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Evaluation of the potential protective effects of *ad libitum* black grape juice against liver oxidative damage in whole-body acute X-irradiated rats

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ABSTRACT

Aims: The aim of this study was to evaluate the potential protective effects of *ad libitum* black grape (*Vitis labrusca*) juice against liver oxidative damage in whole-body acute X-irradiated rats.
Main methods: Animals were fed *ad libitum* and drank voluntarily black grape juice or placebo (isocaloric glucose and fructose solution) for 6 days before and 15 days following a 6 Gy X-irradiation from a 200 kV machine.
Key findings: Irradiated animals receiving placebo showed a significant increase in the concentration of thiobarbituric acid-reactive substances (TBARS), a marker of lipid peroxidation, as well as a significant decrease in both Cu/Zn superoxide dismutase (Cu/ZnSOD) and glutathione peroxidase (GPx) activity and reduced glutathione concentration (GSH). Black grape juice supplementation resulted in a reversal of lipid peroxidation, Cu/ZnSOD activity, and GSH concentration, towards values not significantly differing from those in non-irradiated, placebo-supplemented rats. Poly(ADP-ribose) polymerase (PARP-1) and Cu/ZnSOD changes in protein expression were observed for irradiated rats. No change in p53 expression or DNA fragmentation was found.
Significance: Ad libitum black grape juice intake is able to restore the liver primary antioxidant system against adverse effects due to whole-body acute X-irradiation in rats after 15 days post-irradiation. The results support using antioxidant supplements as a preventive tool against radiation-induced harm.

1. Introduction

Whole body exposure to ionizing radiation may trigger in human and animals multiple organ dysfunctions directly related to an increase of cellular oxidative stress due to overproduction of reactive oxidative species from molecule ionization (Mettler and Voelz, 2002; Coleman et al., 2003). Ionizing radiation exposure involves the development of potentially serious health conditions. Acute effects mainly include hematopoietic cell loss, immune suppression, mucosal damage, and potential injury to liver and other tissues. Whole organ irradiation might lead to hepatocyte failure and radiation-induced liver disease such as hepatitis (Khozouz et al., 2008).

Studies looking for safe and effective radioprotective chemicals are relevant and would be required in the event of massive radiological accident, nuclear terrorist attack or prolonged space travel (Wilson et al., 1999; Coleman et al., 2003; Saada et al., 2003, 2009). It has been suggested that polyphenols found in natural foods such as grape and its products could be efficient protectors against ionizing radiation overexposure. *Vitis vinifera* grape seed extract (GSE) shows a radioprotective effect against chromosomal damage in mouse bone marrow exposed to X-rays (Castillo et al., 2000). GSE enhances the antioxidant status and decreases the incidence of free radical induced lipid peroxidation in blood of rats acutely whole-body exposed to 6 Gy X-rays, with a higher efficiency than vitamin E (Enginar et al., 2010). In Wistar rats receiving GSE orally for 7 days before and for 4 days after 8 Gy whole body irradiation antioxidant parameters related to radiation-induced liver toxicity were restored to control values (Cetin et al., 2008). GSE treatment also attenuates oxidative stress in pancreas, a fact associated with a significant improvement in radiation-induced hyperglycemia and hyperinsulinemia (Saada et al., 2009). A recent study found inhibition of oxidative stress, DNA damage, and

Abbreviations: BGJ, black grape juice; CAT, catalase; Cu/ZnSOD, cytosolic superoxide dismutase; GPx, glutathione peroxidase; GSE, grape seed extract; GSH, reduced glutathione; GSSG, oxidized glutathione; MnSOD, mitochondrial superoxide dismutase; PARP-1, poly(ADP-ribose) polymerase; TBARS, thiobarbituric reactive acid substances.

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apoptosis rate in whole-body irradiated rats (8 Gy) under treatment with the constituent of black grape juice resveratrol (Velioglu-Ogünç et al., 2009).

However, previous studies focused on pharmacological approaches instead of analyzing potential beneficial effects of food supplementation. Therefore, the hypothesis that a moderate intake of black grape juice (BGJ) could also have a radioprotective effect is relevant and needs to be tested. In previous studies by our group, Wistar rats under acute whole body exposure to 6 Gy X-rays were treated with BGJ before and after radiation exposure (Ramos de Andrade et al., 2009a,b). Results indicated that *ad libitum* BGJ intake seems to offer radioprotection over selected hematological parameters and organs, with an abrogation of immediate acute radiation syndrome symptoms, remarkably the maintenance of liver weight (Ramos de Andrade et al., 2009a,b). We postulated that this result could be related to differential oxidative stress modulation in liver tissue of rats supplemented with BGJ, a hypothesis that has been tested in the present study.

2. Materials and methods

2.1. Animals, irradiation and food

Sixteen male Wistar rats weighing 200–250 g (Harlan, Barcelona, Spain), housed at the animal house of University of León (Spain), were included in the study. The experimental protocol used was approved by the University of León Ethical Committee, and adhered to the European Community Guiding Principles for the Care and Use of Animals. Four groups were defined as: (NI-GJ) non-irradiated, supplemented with grape juice; (NI-GL) non-irradiated, supplemented with placebo (GL, isocaloric 50:50 glucose/fructose solution); (I-GJ) irradiated, grape juice supplemented; and (I-GL) irradiated, supplemented with placebo (GL). In order to immobilize the animals, anesthesia was induced by intraperitoneal administration of pentobarbital 0.6% in saline (10 ml/kg body weight), at noon, 15 min before irradiation. The animals were placed in decubitus pronus on a plexiglas board. Four animals were irradiated at a time and exposed to a single dose of 6 Gy of total-body X-irradiation from an X-ray machine (200 kV) MAXISHOT 200 (YXLON, Copenhagen, Denmark), at a radiation dose rate of 0.40 Gy/min, with a source-skin distance (SSD) of 50 cm. Uniform total-body X-irradiation distribution was confirmed by dosimetry using isodose curves measurement. A test with a phantom (water layer) was performed in order to check self-shielding without changes in dose distribution profile for the thickness involved.

Animals were fed standard rat chow diet, having free access to water and food. After 1 week adaptation to individual cages, they were allowed to ingest a maximum of 10 ml of test compound (grape juice) or placebo for 6 days before and 15 days following irradiation. Previous trials found that rats were drinking about 4–8 ml BGJ daily, so that a maximum of 10 ml was allowed to be drank. Placebo solution was made using an equimolar mixture of glucose and fructose to be isocaloric with the sugar composition in the grape juice (95 g/L).

Environmental conditions were controlled (12-h photoperiod and 20 ± 2 °C) throughout the experimental period.

2.2. Black grape juice characteristics

Ecologically-produced (organic) BGJ was obtained from *Vitis labrusca* grapes cultivated in 2007, and the juice was prepared the same year. The procedure involved pressing of freshly ripped grapes, allowing the fluid to circulate through coils at 80–90 °C for 1 h (without boiling) to be immediately included into dark glass bottles, tightly sealed afterwards. Brazilian regulations (Instruçao Normativa 007/99-MAPA, to be superseded by Law 10.831/03 in 2011) establish as main criteria to certify a grape juice as organic the exclusive use or natural fertilizers and the total prohibition of minerals and plant protection products (herbicides and insecticides). The vineyard is located at 29°14′18″S, 51°32′5″4W.

Juice samples were analyzed spectrophotometrically for the content of total phenolics, using a modified Folin–Ciocalteu colorimetric method (Chandra and Mejia, 2004). The estimation of phenolic compounds in the crude extract and fractions was carried out in triplicate. The standard curve of gallic acid was prepared in the same manner and total polyphenol concentration was expressed as mean ± SD mg of gallic acid equivalents per milliliter of grape juice. High-performance liquid chromatography (HPLC) analysis was used to quantify the presence of individual phenolic compounds using an HPLC system consisting of a Shimadzu Prominence LC – 20A, completed with LC-20AT quaternary pump, SIL 20 auto sampler – A, DGU-20A5 on-line degasser, CBM-20A integrator and SPD-20AV DAD detector. Trans-resveratrol quantification was performed according to Souto et al. (2001) using an octadecyl column, 250 mm long and 4.6 mm i.d., with 5-μm particle diameter. Simultaneous determination of quercetin and rutin was performed according to Zu et al. (2004) using standard solutions of these flavonoids prepared in ethanol, in a concentration range of 18–280 mg/L for quercetin and from 12.5 to 200 mg/L for rutin. Quantification was carried out by the integration of the peak using an external standard method. The simultaneous determination of gallic acid and caffeic acid was performed as described by Singh et al. (2008).

The concentration (mg/L) of the main phenolic compounds in the grape juice was as follows: resveratrol 3.95 ± 0.01, quercetin 8.95 ± 0.09, rutin 3.75 ± 0.03, gallic acid 81.07 ± 2.03, caffeic acid 30.28 ± 2.00, and total flavonoid content 249.0 ± 2.0.

2.3. Liver tissue biochemical oxidative biomarkers

All molecular-biology grade reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA), except where otherwise noted.

The amount of aldehydic products generated by lipid peroxidation was quantified by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) using malondialdehyde (MDA) as standard (Wills, 1987). One gram of liver in 9 mL of potassium phosphate 0.1 M, pH 7.4 was homogenated, and tubes were prepared including 1 mL fresh homogenate plus 2 ml of a solution containing 15% trichloroacetic acid, 0.37% thiobarbituric acid, and 0.25 N HCl. After 30 min heating at 90 °C, the tubes were cooled and centrifuged at 2000*g*. Supernatants were collected and its absorbance read at 532 nm.

Oxidized glutathione (GSSG) and reduced glutathione (GSH) analyses were performed fluorimetrically (Hissin and Hilf, 1976). Briefly, 250 mg of tissue were homogenized in 0.1 M sodium phosphate, 5 mM EDTA buffer (pH 8.0) with 25% phosphoric acid at a proportion of 1:20. After centrifugation (100,000*g*, 30 min, 4 °C), 500 μL of supernatant were added 4.5 mL of buffer. Two spectrophotometry cuvettes per sample were prepared with 1.8 mL of phosphate–EDTA buffer, 100 μL of supernatant or buffer, and 100 μL of *O*-phthalaldehyde. After incubating for 15 min at 4 °C, a spectrofluorometric reading was obtained using an excitation wavelength of 350 nm and an emission wavelength of 420 nm. To determine the percentage of glutathione corresponding to oxidized and reduced forms, 500 μL of the sample supernatant were incubated with 20 μL of 4-vinylpyridine for 30 min; to this mixture 4.5 mL of 0.1 N NaOH were added and 100 μL of this mixture were then processed as described above to determine GSSG. GSH was obtained by subtracting GSSG from total glutathione.

The measurement of liver enzyme activities involved homogenization of samples at 4 °C and 750*g* for 15 min in ice-cold medium including 250-mM mannitol, 70-mM sucrose and 2-mM EDTA, pH 7.4. Mitochondria were isolated by centrifugation of the supernatant ad 12,000*g* for 15 min. The mitochondrial pellet was washed twice in isolation medium by centrifugation (12,000*g*, 15 min) and finally diluted to yield about 100-mg mitochondrial protein per milliliter. The supernatant fraction of the first 12,000*g* centrifugation was again centrifuged at 105,000*g* for 60 min, and the supernatant was retained as cytosol. Cytosolic superoxide dismutase (referred as Cu/ZnSOD), and mitochondrial SOD (referred as MnSOD) activity measurements were performed spectrophotometrically as described elsewhere (Misra and Fridovich, 1972). This method is based on the inhibition of adenochrome formation in the self-oxidation of epinephrine. Measurements were performed at 480 nm, 0.025 nm/min, and 30 °C. Bicarbonate-ethylenediaminotetraacetate (EDTA) buffer (pH 10.2) and epinephrine 4 mM were used. Catalase (CAT) activity was determined spectrophotometrically based on the consumption of 0.3-M hydrogen peroxide by the sample on 50-mM sodium phosphate buffer (pH 7.0), reading each 5 s at 240 nm within 2 min at 25 °C. Glutathione peroxidase (GPx) activity was measured spectrophotometrically (Paglia and Valentine, 1967). The reaction mixture contained 0.2 mL of 0.4-M phosphate buffer (pH 7.0), 0.1 mL of 10-mM sodium azide, 0.2 mL of tissue homogenate (supernatant; homogenized in 0.4 M phosphate buffer, pH 7.0), 0.2 mL of 40-mM GSH, and 0.1 mL of 0.2-mM hydrogen peroxide. The tubes were incubated at 37 °C for 10 min. The reaction was stopped by adding 0.4 mL of 10% TCA and centrifugation ensued. Spectrophotometric determination of GPx activity (μmol/min·mg prot) was started by addition of 5 μl of 24 mM NADPH, and NADPH oxidation was followed each 30 s for 4 min at 340 nm. Non-enzymatic NADPH oxidation was subtracted from the overall rate.

2.4. Western blot analysis

In order to perform Western blot analysis of Cu/ZnSOD, poly(ADP-ribose) polymerase (PARP-1), and p53, liver tissue was homogenized in 10 mM Tris buffer (pH 7.4) containing 100 mM NaCl and protease and phosphatase inhibitor cocktails (Roche Diagnostics GmbH, Mannheim, Germany) and centrifuged at 13,000*g* for 30 min. Protein concentration of the cytosolic and nuclear liver fractions was measured by the Bradford assay (Bradford, 1976). Equal amounts of protein (10–40 μg/well depending on the protein assayed) were separated by 10–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred electrically to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 30 min at 37 °C and probed overnight at 4 °C with rabbit anti-Cu/ZnSOD, anti-PARP-1 and anti-p53 polyclonal antibodies (1:1200, Santa Cruz Biotechnology, Santa Cruz, CA). Equal loading of protein was demonstrated by probing the membranes with a rabbit anti-β-actin polyclonal antibody (Sigma, St. Louis, MO, USA; 1:1000). After washing with TBST, the membranes were incubated for 1 h

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at room temperature in TBST-containing secondary antibody (Dako, Glostrup, Denmark; 1:4000). Membranes were briefly incubated with ECL detection reagent (Amersham, Buckinghamshire, UK) to visualize the proteins and then were exposed in a cassette for 5 min to an X-ray film (Hyperfilm ECL; Amersham Pharmacia, Uppsala, Sweden). The film was then developed in 16% Ilford Phenisol developer solution and fixed in 16% Ilford Hypam rapid fixer solution (Ilford Imaging UK Ltd., Cheshire, England, UK). Membranes were scanned with a charge-coupling device (CCD) camera. Densitometric analysis was performed using a Scion Image Software (version 4.0.3.2, Scion Corporation, Frederick, MD, USA). Protein expression levels were normalized through the corresponding expression values of β-actin.

2.5. DNA fragmentation

To detect DNA laddering, the liver tissue was suspended in lysis buffer containing 10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 20 mM EDTA, and 1% N-lauroylsarcosine sodium, and incubated with 100 μg/mL proteinase K at 37 °C overnight. Then, equal volumes of phenol/chloroform-isoamylalcohol (CIAA) were added to the sample solution, mixed, and centrifuged at 13,000*g* for 5 min. The resulting supernatants were incubated with 100 μg/mL RNase at 37 °C for 1 h. Phenol/CIAA was added to the sample solutions and centrifuged at 13,000*g* for 5 min. The resulting supernatants were treated with CIAA and then centrifuged again at 13,000*g* for 5 min. DNA in the solution was precipitated with ethanol at −20 °C, dissolved in 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA and then subjected to electrophoresis for 30 min at 100 V using a 3% agarose gel (Agarose-ME, Nacalai Tesque, Kyoto, Japan) containing 40 mM Tris–acetate, pH 7.8, 2 mM EDTA and 0.5 μg/ml ethidium bromide. After electrophoresis, the gels were photographed under ultraviolet light by Bio-Rad Gel Documentation System and quantified by Quantity One software (Bio-Rad, Hercules, CA).

2.6. Statistical analysis

Means and standard error of the mean (SEM) were calculated for all data. Significant differences between means were evaluated by analysis of variance (ANOVA) and in the case of significance the Duncan test was also applied. A difference was considered significant when p was less than 0.05. Calculations were performed with SPSS 13.0 statistical software (Chicago, IL, USA).

3. Results

The daily intake of the glucose/fructose placebo solution was higher when compared to BGJ supplementation within 6 days before radiation exposure, and water intake was also higher in placebo-supplemented animals (Table 1). Water intake was decreased as a result of X-irradiation, irrespective of the supplementation, and the same pattern is observed for the intake of glucose/fructose placebo or BGJ. No significant differences were found among groups in the percentage of intake of BGJ or glucose/fructose solution relative to the total fluid consumption (BGJ or GL plus water) (Table 1).

A different liver oxidative response was observed 15 days following irradiation among rats receiving or not BGJ supplementation and submitted or not to ionizing radiation (Fig. 1). Irrespective of supplementation and irradiation, CAT and MnSOD activities were similar in all four treatments. A significant increase of lipid peroxidation evaluated by TBARS and expressed as MDA equivalents was observed in all irradiated animals (groups I-GL, I-GJ), but this increase was smaller in the group receiving BGJ. Also, decreased Cu/ZnSOD and GPx activities, and GSH concentration were found in the irradiated, non BGJ-treated group when compared to controls (NI-GL group). BGJ supplementation showed a reversal effect on lipid peroxidation, Cu/ZnSOD, activity, GPx activity and GSH concentration in rats exposed to ionizing radiation (I-GJ), these oxidative biomarker values being similar to those observed in the NI-GL group and significantly different from those in the irradiated animals (I-GL) (Fig. 1).

The potential effect of BGJ supplementation on cellular expression of genes related to apoptotic cell death was also investigated by measuring PARP-1 and p53 protein concentrations. No significant differences were observed in p53 protein expression (Fig. 2C). Significant differences were found in PARP-1 expression, which was increased in the irradiated, BGJ-supplemented group in comparison with all other groups (Fig. 2B). Additionally, liver Cu/ZnSOD protein expression was analyzed, since significant differences in enzyme activity were found among the different treatments. A decrease in Cu/ZnSOD protein expression was observed in the I-GL group when compared to the other groups, and this decrease was prevented by BGJ treatment (Fig. 2A). Radiation-induced genotoxicity impact was evaluated by analysis of DNA fragmentation (Fig. 3). There were no tracks of DNA laddering among groups.

4. Discussion and conclusion

Over the years, extensive experimental studies of radiation-protective agents have enhanced our knowledge of radiation physics, chemistry, and biology. However, translation of agents from animal testing to use in various scenarios, such as prophylactic adjuncts in radiotherapy or post-exposure treatments for potential victims of radiation accidents/incidents and even in military operations has been slow (Weiss and Landauer, 2009). Possible associated limitations could be related to experimental designs which potentially are not comparable between humans and animal models for all purposes. Generally, evaluation of radioprotective molecules in experimental models is performed under highly controlled conditions in order to avoid too much variation in the responses obtained.

Previous investigations showed radioprotective effects of resveratrol and GSE on bone marrow in mice (Castillo et al., 2000, 2008). *In vivo* studies with GSE (Cetin et al., 2008; Saada et al., 2009) and BGJ (Dani et al., 2008) in rats under controlled conditions (intragastric administration) have found improvements in oxidative stress alterations induced by acute ionizing radiation exposure in liver

Table 1
Average daily grape juice, glucose/fructose solution, water, total fluid intake, and proportion of grape juice or placebo intake relative to the total daily fluid ingestion in rats before and after experimental exposure to X-rays.

	Groups	Water intake (mL/day)	Grape juice or placebo intake (mL/day)	Total fluid intake (mL/day)	Grape juice or placebo intake relative to total fluid intake (%)
Before irradiation	NI-GL	28.22 ± 1.08	9.59 ± 0.12	37.81 ± 1.06	25.68 ± 1.53
	NI-GJ	24.11 ± 0.97*	6.42 ± 0.57*	30.54 ± 0.84*	21.54 ± 2.23
After irradiation	NI-GL	27.27 ± 1.18#	9.24 ± 0.06	36.51 ± 1.19#	26.13 ± 2.40
	I-GL	21.04 ± 0.92*	8.92 ± 0.18	29.99 ± 0.98*	29.49 ± 2.97
	NI-GJ	25.06 ± 1.06#	6.97 ± 0.50*,#	31.73 ± 1.20*,#	22.68 ± 3.62
	I-GJ	19.60 ± 0.63*	6.14 ± 0.59*,#	25.73 ± 0.76*,#	23.06 ± 4.32

NI-GJ: non-irradiated, grape juice supplemented; NI-GL: non-irradiated, placebo supplemented; I-GJ: irradiated, grape juice supplemented; I-GL: irradiated, placebo supplemented. Data are expressed as mean ± SEM of four animals per group.
* Significantly different from NI-GL group ($p < 0.05$).
# Significantly different from I-GL group ($p < 0.05$).

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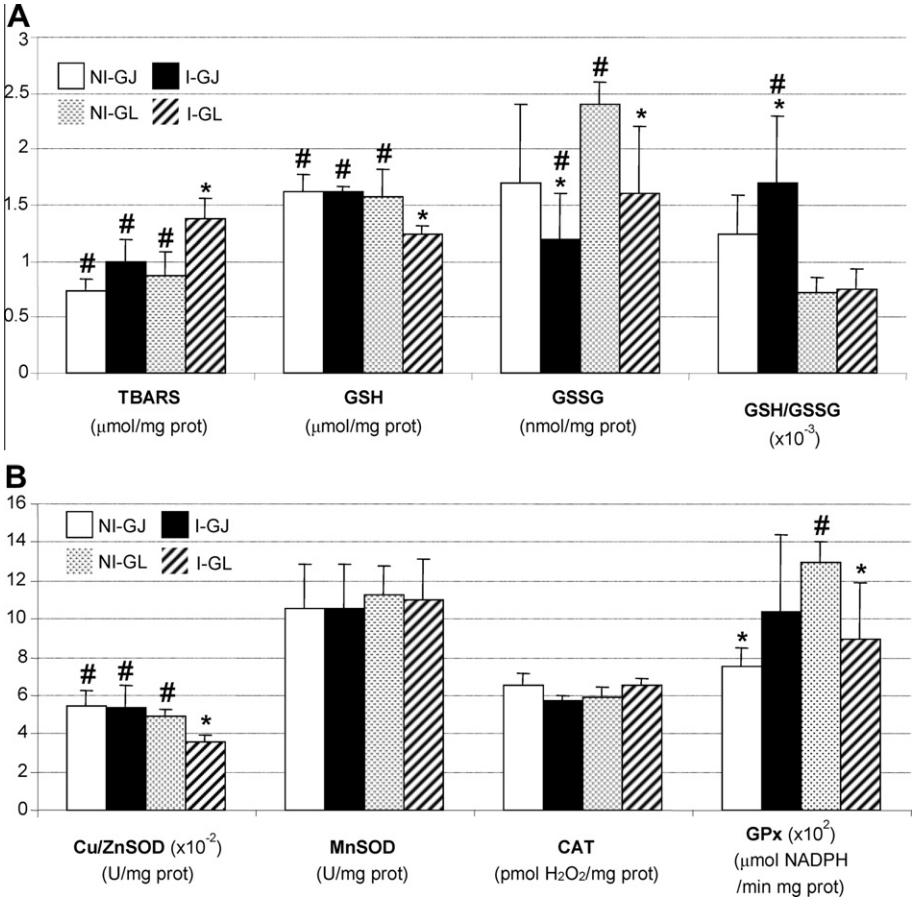

Fig. 1. Oxidative metabolism parameters in liver tissue of rats under ionizing radiation exposure and BGJ supplementation. NI-GJ: non-irradiated, grape juice supplemented; NI-GL: non-irradiated, placebo supplemented; I-GJ: irradiated, grape juice supplemented; I-GL: irradiated, placebo supplemented. Data are expressed as mean ± SEM of four animals per group. GSH/GSSG ratio, Cu/ZnSOD activity, and GPx activity values in the figure have been modified by the factor indicated between brackets. *significantly different from NI-GL group ($p < 0.05$). #significantly different from I-GL group ($p < 0.05$).

(Cetin et al., 2008) and other tissues (Saada et al., 2009), or CCl_4 i.p. injection (Dani et al., 2008). Based on these studies, we tested whether *ad libitum* BGJ ingestion provides radioprotective action in X-irradiated rats. In these experimental conditions we observed that *ad libitum* BGJ intake exerts some protection against oxidative stress caused by ionizing irradiation. Other authors (Dani et al., 2008) have studied the effect of ca. 1.7 ml BGJ given intragastrically twice a day to rats fed *ad libitum*. It has been argued that *ad libitum* rats administered fluids by gavage may result in the aspiration to the lungs of the fluid, since considerable portions of food may remain in the upper gastrointestinal tract, and a stress response vehicle- and dose volume-dependent may also be elicited (Brown et al., 2000), albeit this aspect has been compared to that produced by routine cage housekeeping (Õkva et al., 2006). These inconveniences are avoided by allowing *ad libitum* drinking of the administered fluid, but at the expense of a lesser dosage control.

Pronounced liver radioprotective effect may appear at about 2 weeks post irradiation in rats under acute gamma-irradiation administered silymarin (Kropácová et al., 1998) and mouse on red ginseng extract supplementation (Park et al., 1993). In the same way, our results were obtained 15 days after exposure to radiation, picturing the redox state following a recovery period beyond acute irradiation effects. Glucose supplemented animals (NI-GL and I-GL) used to drink increased quantities of glucose solution when compared to animals supplemented with grape juice (NI-GJ and I-GJ). This finding might be related to flavor differences in the solutions, and not to their caloric content. The energy content of a sweet solution is a more potent factor stimulating drinking than simple flavor, as shown by experiments exposing rats to glucose and sucralose solutions (Martinez Moreno et al., 2009). In our case, the energy content of either grape juice or placebo was the same, but the animals drank less volume of grape juice, which suggest flavor is affecting the voluntary intake of the solutions. These animals also ingested higher amounts of water than the rats receiving BGJ supplementation. However, when we compared the contribution to the *ad libitum* fluid supplementation to the total liquid intake, all groups presented similar percentage values (Table 1). The excess water intake by placebo-supplemented animals could be explained by the insulin-inducing effect of the extra glucose ingested by these animals. It is long known that insulin can stimulate drinking in a way related to osmotic and fluid compartment changes by increasing efflux of vascular water leading to hypovolemia (Booth and Pitt, 1968; Waldbillig and Bartness, 1981). In spite of that variations in drink volume could have an effect on body weight, this has been ruled out since we have previously shown that body and liver weight were significantly less in X-irradiated rats receiving placebo, but not in X-irradiated rats receiving grape juice (Ramos de Andrade et al., 2009b). Therefore, in our experimental set up we found an influence of radiation exposure decreasing total daily liquid intake, a fact that has also been found in other studies (Pradeep et al., 2008). This decreased liquid intake by irradiated rats was also found for food intake (data not shown), especially during the first 3 days post-irradiation, and might be related to the reported decrease in intestinal function due to irradiation (MacNaughton, 2000; Molla and Panes, 2007).

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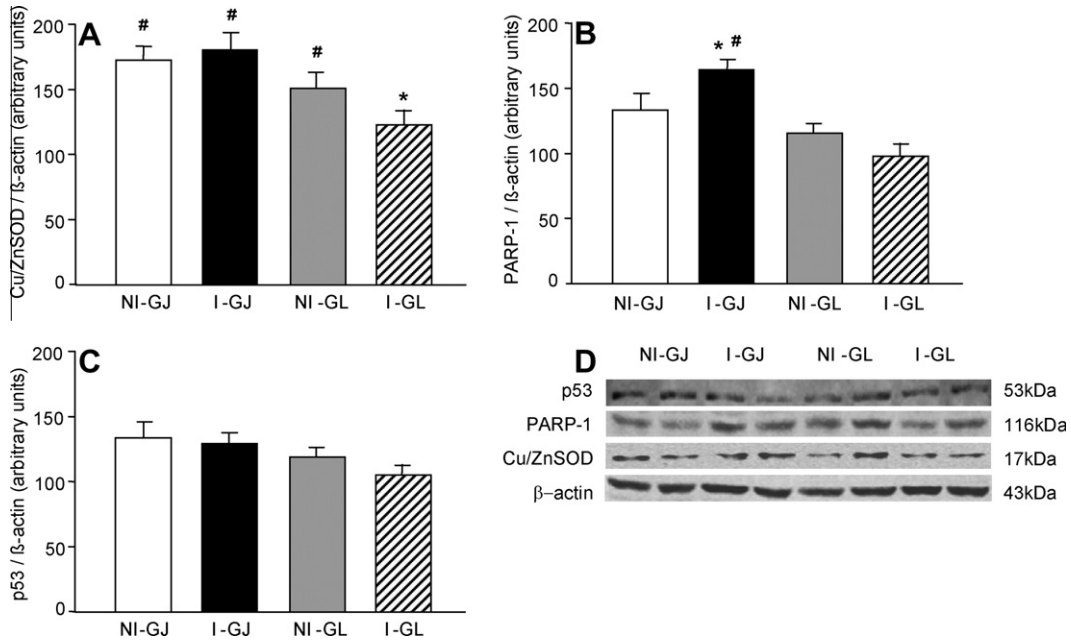

Fig. 2. Effect of total-body X-irradiation and grape juice on the expression of (A) Cu/ZnSOD, (B) poly(ADP-ribose) polymerase (PARP-1), and (C) p53 in rat liver. Panel (D) shows representative western blot photographs. NI-GJ: non-irradiated, grape juice; NI-GL: non-irradiated, placebo; I-GJ: irradiated, grape juice; I-GL: irradiated, placebo. Data are expressed as mean ± SEM of four animals per group. *significantly different from NI-GL group ($p < 0.05$). #significantly different from I-GL group ($p < 0.05$).

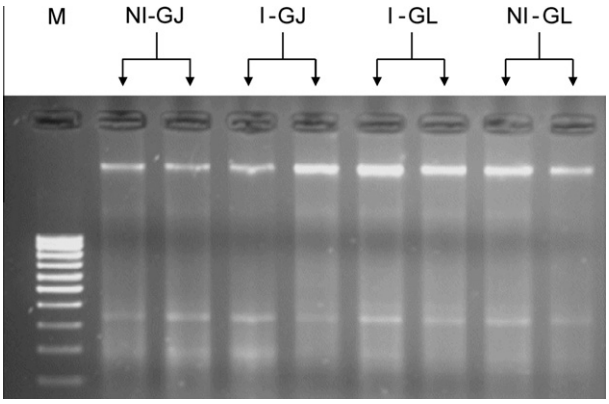

Fig. 3. Effect of total-body X-irradiation and grape juice on tissue DNA fragmentation test. Photograph of agarose gel containing electrophoretically separated low-molecular-weight DNA fragments. M: marker; NI-GJ: non-irradiated, grape juice; NI-GL: non-irradiated, placebo; I-GJ: irradiated, grape juice; I-GL: irradiated, placebo.

Before sacrifice, one animal from each group was randomly picked up in order to check blood glucose levels, and all of them presented normal levels. Since a 50:50 glucose:fructose solution isocaloric with the grape juice sugar content was used and the differences in the intake of these solutions were compensated by the total liquid intake, we can observe that the overall sugar concentration ingested was the same for all groups. This fact strongly contributes to eliminate possible energetic unbalances. Therefore, further liver effects could be attributed mainly to the flavonoid content present in BGJ.

The oxidative status in liver was assessed, since liver is considered a radiosensitive organ with poor tolerance to radiation exposure (Liang et al., 2006; Velioğlu-Oğünç et al., 2009), and in a previous study an effect of BGJ ingestion on liver size was found (Ramos de Andrade et al., 2009a). We observed that lipid peroxidation was higher in irradiated rats without BGJ supplementation when compared to irradiated controls. The increased MDA values in the I-GL group compared with I-GJ animals are suggestive of a protection offered by BGJ intake against liver lipid peroxidation. Our results agree with previous studies on intragastrically administered GSE which have found a protection from protein and lipid oxidation in liver of irradiated rats (Cetin et al., 2008). Research has also reported GSE positive effects in cardiac and pancreatic tissue (Saada et al., 2009) or blood lymphocytes (Enginar et al., 2010). Therefore, considering the influence of grape products on lipid peroxidation, our results suggest that oral *ad libitum* BGJ ingestion has some protective effects similarly to that observed in dose-controlled investigations.

The liver antioxidant system was clearly affected by X-irradiation. A considerable reduction in Cu/ZnSOD activity was observed in the I-GL group, suggestive of failure on managing unbalances from superoxide ($O_2^{\cdot-}$) production, which represents the first wave of free radical generation. This was further supported by the significant increase of Cu/ZnSOD protein expression in the I-GJ group when compared to I-GL and control NI-GL groups. Similar results have been described by others (Cetin et al., 2008), who found decreased total SOD activities in liver homogenates following 8 Gy total body irradiation which were significantly reversed by GSE treatment. Overall, the literature reflects the distinct effects of different doses of ionizing radiation on antioxidant enzyme expression. Exposure of mice to 0.5 Gy for 23 days increased 2.5 times the expression of CAT and MnSOD, but higher doses (1.3 Gy) at the same dose rate yielded gene expression not significantly differing from controls (Otsuka et al., 2006). Time after irradiation is also affecting the expression and/or activity of antioxidant enzymes. Peltola et al. (1993) reported about 20% decrease in rat liver SOD activity at 24 h post-irradiation (3 Gy), which was undistinguished from controls after 7 days, and Bogojevic et al. (2010) found increased liver MnSOD values after 6.7 Gy total-body X-irradiation, peaking at 7 days post-irradiation, to be decreased afterwards. Most reports reflect the decrease in total SOD liver activity by ionizing radiation, without separating between manganese- or copper/zinc-dependent enzyme forms. In the present work, the lack of changes in MnSOD is suggestive that harm is probably done at cytoplasmic level. CAT activity was similar in all experimental

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groups, which supports that the problem is at the superoxide phase of oxidative stress, BGJ seeming capable of acting at the first wave of free radical production in liver. Oxidative stress induced by X-irradiation resulted in an increased utilization of GSH and subsequently a decreased level of GSH was observed in the liver tissues of non-supplemented irradiated animals (I-GL). Depletion of GSH *in vitro* and *in vivo* is known to cause an inhibition of the GPx activity and has been shown to increase lipid peroxidation (Jagetia and Reddy, 2005; Pradeep et al., 2008). The GPx activity in the BGJ-supplemented group (I-GJ) did not change when compared with control (NI-GL), suggesting a protective action of BGJ over the primary antioxidant system.

While the non-enzymatic component of the antioxidant system GSH was found to decrease in the non-supplemented irradiated I-GL group, its levels returned to normal in the BGJ supplemented group. The amount of GSSG became lower in the I-GL group as well. A decrease in GSSG is possibly linked to the GSH and GPx reduction in I-GL animals. The redox index GSH/GSSG ratio was similar to control values in the I-GL group. On the other hand, the group supplemented with BGJ showed an increase in the GSH/GSSG ratio, due to the lower amount of GSSG in the I-GJ group. These animals seem to increase the amount of intracellular GSH in the liver, which becomes available to serve as GPx cofactor, increasing free radical scavenging.

In order to investigate the extension of radiation-induced damage taken by the liver tissue, p53 and PARP-1 were evaluated as cell death markers. Unfortunately, we did not find similar studies investigating the effect of grape products on these biomarkers. Regarding PARP-1, I-GJ rats differed from those in the other groups, suggesting a possible pro-apoptotic trend. However, p53 protein expression in liver was the same for all groups and no differences in DNA damage among treatments were found. These results might indicate that in the early phase of acute whole-body radiation exposure investigated here there was no detectable apoptotic activity or DNA damage. However, complementary studies need to be performed in order to evaluate protective long-term effects of BGJ ingestion on these biological parameters.

Our data are suggestive of a liver radioprotective effect of *ad libitum* BGJ intake under whole-body acute X-irradiation in rats, as supported by the decrease in liver lipid peroxidation and the increase in Cu/ZnSOD and GPx antioxidant enzyme activities in BGJ supplemented rats. These results bring a set of possibilities for the use of antioxidant supplements as a preventive tool against radiation-induced harm. However, additional and complementary studies need to be performed to confirm data obtained.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Bogojevic, D., Ponznanovic, G., Grdovic, N., Grigorov, I., Vidakovic, M., Dinic, S., Mihailovic, M. 2010. Administration of rat acute-phase protein alpha 2-macroglobulin before total-body irradiation initiates cytoprotective mechanisms in the liver. Radiat. Environ. Biophys. doi: 10.1007/s00411-010-0331-z.

Booth, D.A., Pitt, M.E., 1968. The role of glucose in insulin induced feeding and drinking. Physiol. Behav. 3, 447–453.

Bradford, M.M., 1976. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brown, A.P., Dinger, N., Levine, B.S., 2000. Stress produced by gavage administration in the rat. Contemp. Top. Lab. Anim. Sci. 39, 17–21.

Carsten, R.E., Bachand, A.M., Bailey, S.M., Ullrich, R.L., 2008. Resveratrol reduces radiation-induced chromosome aberration frequencies in mouse bone marrow cells. Radiat. Res. 169, 633–638.

Castillo, J., Benavente-Garcia, O., Lorente, J., Alcaraz, M., Redondo, A., Ortuno, A., Del Rio, J.A., 2000. Antioxidant activity and radioprotective effects against chromosomal damage induced in vivo by X-rays of flavan-3-ols (Procyanidins) from grape seeds (*Vitis vinifera*): comparative study versus other phenolic and organic compounds. J. Agric. Food. Chem. 48, 1738–1745.

Cetin, A., Kaynar, L., Kocyigit, I., Hacioglu, S.K., Saraymen, R., Ozturk, A., Orhan, O., Sagdic, O., 2008. The effect of grape seed extract on radiation-induced oxidative stress in the rat liver. Turk. J. Gastroenterology 19, 92–98.

Chandra, S., Mejia, E.G., 2004. Polyphenolic compounds, antioxidant capacity, and quinone reductase activity of an aqueous extract of *Ardisia compressa* in comparison to mate (*Ilex paraguariensis*) and green (*Camellia sinensis*) teas. J. Agric. Food Chem. 52, 3583–3589.

Coleman, C.N., Blakely, W.F., Fike, J.R., MacVittie, T.J., Metting, N.F., Mitchell, J.B., Moulder, J.E., Preston, R.J., Seed, T., Stone, H.B., Tofilon, P.J., Wong, R.S., 2003. Molecular and cellular biology of moderate-dose (1–10 Gy) radiation and potential mechanisms of radiation protection: report of a workshop at Bethesda, Maryland, December 17–18, 2001. Radiat. Res. 159, 812–834.

Dani, C., Oliboni, L.S., Pasquali, M.A.B., Oliveira, M.R., Umezu, F.M., Salvador, M., Moreira, J.C.F., Henriques, J.A.P., 2008. Intake of purple grape juice as a hepatoprotective agent in Wistar rats. J. Med. Food 11 (1), 127–132.

Enginar, H., Cemek, M., Karaca, T., Unak, P., 2010. Effect of grape seed extract on lipid peroxidation, antioxidant activity and peripheral blood lymphocytes in rats exposed to x-radiation. Phytother. Res. 21, 1029–1035.

Hissin, P.J., Hilf, R., 1976. A fluorimetric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74, 214–226.

Jagetia, G.C., Reddy, T.K., 2005. Modulation of radiation-induced alteration in the antioxidant status of mice by narrigin. Life Sci. 77 (7), 780–794.

Khozouz, R.F., Huq, S.Z., Perry, M.C., 2008. Radiation-induced liver disease. J. Clin. Oncol. 26, 4844–4845.

Kropácová, K., Misúrová, E., Haková, H., 1998. Protective and therapeutic effect of silymarin on the development of latent liver damage. Radiats. Biol. Radioecology 38 (3), 411–415.

Liang, S.X., Zhu, X.D., Xu, Z.Y., Zhu, J., Zhao, J.D., Lu, H.J., Yang, Y.L., Chen, L., Wang, A.Y., Fu, X.L., Jiang, G.L., 2006. Radiation-induced liver disease in three-dimensional conformal radiation therapy for primary liver carcinoma: the risk factors and hepatic radiation tolerance. Int. J. Radiat. Oncol. Biol. Phys. 65, 426–434.

MacNaughton, W.K., 2000. Review article: new insights into the pathogenesis of radiation-induced intestinal dysfunction. Alim. Pharmacol. Ther. 14, 523–528.

Martinez Moreno, A.G., López-Espinoza, A., Díaz Reséndiz, F.J., Valdés Miramontes, E., 2009. Consumo de soluciones endulzadas en ratas albinas: sabor vs calorías. Psicothema 21 (2), 191–198.

Mettler Jr., F.A., Voelz, G.L., 2002. Major radiation exposure – what to expect and how to respond. N. Engl. J. Med. 346, 1554–1561.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.

Molla, M., Panes, J., 2007. Radiation-induced intestinal inflammation. World J. Gastroenterol. 13, 3043–3046.

Otsuka, K., Koana, T., Tauchi, H., Sakai, K., 2006. Activation of antioxidative enzymes induced by low-dose-rate whole-body gamma irradiation: adaptive response in terms of initial DNA damage. Radiat. Res. 166 (3), 474–478.

Õkva, K., Tamoseviciute, E., Ciziute, A., Pokk, P., Ruksenas, O., Nevalainen, T., 2006. Refinements for intragastric gavage in rats. Scand. J. Lab. Anim. Sci. 33 (4), 243–252.

Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 158–169.

Park, Y.S., Kim, Y.G., Chang, J.C., Kim, D.Y., 1993. Radioprotective effects of red ginseng extracts on antioxidants and lipid peroxidation of the liver in gamma-irradiated mice. Korean Biochem. J. 26, 184–191.

Peltola, V., Parvinen, M., Huhtaniemi, I., Kulmala, J., Ahotupa, M., 1993. Comparison of effects of 0.5 and 3.0 Gy X-irradiation on lipid peroxidation and antioxidant enzyme function in rat testis and liver. J Androl. 14 (4), 267–274.

Pradeep, K., Park, S.H., Ko, K.C., 2008. Hesperidin a flavanoglycone protects against γ-irradiation induced hepatocellular damage and oxidative stress in Sprague–Dawley rats. Eur. J. Pharmacol. 587, 273–280.

Ramos de Andrade, E., Da Costa Escobar Piccoli, J., Manica da Cruz, I.B., Teixeira Rocha, J.B., Andrade, V.V., González, P., Bauermann, L.F., Barrio, J.P., 2009a. Effect of black grape juice intake on liver lipoperoxidation and body weight loss in whole body X-irradiated rats. J. Med. CBR. Def. 7, 1–12.

Ramos de Andrade, E., Da Costa Escobar Piccoli, J., Manica da Cruz, I.B., Teixeira Rocha, J.B., Garzo, E., Marina, R., Mauriz, J.L., González, P., Barrio, J.P., 2009b. Radiomodifying effect of organic grape juice supplementation on hematological parameters and organ weight in whole-body X-irradiation in rats. Nutr. Hosp. 24, 297–303.

Saada, H.N., Said, U.Z., Meky, N.H., Abd El Azime, A.S., 2009. Grape seed extract *Vitis vinifera* protects against radiation-induced oxidative damage and metabolic disorders in rats. Phytother. Res. 23, 434–438.

Saada, H.N., Ussama, Z.S., Mahdt, A.M., 2003. Effectiveness of Aloe vera on the antioxidant status of different tissues in irradiated rats. Pharmazie 58, 929–931.

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Singh, U., Suman, A., Sharma, M., Singh, J., Singh, A., Maurya, S. 2008. HPLC analysis of the phenolic profiles in different parts of chilli (*Capsicum annum*) and okra (*Abelmoschus esculentus* L.) Moench. The Internet Journal of Alternative Medicine 5(2).

Souto, A., Carneiro, M.C., Seferin, M., Senna, M.J.H., Conz, A., Gobbi, K., 2001. Determination of trans-resveratrol concentrations in Brazilian red wines by HPLC. J. Food Comp. Anal. 14, 441–445.

Velioğlu-Oğünç, A., Sehirli, O., Toklu, H.Z., Ozyurt, H., Mayadagli, A., Eksioglu-Demiralp, E., Erzik, C., Cetinel, S., Yegen, B.C., Sener, G., 2009. Resveratrol protects against irradiation-induced hepatic and ileal damage via its anti-oxidative activity. Free Radical Res. 43, 1060–1071.

Waldbillig, R.J., Bartness, T.J., 1981. Insulin induced drinking: an analysis of the hydrational variables. Physiol. Behav. 26, 787–793.

Weiss, J.F., Landauer, M.R., 2009. History and development of radiation-protective agents. Int. J. Rad. Biol. 85, 539–573.

Wills, E.D., 1987. Evaluation of lipid peroxidation in lipids and biological membranes. In: Snell, K., Mullock, B. (Eds.), Biochemical Toxicology: A Practical Approach. IRL Press, Oxford, pp. 407–420.

Wilson, J.W., Cucinotta, F.A., Shinn, J.L., Simonsen, L.C., Dubey, R.R., Jordan, W.R., Jones, T.D., Chang, C.K., Kim, M.Y., 1999. Shielding from solar particle event exposures in deep space. Radiat. Res. 30, 361–382.

Zu, Y., Li, C., Fu, Y., Zhao, C., 2004. Simultaneous determination of catechin, rutin, quercetin, kaempferol and isorhamnetin in the extract of sea buckthorn (*Hippophae rhamnoides* L.) leaves by RP–HPLC with DAD. J. Pharm. Biomed. Anal. 41, 714–719.

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