452 FAO/WHO/UNU Expert Consultation. WHO Technical Report Series no. 724. Geneva:
453 WHO.
454 [24]. Matthaiou CM, Goutzourelas N, Stagos D, et al. (2014) Pomegranate juice consumption
455 increases GSH levels and reduces lipid and protein oxidation in human blood. Food Chem
456 Toxicol 73, 1-6. DOI: 10.1016/j.fct.2014.07.027
457 [25]. Trinder P (1969) Determination of blood glucose using an oxidase-peroxidase system
458 with a non-carcinogenic chromogen. J Clin Pathol 22, 158–161.
459 [26]. Naito H & David J (1984) Laboratory considerations: determination of cholesterol,
460 triglyceride, phospholipid, and other lipids in blood and tissues. Lab Res Methods Biol Med
461 10, 1–76.
462 [27]. Bucolo G & David H (1973) Quantitative determination of serum triglycerides by the
463 use of enzymes. Clin Chem 19, 476–482.
464 [28]. Weisshaar D, Gossrau E, Faderl B (1975) Normal ranges of alpha-HBDH, LDH, AP,
465 and LAP as measured with substrate-optimated test charges. Med Welt 26, 387–392.
466 [29]. Levine R, Williams J, Stadtman E, et al. (1994) Carbonyl assays for determination of
467 oxidatively modified proteins. Methods Enzym 233, 346–357.
468 [30]. Laporta O, Funes L, Garzón MT, et al. (2007) Role of membranes on the antibacterial
469 and anti-inflammatory activities of the bioactive compounds from Hypoxis rooperi corm
470 extract. Arch Biochem Biophys 467, 119–131. DOI:10.1016/j.abb.2007.08.013
471 [31]. Sejersted OM & Sjøgaard G (2000) Dynamics and consequences of potassium shifts in
472 skeletal muscle and heart during exercise. Physiol Rev 80, 1411–1481.

[32]. Nielsen OB, de Paoli F, Overgaard K (2001) Protective effects of lactic acid on force production in rat skeletal muscle. J Physiol 536, 161–166. DOI: 10.1111/j.1469-7793.2001.t01-1-00161.x

[33]. Banfi G, Colombini A, Lombardi G, et al. (2012) Metabolic markers in sports medicine. Adv Clin Chem 56, 1–54.

[34] Rosenblat M, Volkova N, Abassi Z, Britton SL, Koch LG, Aviram M (2015). High intrinsic aerobic capacity and pomegranate juice are protective against macrophage atherogenecity: studies in high- vs. low-capacity runner (HCR vs. LCR) rats. J Nutr Biochem DOI: 10.1016/j.jnutbio.2015.04.001. [Epub ahead of print]

[35] Rosenblat M, Volkova N, Borochov-Neori H, Judeinstein S, Aviram M (2015). Anti-atherogenic properties of date vs. pomegranate polyphenols: the benefits of the combination. Food Funct 6, 1496-1509. DOI: 10.1039/c4fo00998c.

[36]. Mazani M, Fard AS, Baghi AN (2014) Effect of pomegranate juice supplementation on matrix metalloproteinases 2 and 9 following exhaustive exercise in young healthy males. J Pak Med Assoc 64, 785-90.

[37]. Barrajón-Catalán E, Herranz-López M, Joven J, et al. (2014) Molecular promiscuity of plant polyphenols in the management of age-related diseases: far beyond their antioxidant properties. Adv Exp Med Biol 824, 141–159. DOI: 10.1007/978-3-319-07320-0_11.

[38]. Halliwell B, Rafter J, Jenner A (2005). Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? Am J Clin Nutr 81, 268S-276S.

[39]. Budak HN & Guzel-Seydim ZB (2010) Antioxidant activity and phenolic content of wine vinegars produced by two different techniques. J Sci Food Agric 90, 2021–2026. DOI: 10.1002/jsfa.4047.

[40]. Kara E, Ozal M, Gunay M, et al. (2011) Effects of exercise and zinc supplementation on cytokine release in young wrestlers. Biol Trace Elem Res 143, 1435-1440. DOI: 10.1007/s12011-011-9005-1

[41]. Constans J & Conri C (2006) Circulating markers of endothelial function in cardiovascular disease. Clin Chim Acta 368, 33–47. DOI:10.1016/j.cca.2005.12.030

Table 1: Anthropometric values of each group at day 0 (d0) and day 22 (d22)..

Group	**C (n=8)**		**PJ (n=6)**		**PJD (n=6)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Age (years)	33.3	9.0	35.2	8.5	37.5	11.4
Height (m)	1.7	0.1	1.7	0.1	1.7	0.1
Weight (kg) (d0)	71.3	11.8	67.2	3.4	70.0	12.2
Weight (kg) (d22)	70.3	11.7	66.8	3.8	70.1	12.1
% Fat mass (d0)	14.2	4.4	15.7	6.0	16.3	5.4
% Fat mass (d22)	13.1	4.2	14.5	5.2	15.7	4.8
% Muscle mass (d0)	46.1	4.7	46.4	4.0	43.9	5.3
% Muscle mass (d22)	46.3	4.6	46.7	4.2	43.3	4.8

Abbreviations used: C, control group not consuming pomegranate juice; PJ, group consuming pomegranate juice; PJD, group consuming pomegranate juice diluted 50% with water; d, day.

Table 2: Energy and macronutrient composition of diets used in the study.

Weight range (kg)	kJ/day	% CHO	g/kg·day CHO	% P	g/kg·day P	% F	g/kg·day F
55 – 59	9,630		6.3 – 6.7		1.4 – 1.5		1.2 – 1.3
60 – 65	10,467	65	6.3 – 6.8	15	1.4 – 1.5	20	1.1 – 1.2
66 – 70	10,886		6.0 – 6.4		1.5		1.1 – 1.2
71 - 75	11,723		6.0 – 6.3		1.4-1.5		1.0 – 1.1

Abbreviations used: CHO, carbohydrate; P, protein; F, fats.

Table 3: Significant changes in plasma parameters of each group comparing day 0 and 22

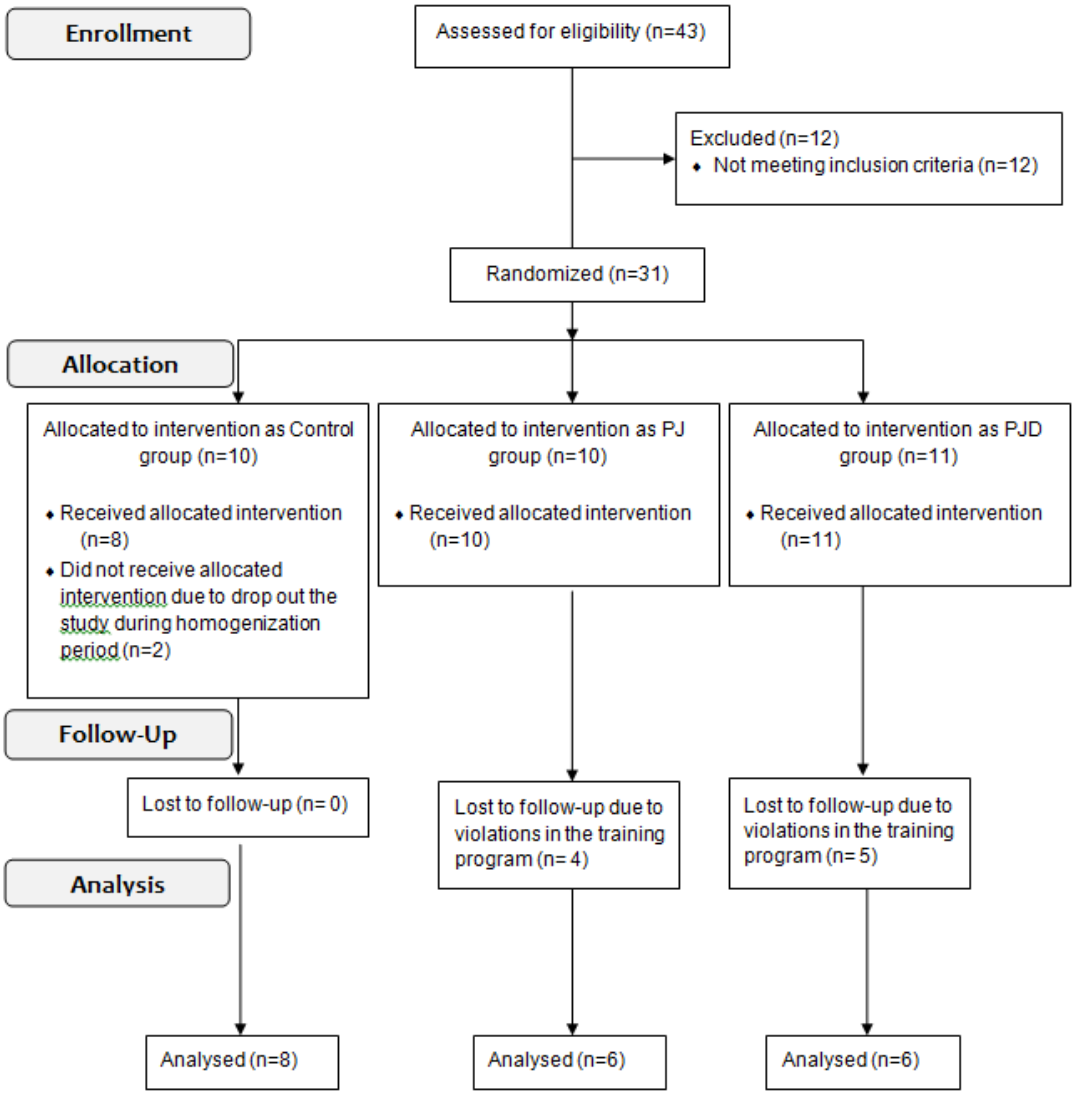
Group	**C (n=8)**				**PJ (n=6)**				**PJD (n=6)**			
Day	**0**		**22**		**0**		**22**		**0**		**22**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Glucose (mg/dL)	74.4	9.5	80.5	13.0*	79.0	8.0	80.8	9.7	73.3	8.0	83.3	5.3*
HDL (mg/dL)	41.9	4.6	42.9	6.5	53.3	10.2	54.3	11.1	48.8	7.3	51.0	7.0*
AST (U/L)	29.5	5.8	23.5	4.3*	22.5	2.7	24.5	3.4	24.2	4.0	29.2	15.7
ALT (U/L)	21.2	5.9	18.4	5.2	17.0	3.0	22.0	11.4	17.0	3.4	25.2	13.6
K^+ (mEq/L)	4.2	0.2	4.2	0.3	4.1	0.1	4.4	0.2*	4.3	0.3	4.3	0.4
Na^+ (mEq/L)	139.1	1.5	139.1	1.0	139.5	0.7	139.5	0.5	139.2	1.2	139.4	1.0
Lactate (mg/dL)	8.9	3.4	10.1	5.8	10.3	2.0	21.2	4.5 †	7.9	4.9	13.7	9.2
Ferritine (ng/mL)	71.7	39.6	65.1	38.4	69.2	37.4	55.0	36.7*	58.7	27.9	57.6	12.7

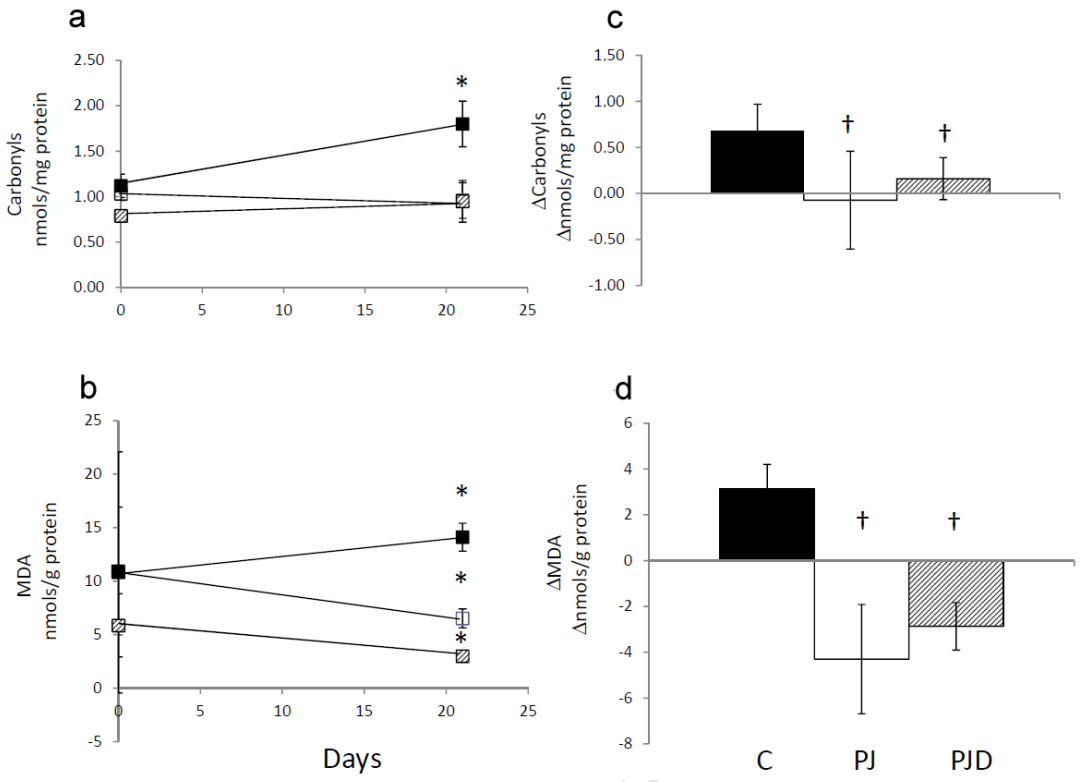
Abbreviations used: C, control group not consuming pomegranate juice; PJ, group consuming pomegranate juice; PJD, group consuming pomegranate juice diluted 50% with water. *$p<0.05$ versus day 0; †$p<0.01$ versus day 0.

Table 4: Significant change scores in biochemical plasma parameters between groups at the end of the study.

Group[a]	**C (n=8)**		**PJ (n=6)**		**PJD (n=6)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
ΔGlucose (mg/dL)	6.1	4.9	1.8	10.0	10.0	6.2
ΔHDL (mg/dL)	1.0	4.0	1.0	2.8	2.2	1.5
ΔAST (U/L)	-6.0	6.1	2.0	3.0	5.0	14.5
ΔALT (U/L)	-2.9	5.6	5.0	10.1	8.2	14.0
ΔK^+ (mEq/L)	0.1	0.2	0.3	0.3	0.0	0.3
ΔNa^+ (mEq/L)	0.0	1.5	0.0	0.9	0.2	0.8
ΔLactate (mg/dL)	1.2	3.9	10.9	6.0*	5.9	8.7
ΔFerritine (ng/mL)	-6.6	20.8	-14.2	3.9	-1.1	18.2

Abbreviations used: C, control group not consuming pomegranate juice; PJ, group consuming pomegranate juice; PJD, group consuming pomegranate juice diluted 50% with water. [a]Δ = (value obtained at day 21) - (value obtained at day 1).* $p<0.05$ C versus PJ.


Enrollment
Assessed for eligibility (n=43)
Excluded (n=12)
Not meeting inclusion criteria (n=12)
Randomized (n=31)
Allocation
Allocated to intervention as Control group (n=10)
Received allocated intervention (n=8)
Did not receive allocated intervention due to drop out the study during homogenization period (n=2)
Allocated to intervention as PJ group (n=10)
Received allocated intervention (n=10)
Allocated to intervention as PJD group (n=11)
Received allocated intervention (n=11)
Follow-Up
Lost to follow-up (n= 0)
Lost to follow-up due to violations in the training program (n= 4)
Lost to follow-up due to violations in the training program (n= 5)
Analysis
Analysed (n=8)
Analysed (n=6)
Analysed (n=6)



Highlights

- The intake of pomegranate juice as antioxidant supplement is proposed.
- The level of oxidative stress markers was measure in endurance-based athletes after supplementation.
- The level of circulating cytokines was measure in endurance-based athletes after supplementation
- MDA and carbonyls decrease signifcantly after 22 days of pomegrante juice supplementation.
- No changes in circulating cytokines levels after 22 days of pomegrante juice supplementation.