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Research Article

Phenolics from purple grape, apple, purple grape juice and apple juice prevent early atherosclerosis induced by an atherogenic diet in hamsters

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Knowledge of the effects of processing on the antioxidant properties of fruits is limited. We investigated the processing of apple (A) and purple grape (PG) and their juices (AJ and PGJ) in hypercholesterolemic hamsters. Five groups of eight hamsters each were fed an atherogenic diet for 12 wk. They received daily by gavage either 7.14 mL/(kg.day) of mashed A or PG, or the same volume of AJ or PGJ, or water as control. Plasma cholesterol, non-HDL cholesterol, liver superoxide dismutase and glutathione peroxidase activities, and thiobarbituric acid reactive substances were efficiently reduced by the fruits and their juices compared with controls, whereas plasma antioxidant capacity was increased and aortic fatty streak area was decreased from 48 to 93%. For each of these parameters, the efficacy was PGJ > PG > AJ > A. The results show for the first time that long-term consumption of antioxidants supplied by apple and purple grape, especially phenolic compounds, prevents the development of atherosclerosis in hamsters, and that processing can have a major impact on the potential health benefits of a product. The underlying mechanism is related mainly to increased antioxidant status and improved serum lipid profile.

Keywords: Apple / Atherosclerosis / Grape / Hamsters / Phenolics

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1 Introduction

The beneficial health effect of high and regular fruit consumption is an important point emphasized by recent epidemiological studies. The protective effect afforded by this dietary supply may be particularly beneficial for pathologies such as coronary heart diseases [1 – 3] and some cancers [4]; however, other degenerative pathologies related to oxidative stress and ageing could also profit from the protection offered by a significant consumption of fruits and fruit juices, rich in vitamins, minerals, and micro-constituents [5, 6]. Especially with regard to cardiovascular diseases (CVD), some studies have shown a relationship between a reduced risk of CVD and fruit consumption [2, 3], but no indubitable evidence was given. These protective effects could come from folic acid, kalium, dietary fibers, and micro-constituents, some of them being phenolic compounds [7]. Most of these phenolics are powerful antioxidants [8] and they might also offer protection against CVD [9]. When not consumed raw, fruits undergo processing (bleaching, concentration, freezing, filtration, high pressures, pasteurization, trimming, drying, thermal processes, *etc.*) that can modify these antioxidant properties in various ways, inducing antioxidant loss, improvement of antioxidant properties of natural compounds, formation of new compounds having antioxidant activity, formation of compounds having pro-oxidant properties, or interactions between different compounds [10]. Processing can also affect the bioavailability of bioactive compounds. This

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Abbreviations: **A,** apple; **AFSA,** aortic fatty streak area; **AJ**, apple juice; **CVD**, cardiovascular diseases; **GSHPx,** glutathione peroxidase; **HDL**, high-density lipoprotein; **HDL-C**, high-density lipoprotein cholesterol; **PAC**, antioxidant capacity of plasma; **PG,** purple grape; **PGJ**, purple grape juice; **SOD**, superoxide dismutase; **TBARS**, thiobarbituric acid reactive substances; **TC,** total cholesterol

aspect has been generally overlooked by nutritionists and epidemiologists. Moreover, food composition tables only present foods that are consumed raw and they do not take into consideration the fact that the biological activities of nutrients and micro-constituents can be modified by technological processes. This is an important aspect since only a small quantity of fruits is consumed in the raw state, whereas the major part needs to be treated to preserve quality as well as for safety and financial reasons.

At a time where scientific knowledge is being used by several authorities and media to encourage fruit consumption, it is interesting to find out whether these different processes affect the physical (stability) and biological properties (prevention of pathologies) of phenolic compounds from fruits and fruit juices. Nowadays, there is limited information on the influence of fruit preservation processes and transforming techniques on the level of phenolics and antioxidant capacity.

The purpose of the present study was (i) to determine the phenolic content of apple (A), purple grape (PG), and their juices (AJ and PGJ) and to evaluate their efficacy before and after technological processing, and (ii) to assess a possible preventive effect of their administration on early atherosclerosis in hypercholesterolemic hamsters. Indeed, these compounds constitute useful markers allowing recognition and evaluation of nutritional quality in fresh and processed products.

2 Materials and methods

2.1 Fruits and juices

Apples, variety Reinette du Vigan, and AJ were obtained from the Société Coopérative Agricole Origine Cévennes de Pont d'Hérault (Sumène, France). AJ was prepared from the same apples and was obtained after pressuring, clarification with pectinase, and flash pasteurization at 90°C for 10 s before being bottled. Nothing was added to the juice during its preparation, it was 100% pure juice. PGs, variety Muscat de Hambourg origin Provence, were purchased from a local market (SICA Edelweiss, Marché Gare 84200 Carpentras, France). PGJ was obtained after two cycles of crushing at 60°C for 30 min, and flash pasteurization at 85°C for 20 s before being bottled. Nothing was added to the juice during its preparation, it was 100% pure juice. AJ contained 116 g total sugars *per* liter and PGJ contained 240 g total sugars *per* liter.

2.2 HPLC analysis

HPLC analysis with UV detection was performed using a Hewlett-Packard Model 1090 and a Nucleosil 100 C18 column (250 × 4 mm, 5 µm particle size). The solvents used for separation [11] were as follows: solvent A, 50 mmol/L ammonium dihydrogen phosphate adjusted to pH 2.6 with orthophosphoric acid; solvent B, 20% A with 80% ACN; solvent C, 200 mmol/L orthophosphoric acid adjusted with ammonia to pH 1.5. Elution was performed with a gradient described previously [12]. Detection was carried out at 280, 313, 365, and 520 nm.

2.3 Animals, diets, and experimental design

Forty weanling male Syrian golden hamsters (Elevage Janvier, Le Genest-St-Isle, France) weighing 80 ± 5 g were randomly divided into five groups of eight animals each. They were maintained in plastic cages in an air-conditioned room (23 ± 1°C) with a 12-h light/dark rhythm and allowed free access to both food and water. They were fed a semipurified atherogenic diet for 12 wk, consisting of 200 g/kg casein and 3 g/kg L-methionine, 393 g/kg corn starch, 154 g/kg sucrose, 50 g/kg cellulose, 150 g/kg lard, and 0.5 g/kg cholesterol. Vitamin and mineral mixes (10 and 35 g/kg, respectively) were formulated according to AIN-93 guidelines [13] and supplied by Scientific Animal Food & Engineering (SAFE, Augy, France); mixes did not contain selenium, vitamin C, or vitamin E. All hamsters additionally received daily by gavage either tap water (control group), apple (group A), apple juice (group AJ), purple grape (group PG), or purple grape juice (group PGJ). Apples (without peel) and grapes were mashed daily using a high-pressure cell disrupter (HAIVA Z Plus, Constant Systems, Daventry, UK). The volume of mashed fruits and fruit juices gavaged was adjusted daily to the weight of the hamsters: it was established by extrapolating 600 g/day average A or PG consumption (*i.e.,* three apples or three bunches of grapes), or 500 mL/day average AJ or PGJ consumption, which is equivalent to about four glasses *per* day for a 70-kg person, for the daily weight of hamsters. This represents a volume of 7.14 mL/(kg body weight.day). For comparison, a sixth group (standard group) of hamsters was fed a non-atherogenic diet (laboratory chow) for 12 wk. Hamsters were handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and the NIH guidelines [14].

2.4 Analytical procedures

At the end of the 12-wk experimental period, blood samples were collected after an overnight fast (18 h) by cardiac puncture with the animals under pentobarbital anesthesia. Plasma was harvested after centrifugation at 2000 *g* for 10 min at 4°C, and plasma total cholesterol (TC) was measured enzymatically [15]. Plasma very-low-density lipoprotein and low-density lipoprotein cholesterol (non-HDL-C) samples were precipitated with phosphotungstate reagent [16] and the supernatant was assayed for HDL cholesterol (HDL-C). Plasma non-HDL-C was calculated from the difference between TC and HDL-C. The antioxidant capacity of plasma (PAC) was assayed with a quantitative colorimet-

ric technique according to the kit supplier's instructions (Kit NX2332; Randox, Mauguio, France) and expressed as Trolox equivalent. The assay is based on the incubation of a peroxidase and H_2O_2 with 2,2′-Azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) to produce the radical cation $ABTS^{\circ+}$. This has a relatively stable blue – green color, which is measured at 600 nm.

The liver was perfused with 0.15 mol/L KCl to remove residual blood, rapidly excised, rinsed in ice cold saline, blotted dry, weighed, sectioned for analyses, and stored in liquid nitrogen. Liver was homogenized in four volumes of ice-cold 0.1 mol/L potassium phosphate buffer (pH 7.4) and the homogenate was spun at 13 000 *g* for 15 min at 4°C. The supernatant was then centrifuged at 105 000 *g* for 60 min at 4°C and cytosols were stored at –80°C for subsequent assay of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activities. Liver SOD activity was determined using the Oxis commercial kit Bioxytech SOD-525 (Bioxytech, Bonneuil/Marne, France). The assay is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo(c)-fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. The activity of Se-GSHPx was measured according to Wendel [17] using 0.2 mmol/L hydrogen peroxide as the substrate and including 1.0 mmol/L sodium azide to inhibit catalase, so that only GSHPx activity was measured. The concentration of hepatic thiobarbituric acid reactive substances (TBARS) was measured on the homogenate by a modification of the method of Sinnbuber and Yu [18]. Homogenate (1 mL) was mixed to 1 mL of 17.5% TCA and 1 mL of 0.6% 2-thiobarbituric acid. This mixture was then added to 70% TCA (1 mL). After centrifugation at 1000 *g* for 15 min, the absorbance of the supernatant was measured at 535 nm. TBARS concentration was expressed as ng/mg protein. Thiobarbituric acid 1,1,3,3-tetraethoxy-propane was used as a TBARS standard. Protein content was determined according to Smith *et al*. [19] and using BSA as standard.

2.5 Quantification of aortic fatty streak area

Following blood collection and liver removal, the intact aorta was first perfused with PBS containing 1 mmol/L $CaCl_2$ and 15 mmol/L glucose for 5 min, then with 0.1 mmol/L sodium cacodylate buffer pH 7.4 containing 2.5 mmol/L $CaCl_2$, 2.5% paraformaldehyde and 1.5% glutaraldehyde for the fixation of the vasculature. The aortic tissue was obtained and processed for fatty streak analysis as previously described [20]. A computerized image analysis system (Image J, Scion Corporation, Frederick, MD, USA) attached to a light microscope was used to measure the total Oil Red O-stained area of each aortic arch. The area covered by foam cells (aortic fatty streak lesion area or AFSA) was expressed as a percentage of the total area surveyed. Equal aortic surface areas were compared.

Table 1. Phenolic levels in fruits and juices (NF, not found)

Phenolic	Apple	Purple grape	Apple juice	Purple grape juice
	[mg/100 g]		[mg/100 mL]	
Total phenols content[a)]	155	166	220	589
Total catechins[b)]	1.07	31.60	5.17	10.28
Catechin	NF	0.76	NF	2.58
Epicatechin	1.07	0.42	5.17	1.31
Dimer B1	NF	3.54	NF	0.74
Dimer B3	NF	23.16	NF	5.62
Dimer B4	NF	3.54	NF	NF
EGC	NF	0.03	NF	0.03
EGCG	NF	0.15	NF	NF
Caftaric acid	0.56	NF	1.83	NF
Caffeic acid	1.22	NF	3.99	NF
Gallic acid	0.04	NF	0.05	0.14
p-Coumaric acid	NF	NF	4.83	NF
Isoquercetin	NF	1.49	NF	1.11
Delphinidin	NF	9.30	NF	3.08
Cyanidin-3-glucoside	NF	1.43	NF	0.58
Peonidin-3-glucoside	NF	7.18	NF	2.29

a) Expressed as gallic acid equivalent
b) Procyanidin dimers B1, B3, B4 + catechin + epicatechin

2.6 Statistical analysis

Stat View IV software was used for statistical evaluations (Abacus Concepts, Berkeley, CA, USA). Data were subjected to logarithmic transformation where necessary to achieve homogeneity of variances. A one-way ANOVA followed by Fisher's Protected Least Significant Difference test was used to analyze all data. All values were expressed as mean ± SEM (n = 8 measurements/group) and statistical significance was set at $P < 0.05$.

3 Results

Phenolic levels (total phenols content, catechins, and individuals phenolics) in fruits and fruit juices are given in Table 1. The level of total phenols was similar in A and PG, and PGJ was about 2.5 times richer than AJ. PG was 31 times richer in catechins (sum of procyanidin dimers B1, B2, B3, and B4 and monomeric catechins) in comparison with A; PGJ was two times richer than AJ. A and AJ were characterized by the absence of anthocyanins. For comparison, in a recent study [21] using HPLC with MS, 5-caffeoylquinic acid and conjugates of the hydroxychalcone phloretin were also found in apples, which were not explored here . The total phenol amount ingested daily for animals mimicked a human consumption of 930 mg A and 996 mg PG (which are quite close in amount), but 1100 mg AJ and 2945 mg for PGJ (which is 2.67 times higher).

There was no significant difference in food intake and body weight gain among the five groups (not shown here).

Table 2. Plasma lipid concentrations and PAC in hamsters fed a non-atherogenic diet (standard) or an atherogenic diet plus water (control), A, AJ, PG, and PGJ by gavage for 12 wk[1)]

Group	Standard[2)]	Control	A	AJ	PG	PGJ
	[mmjol/L]					
TC	3.62 ± 0.22[a)]	8.75 ± 0.20[b)]	7.78 ± 0.49[c)]	6.63 ± 0.13[c)]	6.09 ± 0.26[d)]	5.72 ± 0.17[d)]
HDL-C[c)]	1.95 ± 0.17[a)]	3.47 ± 0.37[b)]	4.07 ± 0.23[b)]	4.29 ± 0.49[b)]	4.19 ± 0.19[b)]	3.55 ± 0.25[b)]
Non-HDL-C	1.67 ± 0.20[a)]	5.28 ± 0.16[b)]	3.71 ± 0.23[b)]	2.34 ± 0.25[d)]	1.90 ± 0.26[a)d)]	2.17 ± 0.20[d)]
TC/HDL-C	1.85 ± 0.21[a)]	2.53 ± 0.22[b)]	1.90 ± 0.30[a)]	1.57 ± 0.22[a)]	1.45 ± 0.24[a)]	1.60 ± 0.22[a)]
PAC	1.25 ± 0.09[a)]	0.85 ± 0.03[b)]	0.96 ± 0.04[c)]	1.08 ± 0.04[c)]	1.24 ± 0.07[a)]	1.37 ± 0.06[a)]

1) Values are means ± SEM, $n = 8$. Data were analyzed by one-way ANOVA followed by the Least Significant Difference test. For each dietary treatment, means in a column with different superscripts differ (a – b), $P < 0.05$
2) Standards were fed a non-atherogenic diet for 12 wk

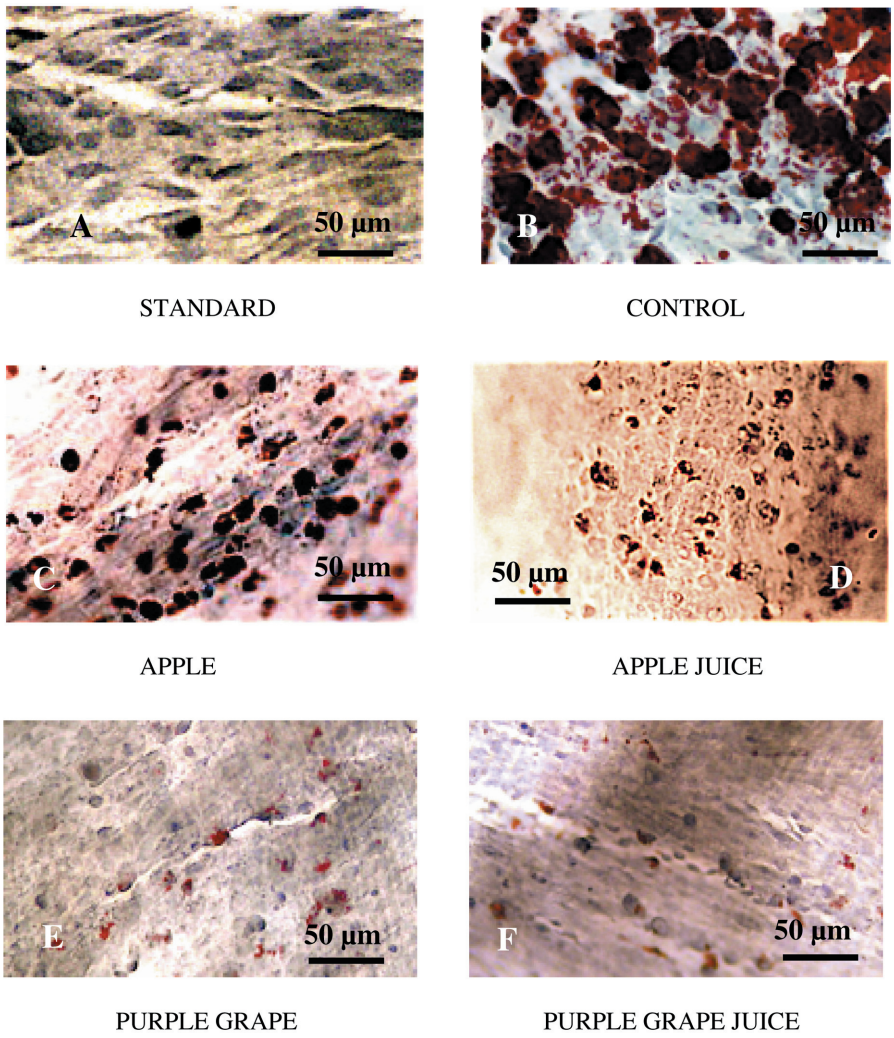

Figure 1. Photomicrographs of typical atherosclerotic lesions of the hamsters' aortic arch (photographed at × 40) after a 12-wk experimentation period. Aortic fat deposits are stained in Oil Red O. (A) From non-atherogenic diet-fed hamsters (standard group). (B) From atherogenic diet-fed hamsters (control group). (C, D, E, F) From atherogenic diet-fed hamsters receiving either apples (C), apple juice (D), purple grape (E), or purple grape juice by gavage.

Table 3. Hepatic antioxidant enzyme activities and TBARS in hamsters fed a non-atherogenic diet (standard) or an atherogenic diet plus water (control), A, AJ, PG, and PGJ by gavage for 12 wks[1)]

Group	Standard[2)]	Control	A	AJ	PG	PGJ
	[units × mg protein^{-1})]					
SOD	2.01 ± 0.44[a)]	10.45 ± 0.4[b)]	7.43 ± 0.76[c)]	7.84 ± 1.16[c)]	4.64 ± 0.53[d)]	2.59 ± 1.38[a)]
GSHPx ($\times 10^{-2}$)	0.74 ± 0.09[a)]	9.28 ± 0.73[a)]	2.69 ± 0.11[c)]	1.42 ± 0.22[d)]	0.61 ± 0.02[a)]	1.23 ± 0.24[d)]
	[ng × mg protein^{-1}]					
TBARS	0.98 ± 0.22[a)]	24.23 ± 4.51[b)]	12.75 ± 2.15[c)]	11.85 ± 1.25[c)]	5.32 ± 0.54[d)]	3.21 ± 0.41[e)]

1) Values are means ± SEM, $n = 8$. Data were analyzed by one-way ANOVA followed by the Least Significant Difference test. For each dietary treatment, means in a column with different superscripts differ (a – d), $P < 0.05$
2) Standards were fed a non-atherogenic diet for 12 wk

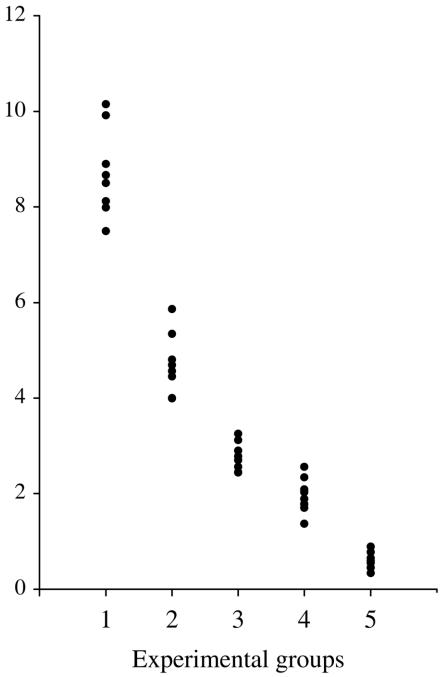

Figure 2. AFSA in hamsters fed an atherogenic diet for 12 wk and receiving daily water (control, 1) or apple (2), apple juice (3), grape (4), or grape juice (5) showing individual data points.

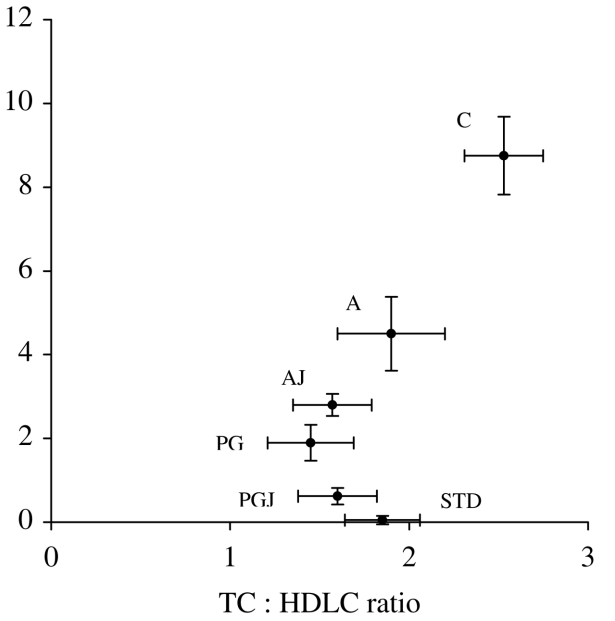

Figure 3. Extent of atherosclerosis plotted against TC/HDL-C ratio in hamsters fed a non-atherogenic diet (STD), or an atherogenic diet for 12 wk and receiving daily water (control, C) or A, AJ, PG, or PGJ.

Table 2 lists the plasma lipid profile of the hamsters. TC levels were significantly lowered in groups fed A (11%), AJ (24%), PG (30%), and PGJ (34%) compared to controls; this decrease was attributed almost solely to markedly reduced levels of non-HDL-C (30%, 55%, 64%, and 59%, respectively). HDL-C was not modified by the fruits and their juices. Consequently, the TC/HDL-C ratio was lowered in hamsters receiving A (25%), AJ (38%), PG (43%), or PGJ (37%). PAC values are also displayed in Table 2; A, AJ, PG, and PGJ significantly prevented the weak PAC induced by the atherogenic diet (13%, 27%, 41%, and 61%, respectively). For comparison, values from a standard group, fed a non-atherogenic diet, are given in Tables 1 and 2. Photomicrographs of representative atherosclerotic lesions of the hamsters' aortic arch after a 12-wk experimental period are shown in Fig. 1, and individual data points for AFSA, measured as the percentage of Oil Red O staining relative to the total area surveyed, are shown in Fig. 2. AFSA was significantly decreased in hamsters receiving A (48%), AJ (60%), PG (78%), and PGJ (93%) as compared to controls, and the fruits were less efficient than their respective juices. Antioxidant enzyme activities of liver tissue are shown in Table 3. SOD activity was significantly reduced after feeding hamsters A (29%), AJ (25%), PG (55%), and PGJ (75%); GSHPx activity significantly decreased when hamsters received A (71%), AJ (85%), PG (93%), and PGJ (87%). The hepatic TBARS concentration

is also shown in Table 3; it was markedly lowered by the consumption of A (47%), AJ (52%), PG (78%), and PGJ (83%) according to the same pattern observed for AFSA. Figure 3 shows the extent of atherosclerosis plotted against the total cholesterol/HDL-cholesterol ratio in hamsters fed a non-atherogenic diet (standard group) or an atherogenic diet for 12 wk and receiving daily water or A, AJ, PG, or PGJ. Because the TC/HDL-C ratio did not differ between the experimental groups (see Table 2), Fig. 3 suggests that the variations observed for AFSA can be mainly attributed to modifications in the antioxidant status.

4 Discussion

This study demonstrates that processing A and PG into juice modifies the protective effect of their phenolics against diet-induced oxidative stress and early atherosclerosis in hypercholesterolemic hamsters. A strong correlation between the concentration of fruit phenolics and the total antioxidant capacity has been reported in several studies [22 – 24], while others found no such relationship [25]. Our findings suggest a relationship between *in vivo* antioxidant activity and total phenolic contents. For example, A had a lower total phenolic content than AJ, and its antioxidant activity (PAC improvement, SOD and GSHPx sparing) was slightly lower than AJ that had a higher total phenolic content. PGJ had a much higher total phenolic content than PG, and its antioxidant activity was higher than PG. Also, there is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidants [26], and antioxidant activity depends mainly on the number and position of hydroxyl groups within their structure [27]. Flavonoids, especially anthocyanins and catechins in PG and PGJ, generally have more hydroxyl groups than phenolic acids found in A and AJ. This could explain why PGJ and PG displayed a better efficacy than A and AJ against early atherosclerosis. Nevertheless, these beneficial effects cannot only be attributed to their phenolic contents, but to the result of the action of different antioxidant compounds present in the fruits (vitamin C, carotenoids, polyphenols) and to possible synergistic and antagonist effects still unknown.

We have shown that A and PG and their juices induced a hypolipemic activity, decreasing TC and non-HDL-C concentrations. Polyphenols are known for their beneficial effects on atherosclerosis, altering the levels of plasma lipids. Indeed, it has been shown that grape juice decreases both TC and LDL-C in hamsters [28]. In addition, it is an established fact that phenolic compounds possess antioxidant properties and they prevent oxidation of LDL [29 – 31] believed to be mainly responsible for the development of atherosclerosis in humans [32]. Nevertheless, it should be kept in mind that the efficiency of phenolics depends on their bioavailability. High-molecular-weight polyphenolics, such as procyanidins, may be effective biological antioxidants, but it seems unlikely they could be absorbed to a large extent. However, low-molecular-weight compounds (which are effectively absorbed), such as catechin, epicatechin, and anthocyanins, are liable to exert antioxidant effects in blood plasma [33, 34]. Recently, it has been shown that daily consumption of grape juice at 70 mL/kg/day (*vs*. 7 mL/kg/day in the present study), rich in phenolics, attenuates TC by 42% and the development of atheroma in rabbits [35]. A recent human study by Castilla *et al*. [36] showed an improvement in the lipoprotein profile and a reduction in the plasma concentrations of inflammatory biomarkers and oxidized LDL with red grape juice, suggesting a reduction in cardiovascular risk. Recent studies have indeed shown that grape juice has the highest antioxidant capacity out of a group of commercial fruit juices including grapefruit, orange, tomato, and apple [37]. The hierarchy of efficacy PGJ > PG > AJ > A emphasized by liver antioxidant enzyme activity and TBARS level, PAC, and AFSA corroborated this finding, although TBARS are not the best biomarkers for oxidative stress.

The antioxidant activity of fruits and fruit juices is known to reduce diet-induced oxidative stress. Here, we showed that apple and grape and their juices prevent early atherosclerosis (< 10% foam cell coverage of aorta) in hypercholesterolemic hamsters, with AFSA development being prevented throughout an improvement of the plasma lipid profile and PAC, sparing of SOD and GSHPx activities, and a decrease in the liver TBARS level. A decreased activity of antioxidant enzymes may be a consequence of the sparing effect of dietary antioxidants, reducing the requirement for enzymatic antioxidant function when elevated concentrations of exogenous antioxidants are present in the circulatory system [38]. Elsewhere, spontaneous hepatic lipid peroxidation has been shown to decrease with an increasing level of dietary antioxidants [39], and supplementation with apples effectively decreases oxidative stress by decreasing malondialdehyde formation in the body [40]. In this study, hepatic TBARS concentration decreased about twofold in groups receiving A and AJ, whereas PG and PGJ were even more efficient. This improvement in the antioxidant status indicates that even a low amount of absorbed phenolics from fruits and juices might decrease lipid oxidation. Thus, our findings suggest that both A and PG, as well as their juices acted by various mechanisms including not only a hypolipemic effect but also, and especially, an antioxidant effect.

Overall, the present results clearly show for the first time that apple and purple grape prevent diet-induced atherosclerosis in hamsters, and that the fruit processing can have a major impact on the potential health benefits of fruit in pathological conditions. These findings, therefore, provide encouragement that fruit and fruit juices may have a significant clinical and public health relevance.

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The authors have declared no conflict of interest statement.

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