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ORIGINAL ARTICLE

Pomegranate juice supplementation in chronic obstructive pulmonary disease: a 5-week randomized, double-blind, placebo-controlled trial

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Objective: The aim of the present study is to investigate the effect of antioxidant polyphenol-rich pomegranate juice (PJ) supplementation for 5 weeks on patients with stable chronic obstructive pulmonary disease (COPD), since the oxidative stress plays a major role in the evolution and pathophysiology of COPD.
Design: A randomized, double-blind, placebo-controlled trial was conducted.
Subjects: A total of 30 patients with stable COPD were randomly distributed in two groups (15 patients each).
Interventions: Both groups consumed either 400 ml PJ daily or matched placebo (synthetic orange-flavoured drink) for 5 weeks. Trolox Equivalent Antioxidant Capacity (TEAC) of PJ, blood parameters (14 haematological and 18 serobiochemical), respiratory function variables, bioavailability of PJ polyphenols (plasma and urine) and urinary isoprostane (8-iso-PGF$_{2\alpha}$) were evaluated.
Results: The daily dose of PJ (containing 2.66 g polyphenols) provided 4 mmol/l TEAC. None of the polyphenols present in PJ were detected in plasma or in urine of volunteers. The most abundant PJ polyphenols, ellagitannins, were metabolized by the colonic microflora of COPD patients to yield two major metabolites in both plasma and urine (dibenzopyranone derivatives) with no TEAC. No differences were found ($P>0.05$) between PJ and placebo groups for any of the parameters evaluated (serobiochemical and haematological), urinary 8-iso-PGF$_{2\alpha}$, respiratory function variables and clinical symptoms of COPD patients.
Conclusions: Our results suggest that PJ supplementation adds no benefit to the current standard therapy in patients with stable COPD. The high TEAC of PJ cannot be extrapolated *in vivo* probably due to the metabolism of its polyphenols by the colonic microflora. The understanding of the different bioavailability of dietary polyphenols is critical before claiming any antioxidant-related health benefit.
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Introduction

The 2002 WHO World Health Report (WHO, 2002) listed chronic obstructive pulmonary disease (COPD) as the fifth leading cause of death in the world (third leading cause in adults over 60 years), with increasing prevalence and mortality in the coming decades (Murray and Lopez, 1997). According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD), 'COPD is a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases'. The main aetiologic factor implicated in the pathogenesis of COPD is smoking (Repine *et al.*, 1997), although air pollution (Perez-Padilla *et al.*, 1996), occupational exposure (Vogelzang *et al.*, 1998) and genetic predisposition (Molfino, 2004) are also involved. Cigarette smoke provides a huge concentration of reactive oxygen species (ROS) that critically contribute to provoke systemic oxidative stress (Rahman *et al.*, 1996). Several studies support the role of ROS in the evolution and pathophysiology of obstructive airway diseases including COPD (Macnee, 2000; Langen *et al.*, 2003; Molfino, 2004). As oxidative stress is increased in patients with COPD (Praticò *et al.*, 1998), antioxidants could be of use in its treatment. In fact, small but significant reductions in exacerbations have been reported upon oral administration of *N*-acetyl cysteine to patients with this disease (Poole and Black, 2001). Moreover, various epidemiological and observational studies demonstrated the positive effect of dietary antioxidants such as vitamins C and E (Devereux and Seaton, 2001) and some polyphenols from fruits and vegetables (Tabak *et al.*, 2001; Watson *et al.*, 2002) on the ventilatory function and clinical manifestations of COPD patients. However, interventional approaches concerning dietary antioxidants and COPD are rather scarce (Devereux and Seaton, 2001). Recently, Culpitt *et al.* (2003) demonstrated *in vitro* the promising use of the polyphenol resveratrol against COPD.

Among dietary antioxidants, plant polyphenols might play a major role in human health. Polyphenols have a number of health-promoting effects including antiplatelet, antioxidant, anti-inflammatory, antitumoral and oestrogenic activities, which might suggest their potential in the prevention and/or amelioration of several diseases including coronary heart diseases and cancer (Arai *et al.*, 2001; Yang *et al.*, 2001; Limer and Speirs, 2004). Pomegranate juice (PJ) is a very rich source of polyphenols, especially ellagitannins (polymeric molecules with ellagic acid (EA) subunits), EA derivatives and anthocyanin pigments (Gil *et al.*, 2000). Pomegranate and derived products have been acknowledged with health-beneficial effects from ancient times (Madihassan, 1984) including hypotensive (Aviram and Dornfeld, 2001), hypocholesterolaemic and antiatherosclerotic activities (Kaplan *et al.*, 2001). However, the impressing antioxidant activity *in vitro* of PJ, higher than that of tea and red wine (Gil *et al.*, 2000), is the main feature that has attracted attention.

Taking into account the above, the aim of the present study is to conduct a randomized, double-blind, placebo-controlled trial to evaluate the effect of daily consumption of polyphenol-rich PJ with high antioxidant activity for 5 weeks on the oxidative stress and clinical features of patients with stable COPD.

Subjects and methods

Chemicals
EA, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™), β-glucuronidase (EC 3.2.1.31; 100 000 units) from bovine liver and sulphatase (EC 3.1.6.1; 10 000 units) from *Helix pomatia* were purchased from Sigma (St Louis, CA, USA). Methanol, diethyl ether, hydrochloric acid and acetic acid were obtained from Merck (Darmstadt, Germany). Ascorbic acid was obtained from Aldrich (Steinheim, Germany). Milli-Q system (Millipore Corp., Bedford, MA, USA) ultra pure water was used throughout this experiment.

PJ preparation and placebo beverage
PJ was obtained from fresh pomegranates (*Punica granatum*, cv Mollar de Albatera) by using a laboratory pilot press according to Cerdá *et al.* (2004). PJ polyphenols were fully stable under refrigeration and protected from light along the present study. Polyphenolic content of the juice was analysed by HPLC–DAD–mass scan (MS)–MS.

Placebo beverage consisted of an orange-flavoured commercial refreshment. The drink contained water, sodium cyclamate, sodium saccharine, vegetable oil, citric acid, synthetic orange flavours and stabilizer (Arabic gum) and lacked antioxidants, fruit and vegetable extracts or vitamins.

Subjects and design
This study conforms to the principles outlined in the 'Declaration of Helsinki' and has been approved by the Committee of Clinical Investigation of the 'University Virgen de La Arrixaca Hospital' (Murcia, Spain). The protocol was fully explained to the volunteers who gave their written consent prior to participation.

The inclusion criteria for participating in this trial were physician-diagnosed COPD, no acute exacerbation of COPD during 8 weeks before the study and no home oxygenotherapy required. Exclusion criteria involved asthma history, positive prick test, atopy, vegetarian diet, weight-reducing dietary regimen, alcoholism, consumption of vitamin supplements or nutraceuticals, diabetes, other respiratory diseases, hyperlipidaemia, history of gastrointestinal disease or any chronic disease besides COPD. From all COPD patients recruited to participate in the present trial, 30 men

with stable COPD and clinical history in the above hospital were finally enrolled (Table 1). The enrolment of patients in clinical trials can be sometimes especially difficult. This is the case of COPD patients whose mean age is over 60 and display inability to carry out many daily tasks. Both age and stage of disability of patients affected the design of the study (urine collection, diet restrictions, intake of juice, etc.).

Patients were randomly distributed in two groups (15 patients each). Both PJ and placebo beverage were supplied to the patients in the same type of bottle (opaque), indistinguishable for the volunteers. The drinks were weekly supplied to both groups. In addition, physicians and researchers involved in the evaluation of clinical features of COPD, clinical chemistry and isoprostane analysis did not know either the type of drink consumed by the patients (double-blind trial).

The volunteers followed a controlled diet (2 weeks before and during the 5-week trial) in which ellagitannin-containing sources such as berries (strawberry, raspberry, blackberry, etc., and derived foodstuffs such as jams), pomegranates, chocolate, nuts and wine were strictly forbidden. During these 7 weeks (2 weeks before and during the 5-week trial), patients were also asked to consume approximately the same amount of other polyphenol-containing sources (fruits, vegetables, tea, coffee, juices and olive oil). The patients provided a diet history with the food and drinks consumed along the trial to monitor the intake of specific foodstuffs that could affect the trial, such as a potential effect on colonic microflora (probiotics, prebiotics, etc.).

Table 1 Clinical characteristics of the COPD groups at the beginning of the trial[a]

Characteristics	*PJ group (n = 15)*	*Placebo group (n = 15)*
Age (year)	60.00 ± 10.90	63.40 ± 8.90
BMI (kg/m^2)	31.40 ± 4.80	30.60 ± 5.80
PaO_2 (mmHg)	72.02 ± 8.36	71.84 ± 10.46
$PaCO_2$ (mmHg)	40.63 ± 4.58	42.37 ± 5.50
FEV_1 (l)	1.46 ± 0.66	0.93 ± 0.35
FVC (l)	2.95 ± 0.84	2.21 ± 0.47
FEV_1 (%)	46.00 ± 16.01	35.10 ± 18.10
FVC (%)	77.02 ± 13.68	70.10 ± 21.90
FEV_1/FVC (%)	46.59 ± 11.69	43.24 ± 10.73
Gold classification (stage of COPD severity)		
Stage I (mild)	0	0
Stage II (moderate)	7	6
Stage III (severe)	6	6
Stage IV (very severe)	2	3
Dyspnoea score[b]		
Degree I	4	4
Degree II	7	6
Degree III	4	5
Smoking		
Current smokers	1	2
Ex-smokers	14	13
Nonsmokers	None	None
Infections	None	None
Therapy		
Inhaled β-adrenergic agonists	All	All
Ipratromium bromide	All	All

[a]Patients were randomly distributed in both groups. Plus–minus values are means ± standard deviation.
[b]According to the British Medical Research Council.

Clinical manifestations and respiratory function variables were evaluated after the randomized distribution of patients in both groups prior to the intervention. In addition, urine and blood samples were taken to determine serobiochemical, haematological and urinary 8-iso-$PGF_{2\alpha}$ baseline values.

Patients consumed 400 ml of their corresponding drinks (either PJ or placebo) daily for 5 weeks. Volunteers were free to consume the juice along the day. Patients attended the consulting room at the hospital in the morning, the same day every week (to determine respiratory function variables, for physical examination and to answer physicians' questions regarding the evolution of the trial: possible problems, accomplishment of the protocol, state of mind, etc.). In addition, the same morning, blood samples were withdrawn and patients also provided 12-h urine volume corresponding to the day before the consultation (i.e. urine sample for the last 12 h before blood withdrawal). Therefore, in the present trial, patients were examined six times (once before drinks supplementation and five times during intervention) and also provided six samples of both urine and blood (one baseline and five during intervention).

Assessment and evolution of respiratory function and clinical symptoms

All the patients were examined before and during the intervention (as mentioned above). All the patients were physically examined. Chest X-ray, spirometry and arterial blood gas determinations were carried out.

Airflow limitation of COPD patients was assessed by spirometry using a Vitalograph 21.70 spirometer (Buckingham, UK) according to the American Thoracic Society guidelines (1994). Forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and FEV_1/FVC (FEV_1 expressed as a percentage of the FVC, which gives a clinically useful index of airflow limitation) were determined before the intervention (baseline values) and once per week along the study (five determinations) as specified above.

Mean values for partial pressure of arterial oxygen (PaO_2) and arterial carbon dioxide ($PaCO_2$) were determined in an ABL 520 gas analyser (Radiometer, Denmark).

Plasma samples

Blood was collected in heparinized tubes and processed according to Cerdá *et al*. (2004). The plasma was analysed by HPLC–DAD–MS–MS. For the enzymatic treatment, the plasma was treated with both β-glucuronidase and

sulphatase as described elsewhere (Cerdá *et al*., 2004). Afterwards, the samples were homogenized with MeOH:0.2 M HCl (1:1, v:v), vortexed for 30 s and centrifuged at 14 000 *g* for 2 min at 4°C. The supernatant was filtered through a 0.45 μm filter, and analysed by HPLC–DAD–MS–MS.

Urine samples
A volume of 40 ml of urine of each sample provided by the patients was filtered through a Sep-Pak solid-phase extraction cartridge (a reverse phase C-18 cartridge; Waters Millipore, Bedford, MA, USA). The cartridges were previously activated with 10 ml of MeOH, 10 ml of water and subsequently emptied with 10 ml of air. After eluting the sample volume, the cartridge was washed with 10 ml of water. The remaining volume in the cartridge was eluted with 2 ml of MeOH. A sample of 100 μl of the methanolic fraction was analysed by HPLC–DAD–MS–MS.

HPLC–DAD–MS–MS analysis
The HPLC–DAD system was equipped with a mass detector from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer (Agilent Technologies) equipped with an electrospray ionization (ESI) system (capillary voltage 4 kV, dry temperature 350°C) for the analysis of nonenzymatically treated samples (blood, urine and PJ). For the analysis of enzyme-treated blood and urine samples, the mass detector was equipped with an atmospheric pressure chemical ionization (APCI) system (capillary voltage 4 kV, dry temperature 350°C, crown voltage 4 kA, APCI temperature 375°C). MS and MS–MS daughter spectra were measured from m/z 150 up to m/z 500 for urine and blood samples and from m/z 200 up to m/z 2000 for PJ. Collision-induced fragmentation experiments were carried out in the ion trap using helium as the collision gas and the collision energy was set at 50%. Mass spectrometry data were acquired in the positive ionization mode for enzyme-treated samples (with APCI) and in the negative ionization mode for the juice and nontreated urine and blood samples (with ESI). Chromatographic separations of PJ and urine samples were carried out on a reverse-phase C_{18} LiChroCART column (25×0.4 cm, particle size 5 μm, Merck, Darmstadt, Germany) according to the protocol of Cerdá *et al*. (2004).

Quantification of PJ polyphenols and related metabolites
Polyphenols in PJ were identified according to their UV spectra as well as ion mass (MS), and daughter fragments (MS–MS) using ion trap (Cerdá *et al*., 2004). Anthocyanins were quantified as cyanidin-3-glucoside at 510 nm (Cerdá *et al*., 2004). Identification of EA was carried out by chromatographic comparisons (UV and MS) with a pure standard of EA. EA and its glycoside derivatives were quantified as EA at 360 nm. Punicalagin isomers (the most abundant ellagitannin in PJ) were quantified at 360 nm using an external standard of punicalagin previously isolated (Cerdá *et al*., 2003). The *in vivo*-generated metabolites derived from pomegranate ellagitannins and EA were identified according to their UV spectra, retention times, ion mass and MS–MS daughter fragments using the corresponding purified metabolites previously isolated from human urine (Cerdá *et al*., 2004). The metabolites were quantified at 305 nm.

Antioxidant assay (ABTS$^{\bullet+}$)
ABTS$^{\bullet+}$ assay was carried out according to Espín and Wichers (2000). *In vitro* antioxidant activity was expressed as Trolox Equivalent Antioxidant Capacity (TEAC) following the nomenclature of Rice-Evans and Miller (1994). Coefficient of variation was always less than 5%.

Haematological and serobiochemical parameters
Biochemical parameters were determined in serum using an automated biochemical auto-analyser HITACHI Modular D + P (Roche Diagnostics, Switzerland). The parameters analysed were: glucose, urea, creatinine, uric acid, total proteins, albumin, total bilirubin, cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and ferritin. Haematological parameters were determined in EDTA-treated blood using an automated haematological analyser (Cell-Dyn 3700 and 4000, Abbott, IL, USA). The parameters analysed were: red blood cells, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets, mean platelet volume, leucocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophils.

Lipidogram
The separation of lipoproteins in human serum was carried out in agarose gels by using the Paragon Lipoprotein Electrophoresis Kit (Beckman Instruments Inc., Palo Alto, CA, USA) according to Cerdá *et al*. (2004). The gel was dried and the lipoprotein pattern visualized by using the Sudan Black B Stain-containing Paragon Lipo Stain (0.07%; w:w) (Paragon®). Lipidogram allowed determining chylomicrons, α-lipoprotein, pre-β-lipoprotein and β-lipoprotein.

Urinary isoprostane
The major F_2-isoprostane, 8-epi-prostaglandin $F_{2\alpha}$ (also known as 15-isoprostane F_{2t}, 8-epi-$PGF_{2\alpha}$ or 8-iso-$PGF_{2\alpha}$), was measured using a commercially available enzyme-linked immunoassay (EIA) kit previously validated by manufacturers using gas chromatography/mass analysis (GC/MS) (Oxford Biomedical Research, Oxford, MI, USA). Briefly, urine samples were mixed with an enhancing reagent that essentially eliminates interferences due to nonspecific

binding, and the 8-iso-$PGF_{2\alpha}$ in the samples or standards competes with 8-iso-$PGF_{2\alpha}$ conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 8-iso-$PGF_{2\alpha}$ coated on the microplate. The HRP activity results in colour development when substrate is added, with the intensity of the colour proportional to the amount of 8-iso-$PGF_{2\alpha}$ bound and inversely proportional to the amount of unconjugated 8-iso-$PGF_{2\alpha}$ in the samples. Absorbance was measured at 450 nm in a STAT FAX-2100 spectrophotometer (Awareness Tech. Inc., Palm City, FL, USA). Urinary 8-iso-$PGF_{2\alpha}$ values were determined before intervention, at the third week and at the end of the study (fifth week).

Statistics
Before beginning the experiment, a statistical analysis was carried out in order to determine the minimum number of subjects (sample size) required to establish significantly statistical conclusions. The number of subjects was based on the construction of a confidence interval (Wonnacott and Wonnacott, 1990). The required sample size to get a confidence interval of 95% was determined for each parameter analysed. Analysis of variance (ANOVA) was used to establish differences in the PJ and placebo groups upon consumption of their corresponding drinks (PJ and placebo, respectively) for 5 weeks. Values are expressed as mean ± s.d. (standard deviation). The normal values range (NVR) establishes the limit between normal and pathological values for serobiochemical and haematological parameters. In the present study, the NVR were those reference values used by the Hospital's laboratory. Differences were considered significant at $P \leqslant 0.05$. Statistic analyses were carried out with the SPSS 11.0.1 program (SPSS Inc., Chicago, IL, USA). Graphs of the experimental data were carried out by using the Sigma Plot™ 6.0 program for Windows™.

Results

The PJ used in the present trial was very rich in the polymeric polyphenols ellagitannins (ETs), especially punicalagin isomers, punicalin, free EA and EA glycosides. In addition, six anthocyanin pigments were identified as delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides. This juice contained 2.4 g/l punicalagin, 1.97 g/l punicalin, 0.61 g/l free EA, 0.60 g/l EA-glycosides and 0.49 g/l anthocyanins. This means that the volume of PJ (400 ml) consumed by COPD patients involved a daily intake of 2.66 g polyphenols (Table 2). As a whole, COPD patients received during this trial 93.1 g total polyphenols, including 61.25 g of ETs. Therefore, according to the *in vitro* antioxidant activity of this juice, COPD patients received 4 mmol/l TEAC daily (equivalent to 2.5 g Trolox/l) and 140 mmol/l TEAC (87.5 g Trolox/l) after 5 weeks, at the end of the trial.

The absorption and metabolism of PJ polyphenols was monitored along the trial in the corresponding group that consumed PJ. Ingested polyphenols (in the molecular form present in the PJ supplied) were not detected either in plasma or in urine. However, two EA-derived metabolites, conjugated with glucuronic acid, that is, 3,8-dihydroxy-6H-dibenzo(b,d)pyran-6-one (also known as 'urolithin A'), and 3-hydroxy-6H-dibenzo(b,d)pyran-6-one ('urolithin B'), were identified in both plasma and urine of COPD patients belonging to the PJ group (Figure 1). A high individual variability was observed in the metabolism of PJ by COPD patients along the trial (results not shown). This was supported by previous reports regarding the metabolism of ellagitannins by humans (Cerdá *et al.*, 2005) and by the no relationship between metabolism of PJ polyphenols and the diet histories provided by the patients (results not shown). At the end of the 5-week intervention period, a mean concentration of the above metabolites, 2.01 ± 1.6 and 2.4 ± 2.3 mg/ml, respectively, was detected in urine. No relation was found ($P > 0.05$) between the metabolism of PJ polyphenols (accumulation of dibenzopyranone metabolites either in plasma or urine) and age of patients, dyspnoea score, COPD stage, respiratory function or any other clinical manifestation associated to COPD.

There were no statistically significant differences in any of the variables or parameters evaluated (serobiochemical, haematological, FEV_1, FVC, FEV_1/FVC, $PaCO_2$, PaO_2) within each group over the 5-week study (Table 3) and thus no differences were observed either in these values as a consequence of PJ supplementation when both groups were compared. No significant differences in any of the variables or parameters evaluated were found between baseline values of the two groups (results not shown).

8-Iso-$PGF_{2\alpha}$ values of COPD patients participating in the present trial, determined with the EIA kit, ranged from 1 to 8.8 pg/mg creatinine in the PJ group and from 1.42 to 8.75 pg/mg creatinine in the placebo group. No relation was found in COPD patients between urinary isoprostane concentration and any of the above parameters evaluated. Mean 8-iso-$PGF_{2\alpha}$ values did not significantly change in both PJ and placebo groups over the 5-week period ($P > 0.05$) (Table 3).

Table 2 Polyphenol content and TEAC of PJ consumed by COPD patients[a]

Polyphenols and TEAC	*Per day (volume: 400 ml)*	*Over 5 weeks (total volume: 14 l)*
Ellagitannins		
Punicalagin isomers (g)	0.96	33.60
Punicalin (g)	0.79	27.65
EA-glycoside derivatives (g)	0.48	16.80
Free EA (g)	0.24	8.40
Total anthocyanins (g)	0.19	6.65
Total polyphenols (g)	2.66	93.1
TEAC (mmol/l)	4	140

[a]Using the ABTS method and according to the nomenclature of Rice-Evans and Miller.

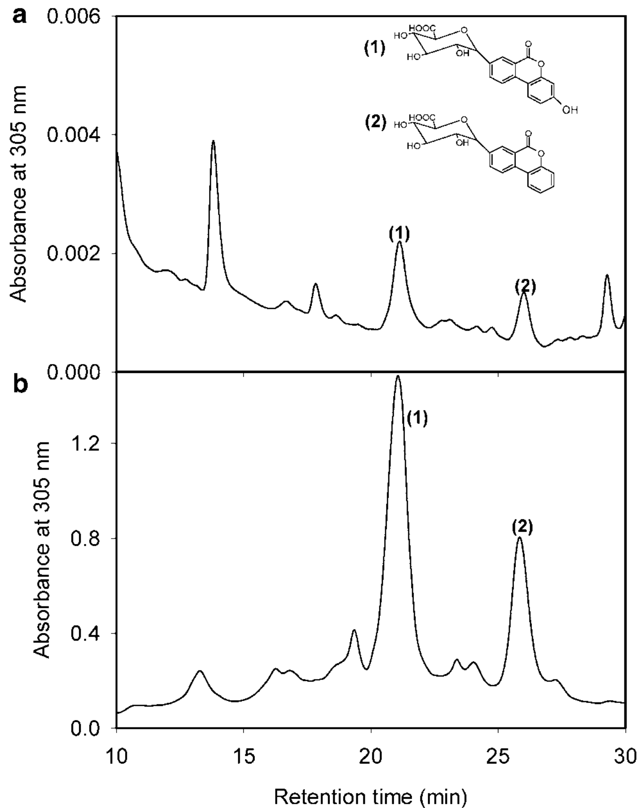

Figure 1 Representative HPLC profiles of plasma (**a**) and urine (**b**) of a COPD patient upon PJ consumption after 1 week. (1) Urolithin A-glucuronide: 3,8-dihydroxy-6H-dibenzo(b,d)pyran-6-one glucuronide ($m/z^{-}=404$; MS/MS = 175, 227), (2) Urolithin B-glucuronide: 3-hydroxy-6H-dibenzo(b,d)pyran-6-one glucuronide ($m/z^{-}=389$; MS/MS = 175, 213).

Although no effect was observed in any of the parameters or variables evaluated upon PJ supplementation, some patients declared in the weekly attendance to the consultation an improvement in their ability to carry out daily tasks. However, these patients belonged to both groups and thus it was an evident 'placebo effect'.

Discussion

PJ has been acknowledged as one of the most antioxidant foodstuffs described so far, with antioxidant capacity *in vitro* about three-fold higher than that of well-known antioxidant beverages such as tea or red wine (Gil *et al*., 2000). PJ owes its impressive *in vitro* antioxidant activity to its polyphenolic constituents, especially to ellagitannins (ETs), very abundant in PJ (Gil *et al*., 2000). A recent study reported that the supplementation of 1 l of PJ to healthy subjects for 5 days had no effect either on plasma antioxidant status or cardiovascular risk markers (Cerdá *et al*., 2004). However, the effect of a longer PJ supplementation in subjects with unbalance oxidative status remained unanswered so far. Therefore, the present trial was conducted to investigate whether polyphenol-rich PJ (with high TEAC) could ameliorate the systemic oxidative stress of COPD patients as a potential dietary complement to standard therapy.

Despite the high daily TEAC (4 mmol/l) provided to the COPD patients who consumed PJ (Table 2), no effect was observed in any serobiochemical and haematological parameter or respiratory function variables (Table 3). Our possible hypothesis to explain this lack of effect is the metabolism of the major antioxidant fraction of PJ, the polyphenols ellagitannins, by the colonic microflora of COPD patients to yield two bioavailable dibenzopyranone derivatives with negligible antioxidant activity as recently reported in the aforementioned report that described the supplementation of PJ in healthy subjects (Cerdá *et al*., 2004). In fact, these dibenzopyranone derivatives have been proposed as biomarkers of human exposure to ellagitannins and EA (Cerdá *et al*., 2005).

Although the 'ideal' biomarker to measure oxidative damage does not exist, F_2-isoprostane is one of the most widely accepted biomarkers to determine lipid peroxidation *in vivo* (Halliwell and Whiteman, 2004). In fact, F_2-isoprostanes (8-Iso-$PGF_{2\alpha}$) have been proposed as potential *in vivo* indicators of oxidative stress in various clinical conditions (Praticò *et al*., 1998, 1999; Proudfott *et al*., 1999). 8-Iso-$PGF_{2\alpha}$ of healthy subjects range from 0.27 to 3.5 pg/mg creatinine (determined with EIA) (Engler *et al*., 2004). These values are usually increased in COPD patients (Praticò *et al*., 1998) since reactive oxygen species (ROS) are involved in the evolution and pathophysiology of this disease (Langen *et al*., 2003). Data regarding the effect of polyphenol antioxidants on isoprostane concentration are apparently contradictory in the literature. For example, lack of effect has been described for polyphenols from chocolate (Öner-Iyidogan *et al*., 2004), red wine (Waddington *et al*., 2004), onions and black tea (O'Reilly *et al*., 2001). However, decrease of isoprostanes upon polyphenol intake has been reported for soy (Wiseman *et al*., 2000) and also for cocoa polyphenols (Wiswedel *et al*., 2004).

In the present trial, PJ supplementation did not significantly alter the urinary 8-iso-$PGF_{2\alpha}$ concentration in COPD patients (Table 3). Therefore, the high potential antioxidant activity ingested by COPD patients belonging to the PJ group (4 mM TEAC) had no effect on systemic oxidative stress of COPD patients, supporting the lack of effect on any respiratory function variable evaluated (Table 3). In the case of lung obstructive diseases such as COPD, the bioavailability of dietary antioxidants to reach lung airways is critical (Barnes and Hansel, 2004). However, no bioavailable polyphenol antioxidants (in the molecular form present in PJ) that could reach lung airways were detected in COPD patients.

Our results suggests that daily supplementation of polyphenol-rich PJ (with high TEAC) is not promising as possible dietary supplement to ameliorate the oxidative stress

Table 3 Differences in parameters and variables evaluated in COPD patients over 5 weeks in the PJ and placebo groups[a,b]

Parameters	*PJ group*			*Placebo group*		
	Baseline	*5 weeks*	P-*value*	*Baseline*	*5 weeks*	P-*value*
Glucose (mg/dl)	114.46±25.53	120.80±40.30	0.54	113.73±51.53	116.40±53.60	0.98
Urea (mg/dl)	38.62±9.95	36.70±11.10	0.74	36.45±9.67	34.10±8.60	0.24
Creatinine (mg/dl)	0.94±0.19	0.97±0.20	0.53	0.89±0.20	0.92±0.21	0.61
Uric acid (mg/dl)	6.40±1.10	6.50±1.15	0.45	5.83±1.81	5.90±1.81	0.89
Total proteins (g/dl)	7.06±0.51	7.09±0.52	0.62	6.95±0.42	6.97±0.39	0.85
Albumin (g/dl)	4.38±0.28	4.49±0.28	0.57	4.38±0.13	4.43±0.21	0.32
Total bilirubin (mg/dl)	0.63±0.33	0.58±0.26	0.63	0.56±0.21	0.55±0.23	0.81
Cholesterol (mg/dl)	205.54±44.89	209.68±39.10	0.83	197.45±33.79	201.34±32.64	0.89
Triacylglycerols (mg/dl)	157.85±210.63	170.68±187.10	0.98	127.55±51.03	137.91±78.22	0.66
HDL (mg/dl)	56.67±13.72	55.05±12.01	0.39	56.09±22.59	56.75±20.81	0.93
LDL (mg/dl)	127.00±39.14	130.48±32.29	0.43	115.91±27.46	116.06±29.14	0.77
GOT (U/l)	18.54±5.01	21.92±10.97	0.16	21.27±8.22	21.37±8.39	0.88
GPT (U/l)	20.00±13.32	26.59±18.46	0.12	20.10±9.46	20.53±9.99	0.98
Ferritin (ng/ml)	234.08±182.18	280.04±210.44	0.82	132.9±94.83	127.47±91.71	0.85
Chylomicrons (%)	0.75±0.63	1.09±1.25	0.24	1.08±1.37	0.87±0.93	0.88
α-Lipoprotein (%)	28.72±8.79	27.02±7.66	0.32	27.51±6.54	28.70±8.25	0.45
Pre-β-lipoprotein (%)	22.89±14.47	25.82±13.70	0.25	26.85±11.71	25.66±9.47	0.95
β-Lipoprotein (%)	46.85±10.07	45.56±10.33	0.29	44.50±8.39	44.29±8.67	0.55
Isoprostane (pg/mg creatinine)	4.46±2.37	4.50±2.97	0.49	4.70±2.87	4.41±2.97	0.44
FEV_1 (l)	1.46±0.66	1.38±0.61	0.74	0.93±0.36	0.91±0.43	0.92
FVC (l)	2.95±0.84	2.94±0.76	0.97	2.21±0.47	2.03±0.48	0.39
FEV_1/FVC (%)	46.60±11.70	45.60±13.28	0.84	43.24±10.73	44.1±12.91	0.44
PaO_2 (mmHg)	72.02±8.36	72.19±8.70	0.96	71.84±10.46	71.67±9.98	0.97
$PaCO_2$ (mmHg)	40.63±4.58	40.35±4.66	0.88	42.37±5.50	42.50±5.26	0.95

[a]Haematological parameters are not shown ($P>0.05$ for all values).
[b]The NVR for serobiochemical parameters were those reference values used by the Hospital's laboratory: glucose 76–110 mg/dl; urea 10–50 mg/dl; creatinine 0.7–1.3 mg/dl; uric acid 3.4–7 mg/dl; total proteins 6.6–8.7 g/l; albumin 3.5–5.3 g/l; total bilirubin 0.1–1.1 g/l; cholesterol 50–230 mg/dl; triacylglycerols 50–200 mg/dl; HDL 45–75 mg/dl; LDL<130 mg/dl; GOT 5–37 U/l; GPT 5–40 U/l; ferritin 30–400 ng/ml; chylomicrons 0–2%; α-lipoprotein 9.8–46.2%; pre-β-lipoprotein 0–29.6%; β-lipoprotein 40.7–71.9%.

associated to COPD pathophysiology. *In vitro* antioxidant activity of PJ has been reported previously (Gil *et al*., 2000; Noda *et al*., 2002; Singh *et al*., 2002) and the antiatherogenic and atherosclerotic effects of PJ have been attributed to its antioxidant properties (Aviram *et al*., 2000). However, the present trial, together with our recent report regarding the supplementation of PJ on healthy subjects (Cerdá *et al*., 2004), critically questions that the potential health benefits of PJ are mediated by its antioxidant activity. Regardless of the not yet well-established relationship between *in vitro* antioxidant capacity and health benefits of PJ, other health-promoting effects of PJ, beyond antioxidant activity, cannot be discarded as suggested already for other polyphenolic-rich foodstuffs (Waddington *et al*., 2004). Whether longer supplementation periods (months or years) with PJ may have some beneficial effects on COPD patients cannot be ruled out and has not been approached in this trial. In fact, from a dietary point of view, a preventive (chronic) rather than therapeutic (acute) effect should be expected for dietary phytochemical-rich foodstuffs.

Very recently (coincident with the submission of the present study), the decrease of urinary isoprostanes (up to 50%) in COPD patients upon consumption of polyphenol-enriched grape extracts (0.5 g polyphenols daily) for 40 days has been reported (Santus *et al*., 2005). Although this study was not placebo-controlled, the diet of COPD patients ($n=11$) was not controlled and polyphenolic analysis as well as absorption and metabolism studies were not carried out; however, the impressing decrease of isoprostanes described together with the improvement of ventilatory functions in COPD patients confirms our hypothesis regarding the critical relationship between the bioavailability and bioactivity of polyphenols. Therefore, our results support previous investigations suggesting that the *in vitro* antioxidant activity of dietary phytochemicals such as polyphenols does not seem to be a valid 'health-promoting index' with direct extrapolation *in vivo*. This will depend on the specific bioavailability and metabolism of each polyphenol. In this context, studies on absorption, metabolism and fate of both ingested phytochemicals and *in vivo*-generated metabolites for each phytochemical-rich foodstuff should precede any antioxidant health claim.

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