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Ana Faria
Rosário Monteiro
Nuno Mateus
Isabel Azevedo
Conceição Calhau

Effect of pomegranate (*Punica granatum*) juice intake on hepatic oxidative stress

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A. Faria (✉) · R. Monteiro · I. Azevedo
C. Calhau
Dept. of Biochemistry (U38-FCT)
Faculty of Medicine of the
University of Porto
Al. Prof. Hernâni Monteiro
4200-319 Porto, Portugal
Tel./Fax: +351-22/551-3624
E-Mail: anafaria@med.up.pt

A. Faria · N. Mateus
Chemistry Investigation Centre
Dept. of Chemistry, Faculty of Sciences
University of Porto
Porto, Portugal

R. Monteiro
Faculty of Nutrition and Food Sciences
University of Porto
Porto, Portugal

Abstract Pomegranate juice (PJ) possesses a high antioxidant activity, which has been related to beneficial health properties. However, *in vivo* confirmation and characterization of these effects on biological systems are lacking and needed. This study was performed in order to investigate the effect of prolonged PJ ingestion on general oxidation status. For this purpose, mice ingested PJ (or water in control group) during four weeks, after which damage to lipids, proteins and DNA were evaluated as oxidative cell biomarkers. Levels of hepatic glutathione and the activities and expression of enzymes involved in its metabolism were determined. Catalase and SOD activities were quantified as these enzymes have a crucial role in antioxidant defence. Protection against protein and DNA oxidation was found in PJ group. There was also a significant decrease in GSH and GSSG, without change in the GSH/GSSG ratio. All studied enzymatic activities (GPx, GST, GR, SOD and catalase) were found to be decreased by PJ treatment. Additionally, RT-PCR results showed that GST and GS transcription were also decreased in this group. These results are compatible with a protective effect of PJ against systemic oxidative stress in mice.

Abbreviations: GPx: Glutathione peroxidase, GR: Glutathione reductase, GS: Glutathione synthetase, GSH: reduced glutathione, GSSG: Oxidized glutathione, GST: Glutathione-*S*-transferase, PJ: Pomegranate juice, REL: Relative expression level, RNS: Reactive nitrogen species, ROS: Reactive oxygen species, SOD: Superoxide dismutase

Key words antioxidant defence enzymes – glutathione – oxidative damage – pomegranate juice

Introduction

It may seem controversial that oxygen can be damaging to living organisms. However, the knowledge that oxygen and oxygen-derived species can cause damage to cellular components is not new [1]. Organisms living under aerobic conditions have evolved several antioxidant defence systems to cope with oxygen derived potentially harmful entities, collectively called reactive oxygen species (ROS). If the generation of reactive species is high and overcomes the effectiveness of the antioxidant defence system, a condition referred to as oxidative stress arises [2]. Damage to cellular components, mainly lipids, proteins and nucleic acids occurs, impairing

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the functions of the involved molecules, which in turn results in metabolic insufficiencies or defects [1, 3]. Oxidative stress plays a critical role in cancer, inflammatory, cardiovascular and neurodegenerative diseases as well as in aging [4, 5].

Endogenous protection against oxidative stress is achieved by enzymes that catalytically remove free radicals and other reactive species. These include superoxide dismutase, catalase and glutathione peroxidase. Another possibility is the existence of low-molecular weight agents that scavenge ROS like glutathione and α-tocopherol.

There is an intimate relationship between nutrition and the antioxidant defence system, as some exogenous low molecular weight antioxidants may be supplied by the diet. These two main systems of the antioxidant defence act in coordination, their levels being regulated by each other, to avoid oxidative stress events [6]. In the past few years, a considerably large group of molecules widespread in plants has come into focus.

Although only recently has pomegranate (*Punica granatum*) been acclaimed for its health benefits, this fruit has long been cultivated and consumed, as a fresh fruit or in the form of beverage, especially in the Mediterranean region. Pomegranate fruit, juice and peel possess a marked antioxidant capacity [7–9] with a high content in polyphenols, in particular, ellagitannins, condensed tannins and anthocyanins [9, 10]. Some of these antioxidant molecules have been shown to be bioavailable and safe [11, 12]. Pomegranate juice has been proposed as chemopreventive, chemotherapeutic, antiatherosclerotic and antiinflammatory [13–17] and accordingly its consumption has grown tremendously [7, 12]. However, it would be important to confirm and characterize PJ *in vivo* effects on biological systems. Therefore, this study was performed in order to investigate the effect of prolonged PJ ingestion on general oxidation status in mice.

Materials and methods

Reagents

2,4-dinitrophenylhydrazine, thiobarbituric acid (Merck, Darmstadt, Germany); 1,2-chloro-2-dinitrobenzene, 2-vinylpyridine, 5,5′-dithio-bis(2-nitrobenzoic acid), 8-hydroxy-2′-deoxyguanosine, cumene hydroperoxide, glutathione reductase, NADPH, malonaldehyde, nitrotetrazolium blue chloride, nuclease P1, oxidized glutathione, reduced glutathione, sodium pentobarbital, xantine, xantine oxidase (all from Sigma, St. Louis, MO, USA); alkaline phosphatase, guanidine hydrochloride (Applichem, Darmstadt, German).

Pomegranate juice preparation

Ten kg of pomegranates (*Punica granatum*) were washed and manually peeled, without separating the seeds. Juice was obtained using a commercial blender (Moulinex, France), filtrated with a buchner funnel and immediately stored at −20°C for no longer than 2 months.

Animals and diets

Twelve male CD1mice (Iberian Harlan, S.L., Barcelona, Spain) weighing 32 ± 2 g were kept under controlled environmental conditions (22–24°C, 12 h light/dark cycles) for one week until the beginning of treatment. Mice were housed three per cage, randomly divided into two groups and fed for four weeks with different beverages: either the control beverage (water) or pomegranate juice (PJ). The juice was supplied on dark water bottles and renewed every 2–3 days. Both groups ingested proper chow for laboratory animals (Iberian Harlan, S.L., Barcelona, Spain) *ad libitum* with the following composition: 16% protein, 5.58% ash, 2.27% fat, 5.20% cellulose, 7 mg/kg copper, 12000 UI/kg of vitamin A, 1400 UI/kg of vitamin D and 60 mg/kg of vitamin E. Chow and fluid intake were monitored and the average amount ingested per animal in each cage was calculated. Their average daily intake of fluid was 13.5 ml and 7.7 ml and chow intake was 4.3 g and 5.5 g per animal in each group, respectively.

Pomegranate juice stability

Pomegranate juice stability was assessed by measuring initial total phenolic and anthocyanins content and evaluating the alterations after 2 and 3 days of exposure to the same conditions as the juice supplied to the animals. Total polyphenol content of the pomegranate juice was 1844.0 ± 29.1 mg/l catechin equivalents, determined following the Folin-Ciocalteu method, as described [18]. Anthocyanin content was 306.1 ± 11.1 μM, evaluated spectrophotometrically at 520 nm. Both parameters were not changed for the evaluated period.

Sample preparation

Animals were anesthetized with sodium pentobarbital (60 mg/kg b.w.) and transcardiac perfusion was performed with ice-cold NaCl 0.9% solution. Liver was extracted, weighed and homogenized using a Thomas-Teflon homogenizer in a solution of 62.5 mM

KH_2PO_4, 50 mM Na_2HPO_4, 0.1% Triton X-100, pH 7.4 (2 ml per g), and kept continuously on ice. This mix was centrifuged at 13000 rpm for 10 min at 4°C and the resultant supernatant was used for all the described assays.

Ex vivo measurements of antioxidant activity

Extent of lipid peroxidation was measured by the assay for thiobarbituric acid reactive substances (TBARS). A volume of the supernatant previously obtained was mixed with 2 volumes of 10% trichloroacetic acid and centrifuged for 2 min at 13,000 rpm. Aliquots of the supernatants were added to an equal volume of 1% thiobarbituric acid; the mixture was heated for 10 min in a boiling water bath and allowed to cool. Absorbance readings were performed at 535 nm. TBARS are presented as malonaldehyde equivalents/mg protein, calculated using a malonaldehyde standard curve [19].

Protein carbonyl content was used as a marker of protein oxidative damage. A volume of liver supernatant was mixed with 2 volumes of 10% trichloroacetic acid and centrifuged for 2 min at 13,000 rpm. Carbonyl content was measured in the resultant pellet, that was treated with 0.5 ml of 2,4-dinitrophenylhydrazine (10 mM in HCl 2 M) or 0.5 ml of HCl 2 M for the blank. Samples were incubated for 1 h at room temperature with vortexing every 10 min. 0.5 ml of TCA 20% was added to each tube and stand for 15 min at 4°C. The resultant pellet was washed 3 times with ethanol-ethyl acetate (1:1), centrifuged at 13,000 rpm for 2 min at 4°C and dissolved in 1 ml guanidine 6 M overnight. The solution was centrifuged at 3,000 rpm for 15 min. Absorbance was read at 340 nm and carbonyl content was calculated using the extinction coefficient of 22 000 M^{-1} cm^{-1}.

DNA oxidative damage was quantified by measuring 8-hydroxy-2′-deoxyguanosine (8-OHdG) as a cellular biomarker. DNA was extracted from liver tissue using a commercial extraction kit (V-gene®, Bioron, Ludwigshafen, Germany) according to the producer's instructions. DNA was dissolved in water and digested with 8 U of nuclease P1 (prepared in 20 mM acetate buffer; pH 5.0) at 37°C for 30 min. The solution was then incubated with 1.3 U of alkaline phosphatase at 37°C for 1 h in 0.1 M Tris–HCl buffer (pH 7.5) [20]. After the incubation, 50 µl of chloroform was added, and samples were vortexed for 10 s and centrifuged at 5000 g for 5 min [21]. Supernatant was collected and analysed by HPLC-EC (Gilson 302, Gilson Medical Electronics, Villiers le Bel, France) on a 25 × 3.2 mm i.d., Superspher® RP18–4 Endcap column (HiCHROM, Merck, Germany); detection was carried out by electrochemical detection (Gilson 141) at 10 V. Solvent was 50 mM KH_2PO_4/K_2HPO_4, 10% (v/v) methanol, pH 5.5 at a flow rate of 0.5 ml/min. 8-OHdG concentration was calculated from a calibration curve, using commercial 8-OHdG as standard.

Biochemical analyses

GSH and GSSG hepatic contents were determined by the 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB)–GSSG reductase recycling assay [22]. The formation of 5-thio-2-nitrobenzoic acid (TNB) was followed for 3 min at 405 nm and compared with a proper standard curve. GSSG was determined using 2-vinylpyridine for derivatization of GSH. The GSH level was calculated by subtracting GSSG content from the total glutathione content (GSH = total GSH—(2·GSSG)). Results were expressed in nmol of GSH or GSSG per mg of protein. Glutathione reductase (GR) activity was assayed spectrophotometrically by following nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) [23]. Enzyme activity was expressed as U of GR/mg of protein (1 unit of GR is defined as the amount of enzyme required to convert 1 nmol NADPH to $NADP^+$ per min). Glutathione-*S*-transferase (GST) was assayed as described [24]. The formation of GSH conjugate with 1,2-chloro-2-dinitrobenzene was monitored for 5 min at 340 nm. GST activity was calculated using an extinction coefficient of 9.6 mM^{-1} cm^{-1} and expressed as U of GST/mg of protein (1 unit of GST is defined as the amount of enzyme required to catalyse the formation of 1 µmol S-2,4-dinitrophenylglutathione per min). Glutathione peroxidase (GPx) activity was assayed by NADPH oxidation at 340 nm when GSSG is reduced back by glutathione reductase [25], using cumene hydroperoxide as substrate. GPx activity was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1} and expressed as U of GPx/mg of protein (1 unit of GPx is defined as the amount of enzyme required to convert 1 nmol NADPH to $NADP^+$ per min). Superoxide dismutase (SOD) activity was assessed using a xanthine-xanthine oxidase system to generate superoxide radicals ($O_2^{\cdot -}$) [26]. The rate of suppression of the reduction of nitrotetrazolium blue by $O_2^{\cdot -}$ was monitored at 550 nm. Enzyme activity was expressed as U of SOD/mg of protein (1 unit of SOD is defined as the amount of enzyme required to inhibit the rate of NBT reduction by 50%). Catalase activity was measured by monitoring the decomposition of H_2O_2 at 240 nm (extinction coefficient 0.00394 ± 0.0002 mM^{-1} mm^{-1}) [27]. Enzyme activity was expressed as U of catalase/mg protein (1 unit of catalase is defined as the amount of en-

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Table 1 Sequences of mouse primers used in RT-PCR experiments. (GR—glutathione reductase; GST—Glutathione-*S*-transferase; GPx—Glutathione peroxidase; GS—glutathione synthetase; GAPDH—glyceraldehydes-3-phosphate dehydrogenase; bp—base pairs)

Primer name	Sequence (5′–3′)	Product size (bp)
GR	TGC GTG AAT GTT GGA TGT GTA CCC [28] CCG GCA TTC TCC AGT TCC TCG	554
GST	TGG CCG GAA GCA CAA CCT G [28] CCC CAC CAA CAC CGG CAC	449
GPx	GCC CTC CCA CTG CAG AAC TCC [28] GCT GCC TGC CGC CTC ATG	193
GS	ACG CTT TTC CCC TCA CCA GTA CC [28] GTC GGT GCA CAG CTG GAG TCC	343
GAPDH	AGT ATG ATG ACA TCA AGA AGG [29] ATG GTA TTC AAG AGA GTA GGG	421

zyme required to hydrolyse 1 μmol of H_2O_2 per min).

RNA extraction

RNA was extracted from the liver using Tripure Isolation Reagent (Roche, Indianapolis, USA), according to the producer's instructions. RNA was dissolved in water (diethylpyrocarbonate-treated) and stored at −80°C.

RT-PCR

Five μg of RNA were used as template for cDNA production through incubation with reverse transcriptase (Reverase, Bioron GmbH) for 1 h at 45°C, in 10 μM random hexamers, 0.375 mM per dNTP, 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris–HCl, pH 8.3, 10 mM dithiothreitol, and 40 units RNase inhibitor (RNaseOUT™; Gibco BRL), followed by 10 min at 95°C to inactivate the enzyme. Samples were incubated for 30 min at 37°C with 0.1 mg/ml RNAse. PCR amplification was performed in the presence of 2 mM of $MgCl_2$, 0.5 mM of each primer (Metabion International, Martinsried, Deutschland) (Table 1), 0.2 mM dNTPs, 2 U of Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron GmbH) and 4 μl of RT product, in a final volume of 50 μl. Simultaneous amplification of the invariant housekeeping gene GAPDH was performed. For all primers, amplification started with denaturation at 94°C for 5 min and 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 45 s and a final elongation at 72°C for 5 min. PCR products were visualized on a 1.6% agarose gel with ethidium bromide staining. The expression of all tested enzymes was normalized to the expression of GAPDH of each sample and compared using Gel Pro Analyser software.

Protein determination

The protein content of liver homogenates was determined as described by Bradford [30], with bovine serum albumin as standard.

Statistical analyses

All the assays were performed in one sample per liver and $n \geq 3$ replicates. Values are expressed as the arithmetic means ± SEM. Statistical significance of the difference between groups was evaluated by Student's *t*-test when the distribution was normal. When the distribution was not normal a Mann–Whitney test was applied. Differences were considered to be statistically significant when $P < 0.05$.

Results

Liver homogenates from mice that ingested PJ or water during four weeks were used to evaluate oxidative cell status. Thiobarbituric acid reactive substances method (TBARS) was used to evaluate lipid peroxidation. There were no significant differences in peroxidation secondary product formation between control group (water) and treated group (PJ) (Fig. 1 a).

To evaluate protein oxidative state, carbonyl groups content was assessed. As can be seen in Fig. 1b, carbonyl content was significantly lower in the group of mice that consumed PJ. This group

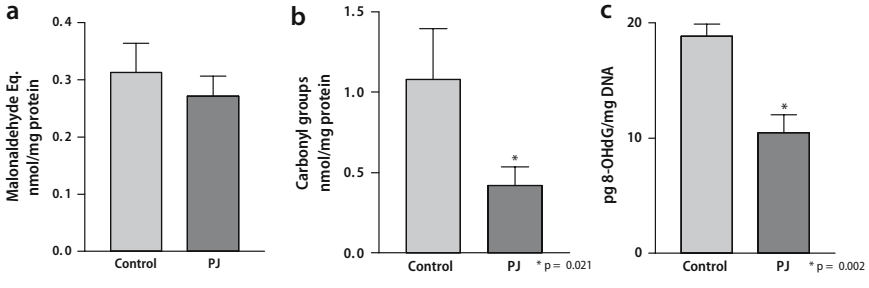

Fig. 1 The effects of pomegranate juice (PJ) consumption or water (control) on hepatic (**a**) lipid peroxidation assayed by TBARS method (Student's *t*-test); (**b**) protein oxidation measured as carbonyl content (Mann–Whitney test); (**c**) DNA oxidation assessed as 8-OHdG content (Student's *t*-test). Each value represents the mean ± SEM ($n = 6$)

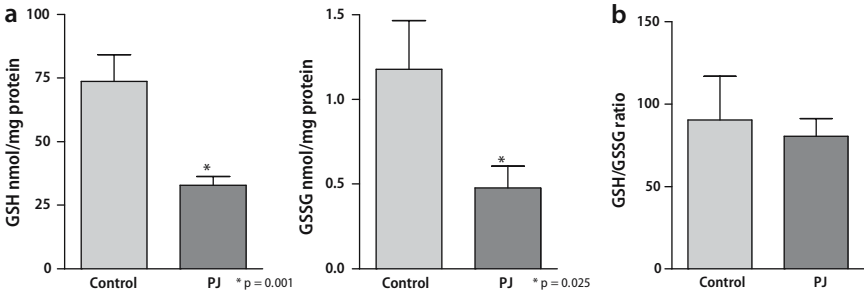

Fig. 2 (**a**) GSH and GSSG hepatic content in mice treated with water (control) or pomegranate juice (PJ), expressed as nmol/mg protein (Mann–Whitney test and Student's *t*-test, respectively). (**b**) Hepatic GSH/GSSG ratio in mice treated with water (control) or pomegranate juice (PJ) (Mann–Whitney test). Each value represents the mean ± SEM ($n = 6$)

presented 0.42 ± 0.1 nmol of carbonyl groups per mg of protein in comparison to 1.08 ± 0.3 for control group.

The extent of oxidative damage to nuclear DNA was measured as pg 8-OHdG/μg DNA in the liver tissue. As shown in Fig. 1c, this metabolite was found to be significantly decreased in the PJ group (10.4 ± 3.5 pg 8-OHdG/μg DNA) compared with the control group (18.8 ± 2.1 pg 8-OHdG/μg DNA).

In order to evaluate endogenous antioxidant defences, glutathione levels were examined. GSH content was significantly reduced (73.7 ± 25.9 nmol/mg protein and 32.9 ± 8.59 nmol/mg protein for control and PJ respectively) in the liver of animals treated with PJ (Fig. 2a). The same has been observed for GSSG content (1.18 ± 0.7 nmol/mg protein for control group and 0.48 ± 0.32 nmol/mg protein for PJ group). When GSH/GSSG ratios were determined, they were found to be not different between control and PJ groups (Fig. 2b).

Table 2 highlights the activity of the scavenger enzymes catalase and SOD along with GSH dependent antioxidant enzymes GR, GPX and GST in the liver of control and PJ treated mice. A consistent and significant decrease was observed in the activity of all glutathione-dependent enzymes and catalase in the liver of treated mice.

Through RT-PCR analysis (Fig. 3) a decrease in GST transcription was confirmed to accompany the decrease in GST activity. It was also observed that glutathione synthetase (GS), enzyme responsible for glutathione synthesis, had its expression significantly reduced after treatment with PJ. The expression of GPx and GR has not been affected by PJ consumption.

Table 2 Effect of pomegranate juice on hepatic antioxidant enzymes glutathione reductase (GR), glutathione-*S*-transferase (GST), glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) of control and treated animals

Enzymes (U/mg protein)	Control	Pomegranate juice
GR	0.0015 ± 0.0002	0.0011 ± 0.0001* ($P = 0.040$)
GST	0.042 ± 0.004	0.026 ± 0.003* ($P = 0.002$)
GPx	0.021 ± 0.001	0.010 ± 0.001* ($P < 0.0001$)
Catalase	259.2 ± 27.8	107.1 ± 9.0* ($P = 0.001$)
SOD	0.565 ± 0.024	0.285 ± 0.029* ($P < 0.0001$)

Results represent mean ± SEM
Statistical test for all enzymes was Mann–Whitney test and for catalase was Student's *t*-test

Discussion

Apart from their antioxidant capacity, there have been numerous reports on the in vivo properties of pomegranates, namely on anti-atherosclerotic capacity [13, 14], anti-proliferative and pro-apoptotic activities of pomegranate tannin extract [10]; anti-inflammatory activity [16], as well as chemopreventive and chemotherapeutical potential towards prostate cancer by PJ [17]. The reduction of platelet aggregation, of atherogenic LDL modifications and of macrophage oxidative state have been demonstrated, establishing a relationship between the consumption of PJ and cardiovascular protection in rats [15, 31]. Still, the lack of whole organism studies on pomegranate and the growing amount of PJ consumption substantiates the need to investigate the effect of prolonged PJ ingestion on general oxidative status.

The first approach was to assess the level of damage to lipids, proteins and DNA in liver homogenates

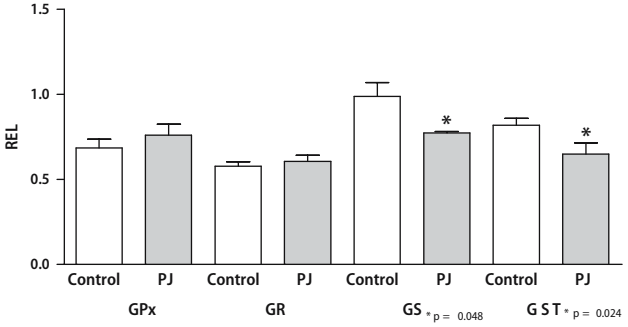

Fig. 3 Effect of pomegranate juice (PJ) consumption on glutathione related enzymes expression in hepatic tissue. The expression of all tested enzymes was normalized by comparison with the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of each sample, under the same conditions (REL—Relative Expression Level; GPx—glutathione peroxidase; GR—glutathione reductase; GS—glutathione synthetase; GST—glutathione-*S*-transferase). Statistical test used was Mann–Whitney test for GS and Student's *t*-test for GST

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of treated and control mice. While no significant difference was found in lipid peroxidation measured as malonaldehyde equivalents, there was a significant decrease both of carbonyls and 8-OHdG content of PJ-treated mice livers, when compared to controls. These results are in good agreement with the already reported antioxidant properties of PJ [7–10].

Another reliable oxidative stress indicator is the measure of glutathione levels. Despite having found a decrease in hepatic GSSG content, a concomitant reduction of GSH levels was also observed, with no difference in GSH/GSSG ratio between PJ and control groups. It has been stated that the ratio of GSH to GSSG is a more sensitive marker of oxidative stress, as small increases in GSSG and/or small decreases in GSH appear amplified by examining the ratio by comparison with measuring either one separately. In our opinion, observed glutathione levels could be translated into one of two hypothetical scenarios: (1) an increase in oxidative stress status supported by reduced GSH levels or (2) interference of PJ with glutathione synthesis or metabolism. The first hypothesis seems unlikely as a decrease in oxidative damage to biomolecules has been observed following PJ ingestion. Both protein and DNA damage were reduced in mice that ingested PJ, which strongly disagrees with this assumption. The second, and more probable explanation needed further biochemical exploration. For this purpose, the activity and expression of hepatic glutathione related enzymes was assessed.

Glutathione reductase is responsible for recycling GSSG formed during oxidation events reducing it back to GSH. After PJ intake, a decrease in GR activity was observed, although no differences were encountered on its transcription between both groups. The reduction of this enzyme's activity could be a result of the decreased total glutathione levels. Less glutathione levels will indubitably require less GR activity. If there was a deterioration of the oxidative status and GR was inhibited, GSSG levels would most probably have been raised and not decreased as they were. It has also been described that certain polyphenols, namely tannic acid and coumarins [9, 32], are able to reduce GR activity [33] and the presence of these or related polyphenols, such as tannic acid, in PJ [10, 16, 32] may account for the GR inhibition observed.

The same pattern of results was found for the GPx: decreased activity after treatment with PJ and no variation in mRNA expression between groups. This enzyme catalyses the reduction of hydroperoxides, at the expense of GSH. Catalase is also a peroxidase, and at the same time the most important enzyme involved in H_2O_2 degradation. Along with GPx inhibition, there was also a decrease in catalase activity in PJ group. As they degrade the same kind of substrates, GPx and catalase activities are often related [34]. This suggests that smaller amounts of hydroperoxides or H_2O_2 are being generated, which is also in agreement with the reduction of SOD activity in PJ treated mice. This enzyme catalyses the dismutation of $O_2^{\cdot -}$, producing H_2O_2, and the reduction on its activity may result from a decrease in superoxide production. It may seem reasonable to believe that endogenous antioxidant defences are lowered as they are no longer required to act on an organism supplied with generous amounts of exogenous antioxidants. The decline in GR, GPx, SOD and catalase activities thus seems more compatible with a general decrease in oxidative stress.

Another glutathione related enzyme is GST, a phase-II enzyme responsible for the detoxification of several substrates. This enzyme can also conjugate altered proteins and mediates protein repair mechanisms. Decreased GST activity and transcription were observed in the group that ingested PJ. GST inhibition could reflect the decrease in protein damage, which will, most likely, translate into less GST activity. Another explanation could be, as for GR, the regulation of this enzyme in a competitive manner by polyphenols from pomegranate (e.g. ellagic acid, tannic acid), as has been previously proposed [35, 36].

Nevertheless, the result that better elucidates the decline in glutathione levels seen in the animals that consumed PJ is the decrease in glutathione synthetase (GS) transcription. GS catalyses the last step on a chain of events that culminates in GSH synthesis. The regulation of this enzyme by PJ ingestion might explain the decrease in total glutathione levels, and more specifically in GSH hepatic content, although the reason for such an effect is not known. However, polyphenols are known to be able to modulate the transcription and expression of proteins related to the endogenous antioxidant defence by interacting with antioxidant response elements in gene promoter regions of genes encoding proteins related to oxidative injury management [37, 38].

It is not clear, however, if the effects of PJ intake result from polyphenols' interference with enzymes and genes or if it is a consequence of a broader and more unspecific action connected to their antioxidant potential. Moreover, many factors need to be taken into account when examining the present results, as the degree of oxidative stress, the polyphenol class and concentration as well as the biological system studied may all introduce other elements of variability in the response to antioxidant ingestion [6].

Thus, the overall condition of the animals after four weeks of PJ ingestion appears to be a state of reduced oxidative stress. This is supported by the decrease of protein and DNA damage, by the decline on GSH and GSSG levels without change of the GSH/GSSG ratio, and by the decrease in antioxidant

endogenous enzymes (GPx, catalase, SOD and GST), most probably in relation with less oxygen reactive species production. However, it should not be forgotten that the inhibition of antioxidant enzymes, along with lower levels of GSH, may lead to vulnerability in the case of exposure to an insult or an aggression, since endogenous antioxidant defences are diminished. To clarify the pathophysiological meaning of these changes will require other approaches.

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