Grape juice concentrate prevents oxidative DNA damage in peripheral blood cells of rats subjected to a high-cholesterol diet

Odair Aguiar Jr [1], Andréa Pittelli Boiago Gollücke[2], Bárbara Bueno de Moraes[1], Gabriela Pasquini[1], Rodrigo Ramos Catharino[3], Maria Francesca Riccio[3], Silvia Saiuli Miki Ihara[4] and Daniel Araki Ribeiro[1,4]*

[1]*Departamento de Biociências, Universidade Federal de São Paulo (UNIFESP), Avenida Ana Costa, 95 Vila Mathias, Santos 11060-001, SP, Brazil*
[2]*Hexalab and Nutrition Department, Catholic University of Santos, Santos, Brazil*
[3]*Department of Clinical Pathology, School of Medical Sciences, State University of Campinas, Campinas, Brazil*
[4]*Departamento de Patologia, Universidade Federal de São Paulo, São Paulo, Brazil*

Abstract

The goal of the present study was to investigate whether subchronic treatment with grape juice concentrate is able to protect liver and peripheral blood cells against cholesterol-induced injury in rats. The effects of the grape juice concentrate treatment on histopathological changes, immunohistochemistry for cyclo-oxygenase-2 (COX-2), and basal and oxidative DNA damage induced by H_2O_2 using a single-cell gel (comet) assay were evaluated. Male Wistar rats (*n* 18) were divided into three groups: group 1 – negative control; group 2 – cholesterol at 1 % (w/w) in their diet, treated for 5 weeks; group 3 – cholesterol at 1 % in their chow, treated for 5 weeks, and grape juice concentrate at 222 mg/d in their drinking-water in the final week only. The results indicated that the treatment with grape juice concentrate did not show remarkable differences regarding liver tissue in group 3 compared with group 2. However, grape juice concentrate was able to decrease oxidative DNA damage induced by H_2O_2 in peripheral blood cells, as depicted by the tail moment results. COX-2 expression in the liver did not show statistically significant differences ($P > 0.05$) between groups. Taken together, the present results suggest that the administration of subchronic grape juice concentrate prevents oxidative DNA damage in peripheral blood cells.

Key words: Cholesterol diet: Rats: Grape juice: Cyclo-oxygenase-2: Oxidative DNA damage

Recently, it has been postulated that there is an increased risk of CVD or atherosclerosis in humans with hypercholesterolaemia, independently of other blood lipid levels[1]. A reduction in the serum cholesterol level in hypercholesterolaemia is therefore desirable, and has mainly been accomplished by inhibiting cholesterol synthesis or by blocking the absorption of dietary cholesterol[2].

Phenolic-rich foods have received increasing attention due to recent findings on their association with disease prevention[3]. Several studies have identified dietary sources of polyphenols as being mainly fruits, fruit juices and beverages, such as wine, tea and coffee[4,5]. They are thought to contribute to the putative beneficial effects. For example, oral administration of grape flavonoids has been shown to confer antioxidant protection, inhibit platelet function, reduce thrombus formation and concentration of inflammatory biomarkers and inhibit the activation of nuclear transcription factor B[6–9].

Accumulating evidence suggests that oxidative DNA damage plays an important role in some chronic degenerative diseases, such as chronic renal failure, hypertension and myocardial infarction[10–12]. In humans, oxidative DNA damage is also considered an important promoter of cancer[13]. It has been recognised as a major cause of cell death and mutations in all aerobic organisms. Some authors have demonstrated that red grape juice ingestion results in increased serum antioxidant capacity and protection of LDL against oxidation, and thus a non-alcoholic red grape extract may have similar beneficial effects to red wine[14]. However, to the best of our knowledge, no studies have addressed whether it also prevents the formation of single- and double-strand breaks induced by hyperlipidaemia.

Cyclo-oxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to PG, which has two isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in many tissues and mediates the synthesis of PG required for normal physiological function. COX-2 is normally undetectable in most of the tissues but is rapidly induced by pro-inflammatory or mitogenic stimuli[15]. Although it has been documented that grape juice polyphenols can prevent inflammation, eliminate excess free radicals and stimulate the regeneration of damaged cells or tissues[16], no studies have revealed the role of COX-2 following the hypercholesterolaemic state treated with grape juice.

Electrospray ionisation-mass spectrometry (ESI-MS) with a direct infusion of the sample has emerged as a robust alternative for the fingerprinting characterisation of foods, with a fast and simple preparation of samples. This new technique has been successfully applied in the typification of several beverages[17], and most recently, in grape juice[18]. ESI-MS fingerprinting is a convenient method for the direct analysis of grape juice concentrate, as most of the molecules bearing acidic or basic sites can be detected. The MS/MS technique with collision-induced dissociation is used for a more detailed structural elucidation of compounds.

Grape juice concentrate has recently been used as an alternative natural food colourant to replace synthetic colours suspected of having certain adverse effects[19]. The product is obtained by nanofiltration of the juice from red grapes (*Vitis labrusca*, mostly of the Concord variety), with subsequent concentration at 68° Brix by evaporation. This results in a concentrated syrup with higher colour intensity, due to greater amounts of polyphenols. Thanks to its phenolic content, one could speculate that grape juice concentrate might exert physiological effects.

As a result, and because of limited evidence, the aim of the present study was to investigate whether grape juice concentrate can modulate the hypercholesterolaemic state in rats, by means of DNA damage and COX-2 expression.

Materials and methods

Animals and experimental design

All experimental protocols involving animals conformed to the procedures described in the Guiding Principles for the Use of Laboratory Animals. The study was approved by the Animal Committee of Federal University of São Paulo, UNIFESP, SP, Brazil.

Male Wistar rats (8 weeks old), weighing approximately 250 g, were obtained from the Centro de Desenvolvimento de Modelos Experimentais, Federal University of São Paulo, SP, Brazil. They were maintained under controlled conditions of temperature (24 ± 2°C), 12 h light–12 h dark cycles and with free access to water and commercial diet (Nuvital, Paraná, Brazil). All animals were acclimatised

for 7 d before the experiment, fed *ad libitum* standard pellet chow and fresh water.

A total of eighteen rats were randomly divided into three groups (six animals per group). To induce a hypercholesterolaemic state, twelve animals were treated with a standard rat chow diet added with 1% (w/w) cholesterol (C8503; Sigma Chemical Company, St Louis, MO, USA) and 0·25% (w/w) cholic acid (C1254; Sigma Chemical Company; cholesterol-enriched diet), according to the methodology described by Manzoni *et al.*[20]. Immediately after weaning, animals were fed a cholesterol-enriched diet for 5 weeks to induce hypercholesterolaemia. Animals were divided into two groups: six animals without any treatment (positive control) and six animals receiving grape juice concentrate, freshly prepared, dissolved in their drinking-water in the final week only (in the final 7 d). A total of six animals served as the negative control group. Body weight was recorded weekly.

At the end of the experimental period, the rats were killed with 0·4% sodium pentobarbital (1 ml/kg, intraperitoneally). The livers were longitudinally bisected for morphological and biochemical examinations. For this purpose, a fragment from the liver was homogenised in two volumes of NaCl at 0·9% to promote cell separation. Then, a volume of 20 μl supernatant was subjected to a single-cell gel (comet) assay[21]. The remainder of the tissues were fixed in 10% buffered formalin (Merck, Darmstadt, Germany), embedded in paraffin blocks and stained with haematoxylin and eosin (Merck, Whitehouse Station, NJ, USA).

Grape juice concentrate intake

Animals were given 222 mg/d of grape juice concentrate (G8000™; Golden Sucos, Farroupilha-RS, Brazil) in their drinking-water. The amount of polyphenols was calculated to be equivalent to four glasses (200 ml each) of natural grape juice[22] and adjusted to the fastest animal metabolism (twice as fast as humans). According to the American Dietetic Association, human consumption of approximately 200–500 ml of grape juice presents moderate to strong evidence of positive physiological effects[23].

Determination of total phenols and *in vitro* antioxidant activity of grape juice concentrate

Total phenols were measured by the Folin–Ciocalteu assay[24], using gallic acid (Sigma-Aldrich, St Louis, MO, USA) for the standard curve, and the results were expressed as mg gallic acid equivalents/kg. The readings (in triplicate) were taken at 740 nm, using a Genesis 2 spectrometer. For the evaluation of *in vitro* antioxidant activity, the 1,1-diphenyl-2-picrylhydrazil (Sigma-Aldrich, Steinheim, Germany) assay was used based on the methods of Brand-Williams *et al.*[25], as modified by Kim *et al.*[26]. Absorbance was measured with a Beckman spectrometer

at 517 nm before the addition of samples and after 30 min. The difference was plotted on a vitamin C (ascorbic acid) (Merck, USA) standard curve. Analyses were carried out in triplicate and the results were expressed in mg vitamin C equivalents/kg.

Characterisation of grape juice concentrate by electrospray ionisation-mass spectrometer fingerprinting

ESI-MS data were collected in the negative-ion mode on an API5000 mass spectrometer (Applied Biosystems/MDS Analytical Technologies, ON, Canada). Typical ESI-MS conditions were as follows: curtain gas, ion source gas and ion transfer voltage were 10 V, 15 and 4 kV, respectively, with a desolvation temperature of 200°C. To each sample (0·1 ml), 1 ml of a methanol–water solution (7:3, v/v) and 0·1 ml of a methanol solution of ammonia (0·1 %, v/v) were added. The diluted solution was then directly infused into the ESI source at a flow rate of 10 μl/min via a microsyringe pump. The mass analyser was set to scan along a m/z range of 50–1000. ESI-MS/MS experiments were carried out by selection of a specific ion by Q1 and then submitting this to collision-induced dissociation with Ar in a collision chamber. The collision energy was optimised for each component, varying from 15 to 50 V. ESI-MS data from the sample were extracted using the Analyst 1.4.1 (Applied Biosystems/MDS Analytical Technologies). Mass spectrum data were accumulated over 60 s. The degree of confidence for this method is 99 %.

Measurement of cholesterol levels

Measurements of total cholesterol were performed through specific enzymatic kits as follows: to determine total cholesterol, the fast colour method was employed with reagents from the Sera Pak-Ames-Analyzer (RAXT-Technicon, Ames, IA, USA).

Histopathological analysis

Histopathological changes, including steatosis and inflammation, were analysed by the semi-quantitative method (Table 1) according to Zhang *et al.*[27].

Table 1. Score criteria for hepatopathological changes (including steatosis and inflammation)[26]

Score	Steatosis area (%)	Inflammation
0	0	0
1	<30	Watering degeneration and some small necrosis
2	<30	Ballooning degeneration, more small necrosis, mallory body and locally PMN infiltration
3	>50	Severe degeneration and necrosis and bridging necrosis

PMN, polymorphonuclear leucocyte.

Single-cell gel (comet) assay and challenge assay

The peripheral blood and liver samples collected from each animal were divided into two aliquots of 30 μl each to study (1) basal DNA damage and (2) DNA damage due to oxidative stress as described elsewhere[21,28]. For this purpose, the first aliquot was immediately processed for the comet assay to assess basal DNA damage. The second aliquot was treated with a known oxidative DNA damage, H_2O_2 (Sigma-Aldrich). On the basis of previous experiments carried out in our laboratory, samples were treated with 10 μM-PBS (at 4°C for 5 min). Samples were then washed three times with PBS to remove any residual mutagen and processed for the comet assay.

The protocol used for the single-cell gel (comet) assay followed the guidelines proposed by Tice *et al.*[29]. Briefly, a volume of 10 μl peripheral blood or cellular suspensions from the liver (approximately 1×10^4 cells) were added to 120 μl of 0·5 % low-melting-point agarose at 37°C, layered onto a pre-coated slide with 1·5 % regular agarose and covered with a coverslip. After brief agarose solidification in a refrigerator, the coverslip was removed and the slides were immersed in lysis solution (2·5 M-NaCl, 100 mM-EDTA – Merck, Darmstadt, Germany; 10 mM-Tris–HCl buffer, pH 10 – Sigma Aldrich; 1 % sodium sarcosinate – Sigma; with 1 % Triton X-100 – Sigma; 10 % dimethyl sulphoxide – Merck) for approximately 1 h. Before electrophoresis, the slides were left in alkaline buffer (0·3 mM-NaOH (Merck) and 1 mM-EDTA (Merck); pH > 13) for 20 min and electrophoresed for another 20 min at 25 V (0·86 V/cm) and 300 mA. After electrophoresis, the slides were neutralised in 0·4 M-Tris–HCl (pH 7·5), fixed in absolute ethanol and stored at room temperature until analysis. In order to minimise extraneous DNA damage from ambient UV radiation, all steps were performed with reduced illumination.

A total of fifty randomly captured comets from each slide[30] were blindly examined at 400 × magnification, using a fluorescence microscope (Olympus, Orangeburg, NY, USA) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments, Haverhill, Suffolk, UK). This computerised image analysis system records images, computes the integrated intensity profiles for each cell, estimates the comet cell components and then evaluates the range of derived parameters. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. To quantify the DNA damage, the tail moment was evaluated. The tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. The comet tail moment is positively correlated with the level of DNA breakage in a cell. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in such a sample.

Immunohistochemistry

Liver sections of 3 μm were deparaffinised in three changes of xylene and rehydrated in a graded series of ethanol to distilled water. For antigen retrieval, slides were placed in 0·01 M-citrate-buffer (pH 6·0) and heated in a steamer for 30 min. Endogenous peroxidases were quenched by incubating in 3 % H_2O_2 for 20 min at room temperature. The sections were incubated overnight at 4°C with a primary antibody: COX-2 mouse polyclonal antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, the sections were incubated with a biotinylated secondary antibody (LSAB, Dakocytomation, Denmark) for 30 min, washed in PBS and incubated with a streptavidin–peroxidase conjugate (LSAB) for 30 min. Finally, the reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma) for 5 min. The slides were briefly counterstained in haematoxylin and dehydrated, and coverslips were added. Negative and positive controls were run simultaneously. Positive controls were represented by mammary tissue. Negative controls were made by eliminating the primary antibody.

Immunostaining was scored by two trained independent observers (B. B. de M. and D. A. R.) without prior knowledge of the histopathological parameters. Discordant cases were reviewed and agreed upon before the data had been statistically analysed. For this purpose, immunohistochemically stained liver sections were analysed for the percentages of immunopositive cells in areas diagnosed as normal and in damaged liver cells. A total of 1000 liver cells were evaluated in three to five fields at 400 × magnification. All values were used as labelling indices. Such a protocol was established in previous studies conducted by our research group[31].

Statistical methods

Initial body weight, weight gain, total cholesterol and comet assay data were analysed by one-way ANOVA followed by Tukey's test. Statistical analyses for immunohistochemistry data and histopathological scores were assessed by the Kruskal–Wallis non-parametric test, followed by post hoc analysis (Dunn's test), using the SPSS software package (version 1.0; SPSS Institute, Chicago, IL, USA). A P value < 0·05 was considered statistically significant.

Results

Chemical analyses

The results of the total phenol content of grape juice concentrate showed a concentration of 45·8 g gallic acid equivalents/kg with an antioxidant activity of 27·3 g vitamin C equivalents/kg. Fig. 1 displays the ESI(−)-MS spectrum of grape juice concentrate, revealing the major components of interest (m/z 200–500). The ESI(−)-MS fingerprints show evidence of characteristic distributions mainly of the following compounds: dimethoxy-flavylium and disaccharide, detected as the deprotonated molecules of m/z 313 and 359, respectively (Table 2). Fatty acids, namely palmitic and linoleic acids, were also present in a relatively significant intensity. This technique also identified caffeoyltartaric acid, fertaric acid and caffeoylquinic acid as the main organic acids, detected as the deprotonated molecules of m/z 311, 325 and 353, respectively.

The most abundant anthocyanidins, identified under this technique, were kaempferol-galactoside, peonidin-glucoside, malvidin-glucoside, petunidin 3-O-acetylglucoside, peonidin 3-p-coumaroylglucoside and malvidin
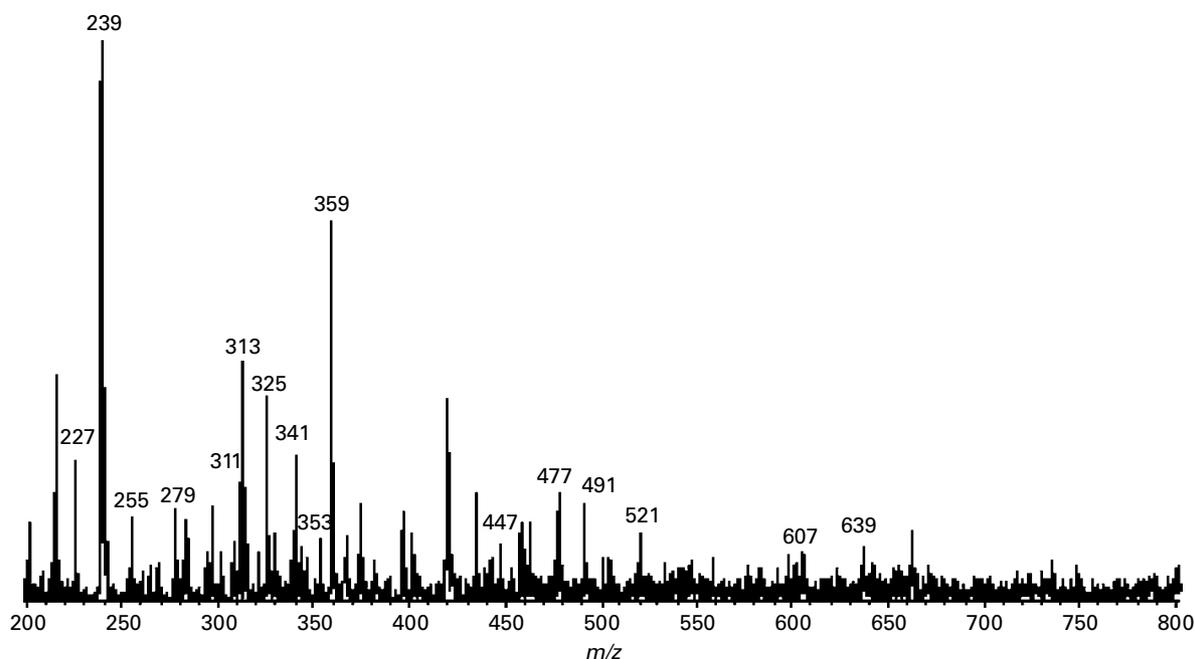

Fig. 1. Electrospray ionisation-MS fingerprinting of the grape juice concentrate.

Table 2. Identified compounds in grape juice concentrate using electrospray ionisation-mass spectrometer/MS

Compounds	Deprotonated ions [M-H]⁻ (*m/z*)	MS/MS ions (*m/z*)	Relative abundances
Resveratrol	227	185	30
Palmitic acid	255	–	17
Linoleic acid	279	–	18
Quercetin	301	151	10
Caffeoyltartaric acid	311	179	28
Dimethoxy-flavylium	313	295	50
Fertaric acid	325	193	47
Caffeoyl glucose	341	179	35
Caffeoylquinic acid	353	191	15
[Disaccharide + H_2O]⁻	359	162	65
Kaempferol-galactoside	447	285	11
Peonidin-glucoside	461	299	8
Quercetin 3-*O*-glucuronide	477	301	26
Malvidin-glucoside	491	329	24
Petunidin 3-*O*-acetylglucoside	521	317	16
Peonidin 3-*p*-coumaroyl glucoside	607	453	11
Malvidin 3-*O*-*p*-coumaroyl glucoside	639	331	11

3-*O*-*p*-coumaroylglucoside, presenting molecules of *m/z* 461, 477, 491, 521, 607 and 639, respectively. Quercetin appeared as a single molecule and in the glucuronide form, detected as the deprotonated molecules of *m/z* 301 and 477, respectively. Resveratrol was also identified as the deprotonated molecule of *m/z* 227 in a relatively significant abundance.

Clinical findings

Neither complications nor behavioural changes were observed. The mean body weights during the experimental period are displayed in Table 3. No statistically significant differences ($P > 0.05$) were observed in either the initial body weight or the weight gain for all groups evaluated. No animals died during the experiment.

Total cholesterol levels

Animals that received the tested combinations of cholesterol and cholic acid had significantly elevated serum cholesterol concentrations. The grape juice concentrate was not able to reduce cholesterol levels, i.e. no statistically significant differences ($P > 0.05$) were observed compared with the negative control group. These findings are shown in Table 3.

Histopathological analysis

Table 4 shows the effect of grape juice concentrate on the degree of histopathological changes in liver tissues in normal and cholesterol-induced rats. Under microscopic evaluation, liver tissues presented hepatic degeneration in the group treated with cholesterol only for 5 weeks

Table 3. Total cholesterol levels, initial and body-weight gain of rats treated with cholesterol and subjected to grape juice concentrate (GJC) therapy

(Mean values and standard deviations, *n* 6)

Groups	Total cholesterol (ng/l) Mean	SD	Initial body wt (g) Mean	SD	Wt gain Mean	SD
G1 (negative control)	1207[a]	135	329·0	30·6	80·2	15·2
G2 (positive control)	1420[b]	120	333·2	29·2	74·4	43·3
G3 (cholesterol and GJC)	1541[b]	200	319·2	24·4	62·6	25·1

[a,b] Mean values with unlike superscript letters were significantly different ($P < 0.05$).

(Fig. 2(b)) compared with the negative control group (Fig. 2(a)). Degenerative vacuoles in hepatic tissues, increased steatosis in lobular central bands, spotty necrosis and focal necrosis with infiltration of inflammatory cells were observed in the majority of animals exposed to cholesterol only. In specimens exposed to cholesterol and grape juice concentrate, no remarkable differences were found (Fig. 2(c)).

Genotoxicity data

The single-cell gel (comet) assay was used to measure DNA damage in peripheral blood and liver cells *in vivo*. DNA strand breaks were represented by tail moment for fifty comets for each sample. Data were expressed as the means and standard deviations from all groups evaluated in the present experimental design. Regarding peripheral blood cells, none of the groups showed any signs of genotoxicity, as depicted by no differences in the tail moment values. These findings are summarised in Fig. 3.

Regarding liver cells, extensive DNA migration was observed in animals treated with cholesterol only compared with the negative control group. The grape juice concentrate was not able to decrease DNA migration because no statistically significant differences ($P > 0.05$) were observed between the evaluated groups (Fig. 4).

When oxidative DNA damage was evaluated, statistically significant differences were noticed among groups in peripheral blood cells. Particularly, grape juice concentrate was able to protect against oxidative DNA damage

Table 4. Total number of rats in all groups according to the degree of histopathological changes in liver tissues*

Groups	Scores 0	1	2	3
G1 (negative control)	6	0	0	0
G2 (positive control)	0	2	2	2
G3 (cholesterol and GJC)	2	2	2	0

GJC, grape juice concentrate.
* Mean values were not significantly different ($P > 0.05$).
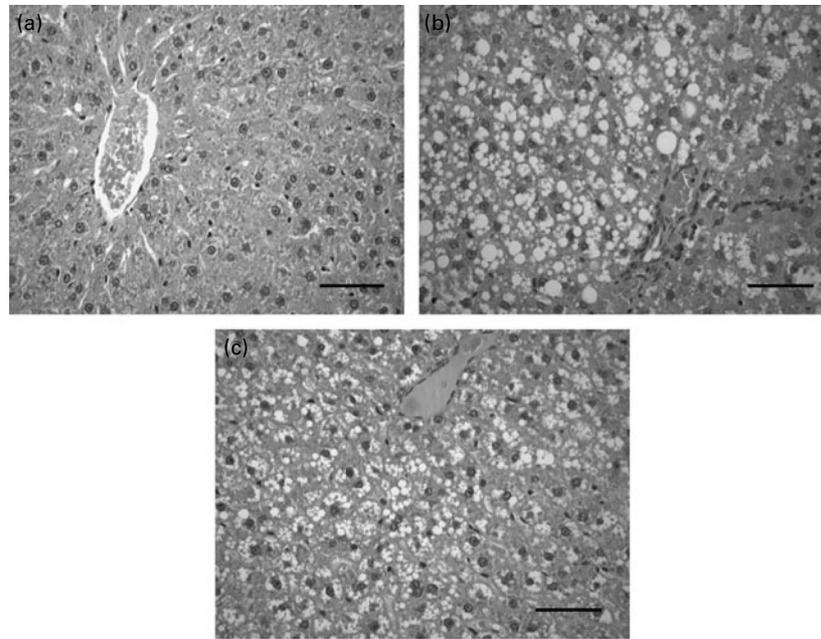
Fig. 2. Histopathological analysis of the liver tissue in rats. (a) Ordinary appearance of the liver in the control rat, (b) rats subjected to liver injury induced by cholesterol and (c) rats treated with grape juice concentrate presenting the same histopathological changes induced by cholesterol only. Haematoxylin and eosin stain; scale bar = 36 μm.

(Fig. 3). In animals exposed to cholesterol, high DNA damage was detected after *in vitro* exposure to H_2O_2 in liver cells when compared to the negative control group. Besides, no statistically significant differences ($P > 0.05$) were found in specimens treated with grape juice concentrate compared with cholesterol-induced liver DNA damage (Fig. 4).

Considering the tail moment parameter for the positive control, it was clearly observed that H_2O_2 caused higher damage in DNA in comparison with the negative control group.

Immunohistochemistry

COX-2 expression was detected in the cytoplasm of liver cells. COX-2 expression was weakly detected in all negative controls. On the whole, the expression was considered negative for the group (Fig. 5(a)). COX-2-positive cells were found in the cholesterol-treated group. The same picture appeared concerning the animals exposed to cholesterol and grape juice concentrate (Fig. 5(b)). No statistically significant differences ($P > 0.05$) were observed in the morphometric analysis between the experimental groups and the negative control group. These findings are shown in Fig. 6.

Discussion

The goal of the present study was to investigate whether subchronic treatment with grape juice concentrate can protect peripheral blood and liver cells against a high-cholesterol diet in rats. The effects of the grape juice

concentrate treatment on histopathological changes, oxidative DNA damage and immunohistochemistry for COX-2 were evaluated. To the best of our knowledge, such an approach has not been addressed yet.

With respect to the liver histopathological examination, cholesterol-treated rats displayed extensive steatosis, presenting liver degeneration with inflammatory cells and necrotic areas. In this regard, concomitant treatment with grape juice concentrate did not alter the pathological features induced by cholesterol, as evident from the
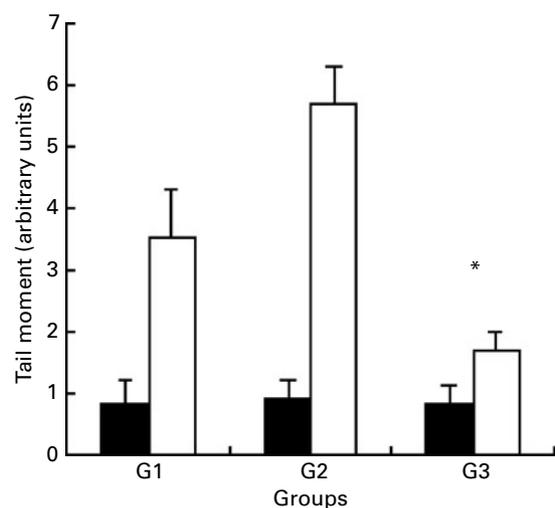
Fig. 3. DNA damage in peripheral blood cells exposed or not exposed to hydrogen peroxide. G1, negative control; G2, cholesterol-treated rats; G3, cholesterol-treated rats exposed to grape juice concentrate during 1 week. Results are expressed as the means and standard deviations. * Mean values were significantly different compared with G1 (positive control) ($P < 0.05$). ■, DNA damage; □, exposure to H_2O_2.
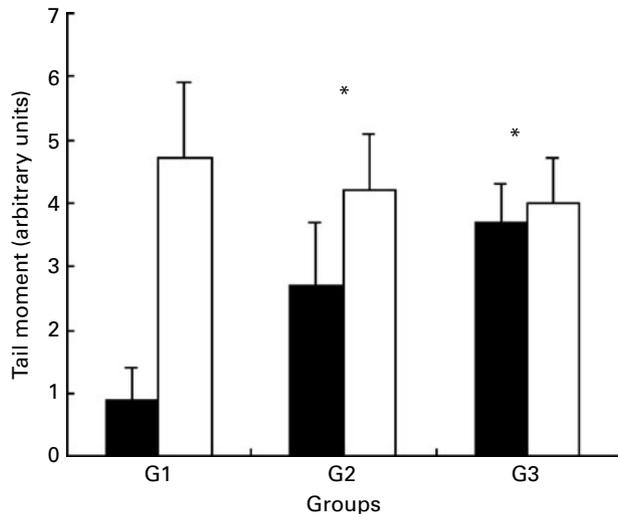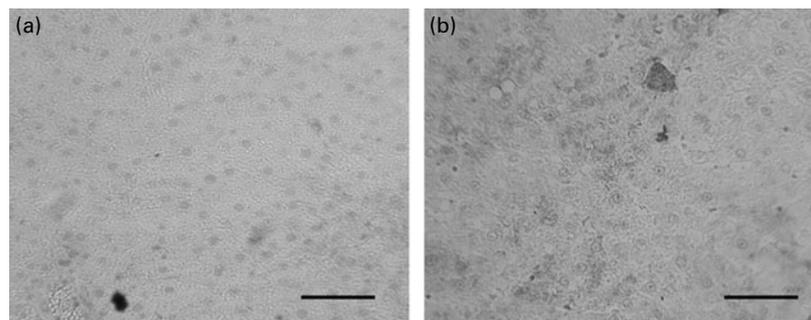
Fig. 4. DNA damage in liver cells exposed or not exposed to hydrogen peroxide. G1, negative control; G2, cholesterol-treated rats; G3, cholesterol-treated rats exposed to grape juice concentrate during 1 week. Results are expressed as the means and standard deviations. * Mean values were significantly different compared with G1 (negative control) ($P < 0.05$). ■, DNA damage; □, exposure to hydrogen peroxide.

histopathological examinations, indicating no protective effect on the liver. In addition, grape juice concentrate did not decrease cholesterol levels, because no statistically significant differences were observed between the groups. In the present study, feeding rats with 1 % cholesterol and cholic acid for 5 weeks increased serum cholesterol by only 18 %. Probably, the amount of cholesterol was not very high compared with other studies[32,33], but statistically significant differences were found between controls. Taken together, it seems that grape juice concentrate is not able to prevent experimental steatosis as well as hypercholesterolaemia under the experimental conditions used in the present study.

Accumulating evidence suggests that antioxidants constitute the foremost defence system that limits the toxicity associated with free radicals[34]. Hence, these antioxidants are expected to be effective against cellular injury induced by several pathological processes. Results from a few studies on animals and human subjects confirmed that

polyphenolic compounds contained in grape juice exert protective effects on lymphocytes, limiting oxidative DNA damage possibly by decreasing free-radical levels[35,36]. The alkaline version of the single-cell gel (comet) assay used in the present study is sensitive to a wide variety of DNA lesions. Among them are single- and double-strand breaks, oxidative DNA base damage, alkali-labile sites, including abasic and incomplete repair sites, and DNA−DNA/DNA−protein/DNA−drug cross-linking in any eukaryotic cell[29]. Tail moment is a virtual measure calculated by a computerised image analysis system, considering both the length of DNA migration in the comet tail and the tail intensity. Such a parameter is one of the best indices of induced DNA damage among the various parameters calculated by this method[29]. The results of the present study indicate that the alkaline single-cell gel (comet) assay, in the experimental conditions used herein, failed to detect the presence of DNA damage after the treatment with cholesterol, as well as with grape juice concentrate in peripheral blood cells. Therefore, cholesterol and grape juice concentrate are not genotoxins in such cells. However, extensive DNA damage was detected in liver cells after the treatment with cholesterol. As a result, grape juice concentrate was not able to prevent DNA damage in liver cells induced by experimental steatosis at a feasible dietary dose. These findings confirmed and extended previously published data concerning genotoxicity induced by liver steatosis[37].

When oxidative DNA damage was induced *in vitro*, with reference to either peripheral blood cells or liver cells, grape juice concentrate was able to prevent oxidative DNA damage induced by H_2O_2 only in peripheral blood cells. This a result is in agreement with other studies[6,38]. Some studies have revealed antioxidant potential exerted by grape juice extracts in peripheral blood cells from haemodialysis patients and healthy subjects[39]. Moreover, recent findings have revealed antioxidant activities from organic purple grape juice in both the plasma and the liver compared with the conventional and control groups. Resveratrol is a polyphenolic compound present in significant amounts in grape juice concentrate evaluated in this setting. It has been postulated that resveratrol exhibits

Fig. 5. Immunohistochemistry for cyclo-oxygenase-2: (a) negative control; (b) cholesterol-treated rats exposed to grape juice concentrate (immunohistochemistry stain, scale bar = 24 μm).
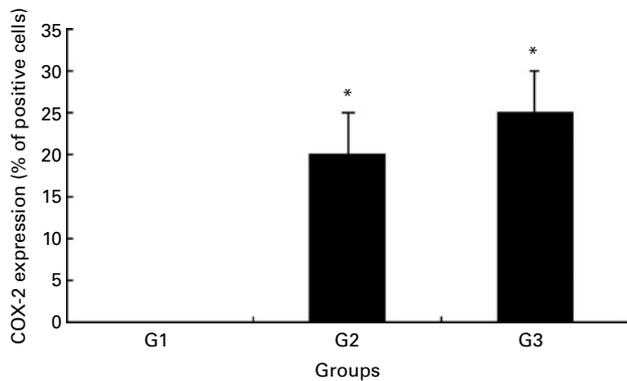
Fig. 6. Cyclo-oxygenase-2 (COX-2) labelling index. G1, negative control; G2, cholesterol-treated rats; G3, cholesterol-treated rats exposed to grape juice concentrate during 1 week. Results are expressed as the means and standard deviations. * Mean values were significantly different compared with G1 (negative control) ($P < 0.05$).

antioxidative, anti-inflammatory and chemopreventive properties in mammalian systems[35]. Conversely, Azmi et al.[40] have demonstrated that resveratrol also catalyses oxidative DNA degradation in vitro in the presence of transition metal ions, such as Cu[40]; this would at least partially explain the present results. Probably, the realistic grape juice concentrate dose and the short exposure were not enough to produce a positive response in the liver against oxidatively DNA-damaging agent in vitro. Moreover, limited uptake, bioefficacy or even limited bioavailability and efficacy post-metabolism in the liver may influence the positive response. Consequently, further study is required. It is important to stress that the single-cell gel (comet) assay does not necessarily predict the mutagenic potential of the test compound. One possible explanation for the absence of a close relationship to mutagenesis is that the effects seen in the single-cell gel (comet) assay for some substances mainly occur as a consequence of an error-free DNA repair process[41]. In addition, no single test is capable of detecting all genotoxic agents. Thus, for a more detailed judgement on the genotoxic potential of cholesterol as well as anti-genotoxicity exerted by grape juice concentrate, a battery of tests is necessary. Taken as a whole, we assume that grape juice concentrate can protect oxidative DNA damage in peripheral blood cells in rats.

To further elucidate putative mechanisms of action involving grape juice concentrate on rat liver injury, we designed additional experiments by means of immunohistochemistry. It has been postulated that tea polyphenols have been found to prevent inflammation, eliminate excess free radicals and stimulate the regeneration of damaged cells or tissues[42]. The present results demonstrated COX-2 expression in rats with cholesterol-damaged liver. Exposure to grape juice concentrate did not alter the COX-2 levels in liver cells, as depicted by morphometric results. Therefore, it seems that grape juice concentrate was not able to exert any anti-inflammatory activity, as

depicted by COX-2 synthesis, on damaged liver cells. In fact, some studies have postulated that cholesterol exerts complex effects on the liver and that the generation of inflammatory mediators through an increased expression of COX-2 plays an important role in the pathogenesis of liver injury, such as hepatic cirrhosis[43]. By comparison, some authors have demonstrated that resveratrol, at lower doses, has few significant changes in gene expression, as depicted by complementary DNA microarrays in the rat liver[43].

In conclusion, the results of the present study suggest that subchronic grape juice concentrate administration was able to prevent oxidative DNA damage in peripheral blood cells. Further studies, possibly with higher doses, are welcomed to advance on the subject and to elucidate the issue.

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