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ORIGINAL PAPER

Regular Consumption of an Antioxidant-rich Juice Improves Oxidative Status and Causes Metabolome Changes in Healthy Adults

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Abstract An improvement in oxidative status is associated with a reduction in the incidence of several chronic diseases. However, daily intake of antioxidants in Western diets is decreasing. This study evaluates the effect of daily consumption of an antioxidant-rich juice (ARJ) on oxidative status, cardiovascular disease risk parameters, and untargeted plasma and urine metabolomes. Twenty-eight healthy young adults participated in an 8-week clinical trial by drinking 200 mL of ARJ (pomegranate and grape) daily. At the end of the study, the subjects showed a significant decrease (−29 %) in plasma lipid oxidation (malondialdehyde concentration), and a significant increase (+115 %) in plasma antioxidant capacity. Plasma and urine metabolomes were also significantly modified and some ions modified in urine were identified, including metabolites of polyphenols, ascorbic acid and biliary acids. No significant changes were observed in lipid profile, inflammation, blood pressure or glycaemia. These results show that incorporating antioxidant-rich beverages into common diets may improve oxidative status in healthy subjects.

Keywords Oxidative status · Antioxidant capacity · Metabolomics · Fruit juice · Pomegranate · Grape

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Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AC	Antioxidant capacity
ALP	Alkaline phosphatase
ALT/GPT	Alanine aminotransferase/glutamic pyruvic transaminase
ARJ	Antioxidant-rich juice
AST/GOT	Aspartate aminotransferase/glutamic oxaloacetic transaminase
CVD	Cardiovascular disease
FRAP	Ferric reducing-antioxidant power
LC/MS	Liquid chromatography/mass spectrometry
MDA	Malondialdehyde
PCA	Principal component analysis
Q-TOF	Quadrupole time of flight

Introduction

Cumulative scientific evidence associates an improvement in oxidative status with a reduction in the incidence of various chronic diseases or in their associated risk factors. Control subjects exhibit increased plasma antioxidant capacity (AC) or decreased levels of oxidation biomarkers compared to subjects with cardiovascular disease (CVD) [1] or colorectal cancer [2]. Conversely, a reduction in plasma AC is associated with significant increases in markers of endothelial dysfunction [3] and with the aging process [4].

Dietary antioxidants, comprising a wide variety of compounds present in plant foods are major contributors to plasma antioxidant status and to the associated health effects. Thus, it has been observed that adherence to a Mediterranean dietary pattern, rich in antioxidants, increases both dietary antioxidant

intake and plasma AC [5]. It has also been shown that the AC of the diet is independently related to the inflammation marker high-sensitive C-reactive protein [6].

However, the current trend within Western diets is towards a reduced intake of antioxidants, resulting in decreased antioxidant intake and therefore leading to poor *in vivo* oxidative status. For instance, in Spain, where people used to adhere closely to the traditional Mediterranean dietary pattern, there has been a corresponding significant decrease in the AC of the diet over recent decades, that has been estimated to be around 2000 μmol Trolox equivalents (the most common unit for antioxidant capacity determination) per day [7]. This situation has generated interest in increasing daily antioxidant intake, for instance incorporating specific foods or beverages particularly rich in antioxidants into diets.

The present work consists of a pilot clinical trial to elucidate whether daily consumption of an antioxidant rich juice (ARJ) has any beneficial effect on the oxidative status of healthy young subjects, or on parameters related to CVD risk. Preliminary non-targeted metabolomics analysis was also carried out on plasma and urine.

Materials and Methods

Participants A total of 28 (14 men and 14 women) apparently healthy young subjects (mean age 29.5±4.1 years) were recruited through posters and personal contacts. Inclusion criteria for the study were: apparently healthy; age under 45 years; body mass index lower than 29.9 kg/m^2; non-smoker; alcohol consumption lower than 30 g/day. Exclusion criteria were: being on any weight-reducing regimen; being pregnant or lactating; being peri-menopausal or postmenopausal; having been diagnosed with a chronic disease (CVD, inflammatory diseases, diabetes, cancer); taking regularly any medication or nutritional supplements; parallel participation in any other dietary intervention study. This study was a controlled clinical trial approved by the Ethics Subcommittee of the CSIC, Madrid, Spain (2011/08/9). The study was conducted between February 2012 and June 2012.

Study Design The study was a eight week controlled intervention, where the volunteers drank 200 mL of the ARJ per day. The ARJ was delivered to the participants at the beginning of the study and they were instructed to store them in the fridge. They were asked not to modify their diet throughout the study and filled in two 24-hour recalls per week, starting two weeks before the intervention. Baseline blood pressure, body weight and body fat (by bioimpedance) were measured and repeated at week 8. Blood and urine samples were also collected at those times.

The primary outcome variable was plasma MDA (malondialdehyde). Power calculations were based in a 25 % of reduction in MDA. A sample size of 25 was calculated to detect this change with 80 % power and an alpha value of 0.05, using published variances of MDA [8]. This number was increased to 28 to ensure statistical power, even if some subjects failed to complete the trial.

Antioxidant-rich Juice In order to select an appropriate antioxidant dietary source, the AC of several commercial fruit juices was evaluated (data not shown). A commercial pomegranate (70 %) and grape (30 %) juice was selected (El Corte Ingles S.A., Madrid, Spain, produced by AMC Grupo Alimentación S.A.) that provided an AC of 1997 μmol Trolox equivalents per 200 mL, as determined by ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay [9]. The following additional determinations were also carried out on the ARJ: total polyphenols by Folin assay [10], polyphenol profiles, according to the HPLC procedure described below for metabolomics analysis; antioxidant capacity by FRAP (ferric reducing antioxidant power) assay [11]; dietary fiber, according to the procedure for beverages [12]; fat, protein and ash by official procedures; carbohydrates by difference. The complete composition of the ARJ is shown in Table S1.

Measurements To determine AC intake, data on the consumption of the different food groups (obtained after processing 24-hours recalls with DIAL System, Universidad Complutense de Madrid, Spain) were multiplied by published data on the AC for food groups –obtained from a mixed of the individual items present in each food group at the proportion in which they are consumed in Spain- as determined by ABTS assay [13].

The height, weight and percentage of body fat (by bioimpedance) of the participants were recorded. Biochemical determinations (glucose, total cholesterol, LDL-C, HDL-C, glucose, hemoglobin, triglycerides, ALT/GPT (alanine aminotransferase/glutamic pyruvic transaminase), AST/GOT (aspartate aminotransferase/ glutamic oxaloacetic transaminase), ALP (alkaline phosphatase) were carried out by routine methods and with commercial kits [14].

For the evaluation of oxidative status, the following measurements were taken in plasma, according to procedures previously described: AC by FRAP assay [11, 15], AC by ABTS assay [9] and lipid oxidation as plasma MDA [16] concentration. Uric acid content, after determination by an enzymatic method [17], was subtracted from AC values, since it may contribute to these values [11] and the aim of this study was to evaluate changes in AC caused by other components, *e.g.*, polyphenols and vitamin C.

For untargeted metabolomics analysis, plasma samples were treated with cold methanol for deproteinization. After centrifugation, the supernatant was concentrated under nitrogen until dry. The residue was then reconstituted into a water/acetonitrile (50/50) mixture with 0.1 % formic acid. Urine

samples were centrifuged, and diluted with milliQ water. Both plasma and urine samples were analyzed with an Agilent 6530 Accurate-Mass Quadrupole Time of Flight (Q-TOF) LC/MS (liquid chromatography/mass spectrometry) with ESI-Jet Stream Technology (Agilent Technologies, Waldbroon, Germany) [18]. Briefly, a 2.7 μm, 15×3 mm, C18 Ascentis Express column (Sigma-Aldrich) was used and separation was achieved with a linear gradient of mobile phases consisting of water (A) and acetonitrile (B) both containing 0.1 % formic acid, at a flow rate of 400 μL/min. The Q-TOF acquisition method was 4 GHz, mass range low 1700 m/z, negative and positive polarity, drying gas 10 L 325 °C, sheath gas 6 L 250 °C, nebulizer 25 psi, capillary voltage 3500 V, nozzle voltage 500 V and fragmentor voltage 100 V. The Molecular Feature Extraction algorithm in the Qualitative Masshunter software (B.04.00) was applied to find the molecular features in each total ion chromatogram and generate data for subsequent statistical analysis.

Statistical Analysis Data are shown as mean value ± SD. Statistical analysis (biochemical and anthropometric data, specific compounds detected by metabolomics), was performed using SPSS (version 13.0 for Windows, Chicago, IL, USA), applying Student's *t*-test for related samples. The multivariate metabolomics data matrix was analyzed using SIMCA-P+12 software (Umetrics, Kinnelon, NJ, USA); unsupervised segregation before and after the treatment was checked by principal component analysis (PCA) using Pareto-scaled data. Differences with $P<0.05$ were considered significant.

Results and Discussion

Effects on Oxidative Status This exploratory clinical trial evaluated the effects of regular consumption of a glass of ARJ, equivalent to 2000 μmol Trolox units in CVD risk factors and oxidative status in young healthy volunteers. The particular antioxidant vehicle for the study (a commercial pomegranate and grape juice), as well as the dose, were chosen based on: a) dietary AC in Spain having decreased approximately 2000 μmol Trolox units per day since the 1960s [7], so this dose would be equivalent to a return to the traditional Mediterranean dietary pattern in terms of AC; b) a commercial processed product providing a standardized antioxidant content; and c) antioxidants from a beverage being more accessible in the small intestine than those from solid food, where a fraction of the polyphenols is associated with other components of the food matrix [19]. It is notable that for the ARJ chosen, this AC corresponds to a glass a day.

Oxidative status was evaluated by different markers, *i.e.*, plasma lipid oxidation as determined by MDA and plasma antioxidant capacity as determined by FRAP and ABTS. Regarding lipid oxidation, plasma MDA values were significantly ($P<0.001$) decreased (−29 %) over the study period (Fig. 1A). This tendency was kept when the subjects were divided by sex ($P<0.001$). Previous studies also reported a decrease in MDA values after supplementation with other ARJs [8].

Regarding plasma antioxidant capacity, FRAP values significantly ($P<0.001$) increased (+115 %) over the study period (Fig. 1B). This tendency was kept when the subjects were divided by sex ($P<0.001$). It is noteworthy that dietary recalls showed that no dietary modifications in foods of vegetable origin, *i.e.*, those providing antioxidants, besides the ARJ supplementation took place over the study period (Table S2). Moreover, dietary AC intake, excluding that provided by the ARJ, did not change (3248±2083 *vs* 3340±1105 μmol Trolox/p/day as determined by ABTS assay). This AC intake is similar to that previously reported for a typical current Spanish diet [7].

Therefore, supplementing common diet with a daily glass of ARJ for 8 weeks causes a sustained improvement in oxidative status, as shown by a significant increase after the supplementation period in plasma antioxidant capacity and a significant decrease in lipid oxidation. Regarding the

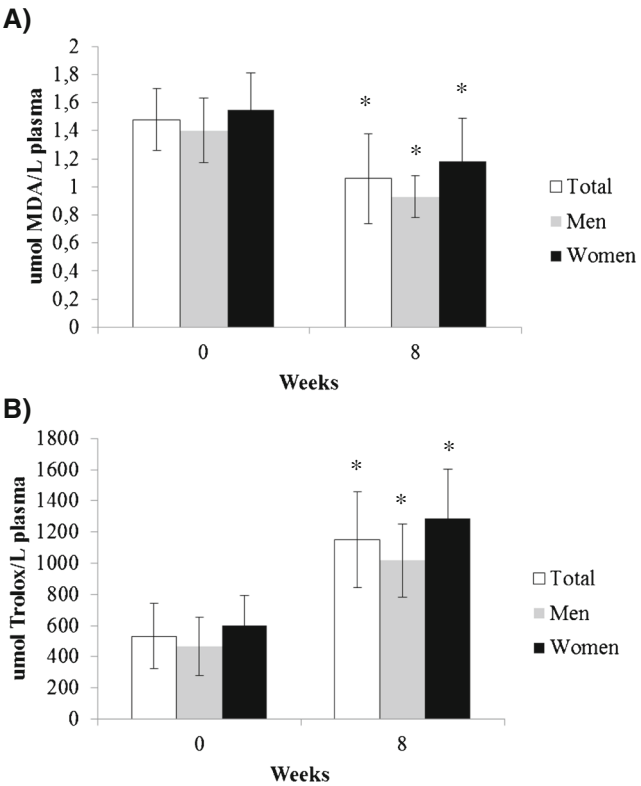

Fig. 1 Effect of 8-week supplementation with ARJ in oxidative status markers: **a**) plasma lipid oxidation (MDA); **b**) plasma AC by FRAP assay after uric acid correction. * indicates significant differences in a group before and after the treatment ($P<0.001$). *ARJ* Antioxidant –rich juice, *MDA* Malondialdehyde, *FRAP* Ferric reducing antioxidant power

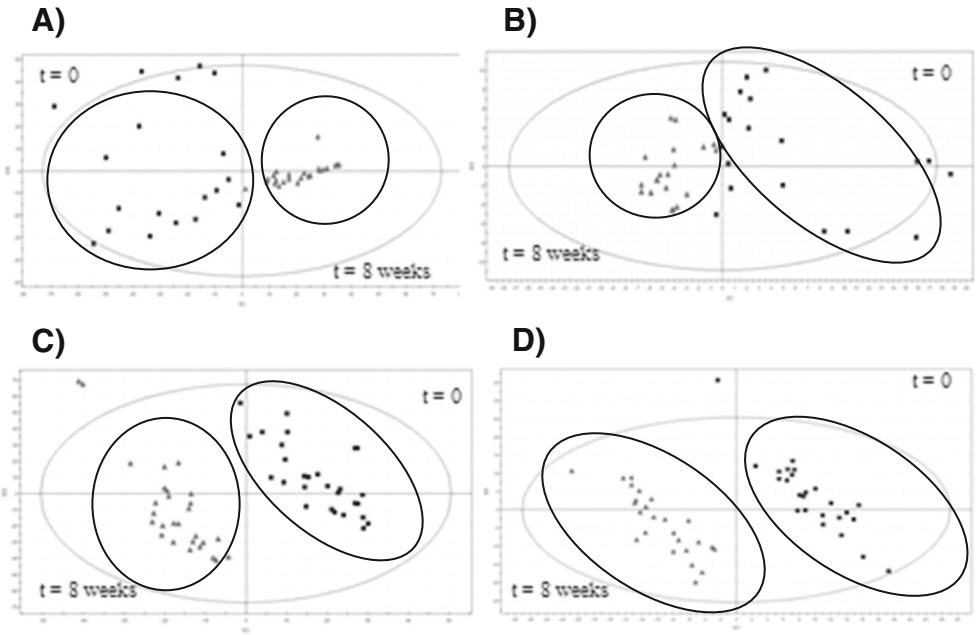

Fig. 2 Principal component analysis (PCA) scores scatter plot showing unsupervised segregation of metabolome before (*boxes*) and after the 2 months of ARJ intake (*triangles*); **a**) ESI +, plasma; **b**) ESI −, plasma; **c**) ESI+ , urine; **d**) ESI-, urine. *ARJ* Antioxidant-rich juice; ESI, electrospray ionisation

compounds responsible of these modifications, dietary antioxidants are complex mixtures of different kinds of compounds, but since the constituents of the ARJ used here – pomegranate and grape- are particularly rich in polyphenols [20, 21], the effects observed in this study may be mostly attributable to polyphenols and, to a lesser extent, to vitamin C, also found in these fruits. Indeed, previous studies have shown an increase in AC and polyphenol metabolite concentrations in plasma after acute intake of either pomegranate or red grape juice [22, 23] or a red grape concentrate rich in both polyphenols and dietary fibre [24].

In contrast to FRAP values, no significant changes in plasma AC as determined by ABTS assay were observed over the study period (2741±285 *vs* 2771± 347 μmol Trolox/L plasma). This tendency remained when subjects were divided by sex. This difference in results is due to the fact that specific AC determination methods evaluate different mechanisms of action [25]. Indeed, a recent meta-analysis on modifications in plasma AC after chronic consumption of antioxidant-rich products concluded that it was more common to detect modification by FRAP than by ABTS assay [26], which agrees with our results.

Metabolomics Modifications Preliminary untargeted metabolomics analysis of blood and urine samples was carried out. The unsupervised PCA plots (Fig. 2), where each dot represents the overall metabolome for a subject either before or after the intervention, show a clear separation between baseline and week 8; the separation was greater in urine than in plasma, and slightly better in negative than in positive mode. This indicates that a chronic supplementation to healthy subjects with an ARJ causes sustained modifications in the circulating metabolites in the organism. Moreover, the fact that the modifications were clearer for both plasma and urine metabolomes in negative MS mode than in positive MS mode suggests that the intake of the ARJ particularly affects the lipid and oxidative response metabolic pathways [27].

Table 1 Tentative identification of compounds modified by supplementation with ARJ, detected by Q-TOF LC/MS untargeted metabolomics analysis in urine with negative mode

Compound	Molecular formula	Rt (min)	$[M-H]^-$ exp	$[M-H]^-$ calc	Error (ppm)	Modification	*P* value
Urolithin A glucuronide	$C_{19}H_{16}O_{10}$	13.7	404.0765	404.0743	−5.3	Up	<0.001
Ascorbic acid sulfate	$C_6H_8O_9S$	2.5	255.9913	255.9889	−9.4	Up	<0.001
Pyrogallol sulfate	$C_6H_6O_6S$	7.8	205.9907	205.9885	−10.6	Up	<0.01
Glycocholic acid/ 3a,7b,12a-trihydroxyoxocholanyl-glycine	$C_{26}H_{43}NO_6S$	16.2	465.3088	465.3090	0.6	Down	0.07

ARJ Antioxidant-rich juice

In contrast to the overall modifications in plasma and urine metabolomes, the incorporation into the diet of the ARJ for 8 weeks did not modify any anthropometrical or biochemical parameters (glycaemia, lipid profile, inflammation markers) in this group of healthy subjects (Table S3). In that sense, it should be remarked that untargeted metabolomics is able to detect subtle modifications before they become apparent *via* common markers [28]. In the healthy subjects participating in this study it would seem reasonable not to expect to find major modifications in common clinical markers, but the modifications observed in metabolomes may indicate that some changes below the level of clinical changes were taking place; such preclinical modifications are especially interesting in the field of nutrition.

Although this was a preliminary qualitative analysis of metabolomics profiles, some ions that significantly increased during the study were identified in urine in negative mode, where there was the clearest separation between the groups (Table 1). Those ions were: urolithin A glucuronide ($P<0.001$), derived from the polyphenol punicalagin, an ellagitannin present in pomegranate [29]; pyrogallol sulfate ($P<0.01$), another polyphenol-derived metabolite previously reported in urine after the intake of green tea [30]; and ascorbic acid sulfate ($P<0.001$), derived from hepatic conjugation of ascorbic acid. The modifications in the concentrations of these compounds before and after the supplementation with ARJ strengthens the hypothesis described above for polyphenols and vitamin C as main responsible for the observed modifications in oxidative status. Also, since ellagitannins are present in a limited number of foods, the increase in urinary urolithin A glucuronide may be considered as a compliance marker of the study.tgroup

Besides, a tendency ($P=0.07$) towards a reduced urinary excretion of a conjugated secondary biliary acid, either glycocholic acid or 3a,7b,12a-trihydroxyoxocholanylglycine was observed; this would imply a decreased absorption of biliary acids, eventually leading to beneficial effects on the plasma lipid profile.

Conclusions

This study shows that daily supplementation of healthy young adults with a glass of ARJ for 8 weeks significantly improves oxidative status (increasing plasma AC and decreasing plasma lipid oxidation), together with significant modifications of the plasma and urine metabolomes. Therefore, incorporating ARJ into common diets may be a way to improve antioxidant status in Western populations. Further studies are needed to identify the metabolomics modifications precisely, as well as to evaluate the effect of this supplementation on aged subjects or subjects at high risk of CVD.

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Conflict of Interest The authors declare no conflict of interest.

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