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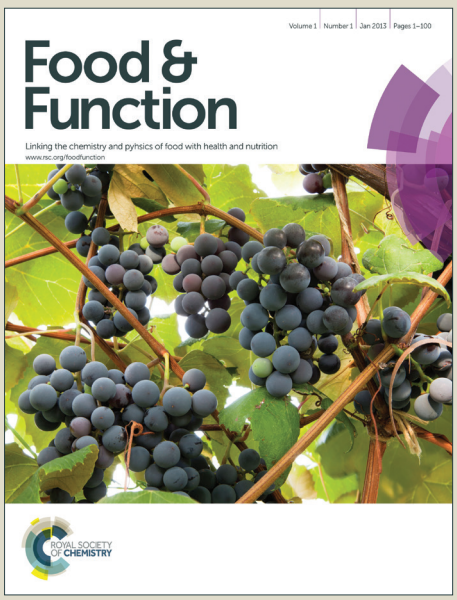

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FULL PAPER

Intake of heat-expanded amaranth grain reverses endothelial dysfunction in hypercholesterolemic rabbits

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This study reports the new functional property of amaranth grain against diet-induced endothelial dysfunction in rabbits. Twenty-eight New Zealand rabbits were fed either a standard diet (SD/G1) or a hypercholesterolemic diet (Hichol) for 28 days. On day 29, the Hichol group was subdivided into four groups and begun receiving the following diets for 21 days: G2: SD + amaranth, G3: Hichol + amaranth, G4: SD alone, and G5: Hichol alone, while G1 continued to receive SD for 21 days. Amaranth intake restored endothelial function (G2, G3) to nearly normal during the 21-day recovery besides substantially lowering total and LDL blood cholesterol levels. This effect was not seen by simply correcting the diet (G4). Upon continuance of Hichol, however, amaranth supplementation did show some contribution to the cholesterol-lowering effect (G4 *vs*. G3). On day 49, feeding Hichol without the help of amaranth, harm was further magnified by lowering HDL-cholesterol (G5). Fecal cholesterol was found increased in groups that ingested amaranth (G2, G3), but no significant impact from either supplementation or diet reversal was found in fecal bile acids or C-reactive protein. Amaranth supplementation granted some protection against tissue cholesterol (G5) and tissue peroxidation (G3). It is concluded that even in concurrence with a hypercholesterolemic diet, intake of heat-expanded amaranth can revert an associated endothelial dysfunction besides incrementing fecal cholesterol excretion and lowering blood and tissue cholesterol oxidation in dyslipidemic rabbits. These results supported the notion of a lipid peroxidation process occurring with high cholesterol intakes.

1. Introduction

Dyslipidemias are disorders of lipid transport and distribution resulting from metabolic abnormalities in the synthesis or degradation of plasma lipoproteins, altering the plasma concentrations of different components.[1]

Amaranth grain has an unusual nutrient composition sharing some characteristics with cereals and legumes thus offering a higher nutritive value as a single food than either cereals or legumes. It is increasingly being used as an alternative food and is particularly known for its cholesterol-lowering property. The expanded grain is one of the preferred forms of consumption because of its appearance, texture and flavor. The effect of amaranth on lipoprotein metabolism, total cholesterol, and triacylglycerols has been assessed in various animal models, including rats, hamsters, rabbits, birds, and humans[2]. Several hypotheses have been formulated to explain the cholesterol-lowering effect of amaranth, including its amino acid profile, the amount of total and soluble fibers, the unsaturated fatty acid content, and the presence of such phytochemicals as tocopherols, tocotrienols, phytosterols, and squalene.[2]

Diet induced dyslipidemias are commonly characterized by abnormally high blood levels of triacylglycerols and (or) cholesterol and may be aggravated by genetic predisposition. The condition is known to be a risk factor for cardiovascular diseases Since the hypercholesterolemic state reduces endothelial function and rabbits[3] rapidly develop atherosclerosis and atherosclerotic lesions[4] we chose to assess if the benefit of consuming heat-expanded amaranth grain were extensible to the aorta endothelial function and lipid peroxidation using the hypercholesterolemic rabbit model.

2. Materials and methods

2.1. *Amaranthus caudatus*

The heat-expanded amaranth grain used (*A. caudatus*) was purchased from Bolivia. A daily dose of 5 g (0.66 g protein) of amaranth, based on acceptable human intakeswhere the grain is traditionally consumed, was well tolerated by the rabbits, causing no adverse reactions. Once a day, the expanded amaranth was finely ground, every dose suspended in 30 mL of water and gavaged to groups G2 and G3 after hypercholesterolemia was induced.

2.2. Rabbits and treatments

Twenty-seven16-week old, male New Zealand rabbits (initial weights between 2.1 and 2.5 kg) were randomly assigned to individual cages (20 - 22 ºC; normal light 12-hr cycle). Food and water were always available and weights were recorded weekly to note body weight changes. Two rabbits in G4 were lost during the assay.

For 28 days, the standard diet was fed only to the normocholesterolemic animals (G1; Figure 1), while the other groups (G2-G5) received a hypercholesterolemic diet made by supplementing the standard chow (Nutricoelho, Purina, Paulínia, Brazil) with 0.5% cholesterol (code 967, from Vetec Química Fina Ltda, Rio de Janeiro, Brazil) and 10% coconut oil. The supplemented diet provided 70% saturated, 17% monounsaturated and 13% polyunsaturated fatty acids. Supplementation was done by dissolving 5 g of

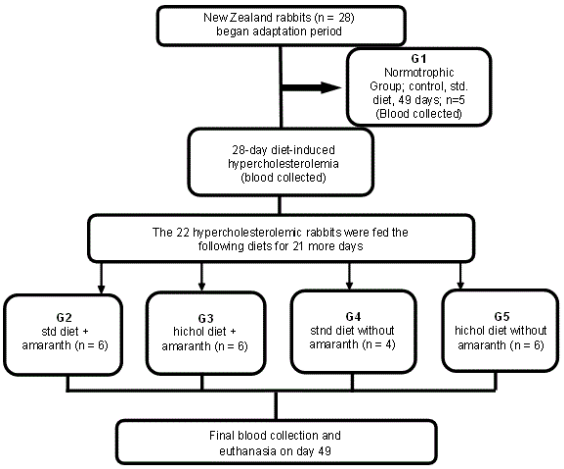

Figure 1.Feeding scheme and definition of experimental groups.

cholesterol in 100 g of heated coconut oil and blending this mixture with 1 kg of standard chow until the pellets completely absorbed the lipids. The diet was prepared weekly.

After 28 days, when the diet-induced hypercholesterolemia was installed, the rabbits were divided into 4 groups (G2-G5), and started receiving either the standard diet supplemented with popped amaranth (G2) or the standard alone (G3), or continued to receive the hypercholesterolemic diet plus the amaranth supplement (G4) or the hypercholesterolemic alone (G5) for 21 more days, as shown in Figure 1. Prior to defining the groups, blood cholesterol was determined and the animals distributed according the proximity of their cholesterol levels. Five and six rabbits per group have been considered acceptable for endothelial studies.[5] The experimental protocol was approved by the Ethics Committee for Animal Experimentation, of the Institute of Biology at the University of Campinas, Brazil.

2.3. Blood collection and euthanasia

Blood sample collection and animal euthanasia were performed after a fasting time of 16 hours. Blood was collected at the start of the experiment from the marginal ear vein to determine the initial level of total cholesterol. The animals were euthanized at the end of the experiment by excessive anesthesia with a solution of 20 mg sodium thiopental (Aspen Pharma®) per kg weight, 25 mg ketamine (Ketalar, Pfizer®) per kg weight, combined with 5 mg xylazine hydrochloride (Rompun, Bayer®) per kg weight, in 1 mg/kg and 10 mg/kg weight ratios, respectively. Blood was collected by cardiac puncture and stored in tubes containing EDTA-disodium to prevent coagulation and oxidation. Within 1 hour from collection, the blood samples were centrifuged for 15 minutes at 1,500xg at room temperature, and the plasma was separated for subsequent analyses. All analyses were performed in duplicate, and the values were averaged.

2.4. Plasma lipid analysis

At the end of the hypercholesterolemia induction period and after euthanasia, total cholesterol and its fractions (HDL-c, LDL-c, VLDL-c) and triacylglycerols were determined in the plasma of the five groups by using enzymatic kits (Laborlab, Brazil).

2.5. Quantification of excreted cholesterol and bile acids

Fecal cholesterol and bile acid excretion were also assessed. Fecal cholesterol was determined by an HPLC method[6] (Shimadzu Corporation chromatograph, Prominence model) with diode array detector SPD-M20A, at 210 nm, using an RP-C18 column (12.5 x 4.6 mm). Bile acids were extracted by a classical procedure.[7] The desiccated feces were extracted with 50% *tert*-butanol for 15 min at 37ºC and centrifuged at 10,000 x g for 2 min (3-18-K- Sigma Laborzentrun, Germany). Quantification was performed using a DZ042A enzymatic colorimetric kit (Dyazime, San Diego, USA).

2.6. Assessment of tissue cholesterol

Cholesterol in aortic tissue segments were homogenized in Tris buffer pH 7.8 and it was added Folch reactive to separate the left aortic cholesterol for 24 h. On the next day, the supernatant was dried under a stream of nitrogen gas, resuspended in isopropyl alcohol and the cholesterol determined with enzymatic kits (Laborlab, Brazil). The reading was done in a spectrophotometer (Genesys 10 UV, wavelength set at 500 nm and the results expressed in mg/g tissue.[8]

2.7. Assessment of lipid peroxidation

The occurrence of tissue lipid peroxidation was assessed in segments of the aortic arch by determining the amount of malondialdehyde (MDA).[9] Samples of tissue (1 g) were homogenized in trichloroacetic acid (10 vol. of 20% TCA) to deproteinize. After removal of the protein, a mixture of 25% TCA with 75% thiobarbituric acid was added, and the tube heated at 100 ºC for 20 min. The concentration of MDA was measured in a spectrophotometer at 532 nm. For calculating the concentration, the molar extinction coefficient of $1.49\times10^{-5.0}$ was used and the results expressed in nmol/mg tissue X $10^{-7.0}$ M.

2.8. Assessing the endothelial function

A well preserved segment of the proximal region of thoracic aorta was cleared from conjunctive tissue and used for evaluating the endothelial function according to a standardized technique.[10] The segment was suspended in a 10 mL bath of Krebs-Henseleit solution, pH 7.4, with the following composition (mmol/L): NaCl, 113; $CaCl_2$, 21.9; $NaHCO_3$, 25; $MgSO_4$, 0.44; KH_2PO_4, 1.18; EDTA, 0.03; glucose, 11. All aortic rings underwent normal contraction with 10^{-7} M noradrenalin (Sigma, USA), equivalent to sub-maximal dose and stabilized after contraction. Relaxation was accomplished by cumulative addition of acetylcholine (10^{-8} to $10^{-5.5}$ M) to produce the concentration effect. After completing the concentration-response, the perfusion fluid was replaced every 15 min and the voltage returned to baseline. After 30 min, the rings were contracted with noradrenaline and another concentration effect curve was obtained with sodium nitroprusside. The experiment utilized the physiograph (Narcotrace 40, USA).[10,11]

2.9. Statistical analysis

Significant differences were assessed after repeated measures and ANOVA with Tukey's *post-hoc* test and the contrast profile test, adopting $p<0.05$ as the threshold for statistical significance. Comparative analysis of the data from the 5 groups of rabbits at a single time point (endothelial function) was performed using a one-way ANOVA with Tukey's *post-hoc* test for multiple comparisons. The SAS for Windows, version 8.02 (Cary, NC, USA) was used to perform the statistical analyses.[12]

3. Results

The rabbits in all groups exhibited similar weight increases thus suggesting that responses to both the hypercholesterolemic diet and to the force-feeding routine were normal. The variation of all parameters studied can be found and compared in Table 1 and Figure 2.

3.1. Plasma lipids

As expected, treatment with the hypercholesterolemic diets resulted in considerable increases of total, fecal and tissue cholesterol levels in groups G2 through G5 (Figure 2). The increases were generally explained by the elevation of LDL-c. Although the levels of total cholesterol remained high during the 21 days of the second phase of the experiment, significant differences were seen between the LDL-c in animals fed amaranth, regardless of whether they ingested the diet with or without excess cholesterol (Figure 2, G2 through G4), and those that continued to ingest the harmful diet, but without the protection of amaranth (G5). The triacylglycerols (Figure 2) did not significantly vary among the groups.

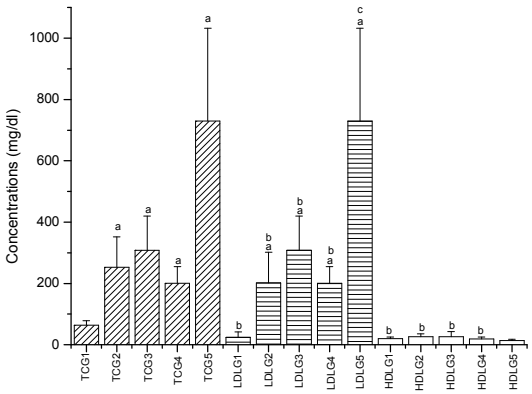

Figure 2. Total, LDL and HDL-cholesterol for each group G1 through G5.

3.2. Fecal cholesterol

In agreement with the low results for plasma cholesterol, fecal cholesterol levels were found increased in the groups (G2 and G3) that consumed amaranth in phase 2 of the experiment (Table 1).

3.3. Tissue Cholesterol and tissue peroxidation

The data in Table 1 also show increases in tissue cholesterol due to the high cholesterol intake during phase 1 for groups G2 through G5, although G5 stood out as the group that accumulated the most. After feeding either the normal diet or the hypercholesterolemic diet supplemented with amaranth for three weeks, the extents of tissue peroxidation became indistinguishable from that of G1. In G5, however, peroxidation continued to mount throughout the three weeks of phase 2.

3.4. Endothelial function

The results of maximum endothelial relaxation detected significant decreases in the capacity of only the rabbits that did not ingest the amaranth (groups G4 and G5).

4. Discussion

Studies have shown that diets enriched with cholesterol at concentrations higher than 0.15% for various periods of time can induce endothelial dysfunction and increase plasma lipids and cholesterol in the aorta of rabbits.[13-15] In the present study, supplementation with 0.5% cholesterol and 10% coconut oil induced hypercholesterolemia in 4 weeks.

Although experimental conditions vary and conclusions on its efficacy may differ among reports, the volume of evidence indicates that amaranth grain carries components capable of lowering blood cholesterol to variable extents in experimental animals and often in humans. The type of processing, for instance, seems to influence the health functional properties of the grain. In a study with hamsters consuming a diet containing 20% *A. cruentus* grains, a reduction was observed of 15% (in total-) and 22% (in non-HDL) cholesterol, coupled with a concomitant increase in HDL-c.[16] However, when flakes of amaranth were fed, the effect was found to be not as potent.[17]

Other works with focus on different fractions of the grain have given diverging results obviously explicable by the different nature and components of each fraction, as has been reported when feeding rabbits a diet of extruded amaranth and amaranth oil.[18] Furthermore, other authors[19,20] have reported a decrease in the level of total cholesterol in rats when they were fed either a diet with amaranth flour or isolated squalene. Still another study using amaranth protein isolates also resulted in a significant decrease of total cholesterol in rats.[21]

Our results showed that supplementing a hypercholesterolemic diet with amaranth grain resulted in significant mitigation by reducing the levels of plasma total- and LDL-cholesterol. This could be gathered from comparing groups G2 through G4 with the high levels of G5; the latter expressing the effect of continued exposure to the harmful diet for the entire 49 days without the protection from amaranth. It should be pointed out, however, that after reaching the hypercholesterolemic state at the end of phase 1, the effect of supplementation was no different from simply going back to the standard diet for three weeks; an observation that has not been made in previous studies. It was also noted that the large individual variations of serum LDL-cholesterol do occur in response to the supplement, thereby determining the variation size in total cholesterol (Figure 2).

With regard to the lipoprotein cholesterol fractions and triacylglycerols, significant increases in LDL-c, triacylglycerols, and VLDL-c, resulted from the harmful diet (G2-G4) compared to group G1. The higher values of group G5 were explained by the continued exposure to the hypercholesterolemic diet without the protection of amaranth until the end of the experiment, while no significant variations of HDL-c relative to group G1 were detected.

The response of HDL-cholesterol has its own pattern. Rabbits fed extruded amaranth showed a decrease in serum LDL-c, but not in HDL-c levels.[18] Since in rabbits, only 10-15% of the cholesterol is found as HDL-c,[22] it is understandable that large reductions in total cholesterol are accompanied by imperceptible changes in HDL-c, as has been observed with amaranth.[23,24]

An increase in fecal cholesterol excretion was found in groups G2 (hypercholesterolemic diet, followed by the standard diet + amaranth) and G3 (hypercholesterolemic diet, followed by hypercholesterolemic diet + amaranth), but only in group G2was the increase in fecal cholesterol significant. This result is consistent with the notion of a multi-factorial mechanism for the hypocholesterolemic effect of heat-expanded amaranth grain in rabbits. The decrease in plasma cholesterol caused by the action of plant proteins, such as soy protein, was reported to occur in rats and rabbits because of the greater excretion of fecal bile acid and cholesterol.[25,26]

An increase in fecal cholesterol was initially thought to be a compensating response to rising plasma lipids in amaranth-fed animals, but it was later suggested to result from the antioxidant activity of amaranth, the nature of its fibers or its proteins.[18,27] Conversely, in the absence of factors favoring the excretion of bile acids, the intestine reabsorbs most of them thus leading to low fecal bile acid excretion.

In the present study, high levels of fecal deoxycholic acid excretion were detected in groups G2 and G3 (although without significant differences), which received the heat-expanded amaranth. This finding suggests that digestion residues of heat-expanded amaranth can bind to bile acids thus promoting their excretion. In that respect, researchers have also shown that amaranth products have the ability to bind bile acids *in vitro*, including the toxic secondary deoxycholic acid.[28] Amaranth has also been found to increase cholesterol excretion and decrease fecal bile acid excretion, without finding any hypocholesterolemic effect, but emphasizing the diminishing levels of fecal bile acid excretion.[29] Another study with hamsters reported a decrease in fecal bile acid excretion, a decrease in plasma cholesterol, and an increase in fecal cholesterol excretion in a group fed isolated amaranth protein relative to the control group.[21]

Partial recovery of the animals from the hypercholesterolemic state occurred indistinctly whether by adding amaranth to the harmful hypercholesterolemic diet (groups G2 and G3) or by simply switching back to the standard diet without amaranth (G4; Figure 2). This is to say that discontinuation of the hypercholesterolemic diet was sufficient to decrease the plasma total- and LDL-cholesterol levels, and increase fecal cholesterol (G3); which made the therapeutic addition of amaranth equivalent to correcting the diet (G2).

The increases in tissue cholesterol caused by the hypercholesterolemic diet were not eliminated by either suspension of the harmful diet or the amaranth intervention. Tissue metabolism is known not to spontaneously change following plasma lipid changes that occur upon discontinuation of the causative diet.[15,30]

Lipid peroxidation in group G3 was lower compared to the other groups, yet not significantly different from control G1. This result may indicate that there was no correlation between tissue lipid peroxidation and tissue cholesterol levels. Nonetheless, it has been reported that increases in lipid peroxidation and tissue cholesterol can be related and responsible for the endothelial dysfunction observed.[15]

However, other mechanisms may be involved and explain the results of the present study. It would be noticed that the rabbits consuming amaranth (G2 and G3) reversed endothelial dysfunction to nearly G1 levels, while the others exhibited substantially diminished endothelial function. These findings are consistent with earlier literature reports suggesting that hypercholesterolemia enhances lipid peroxidation and impairs endothelial function.[14,31,32]

Moreover, in a study conducted with rabbits fed a standard diet supplemented with 1% cholesterol for 45 days and subsequently treated for 30 days with 150 mg amaranth extract, the data showed significant decreases in total cholesterol, LDL-cholesterol, and lipid peroxidation. Those authors concluded that their results were due to an improvement of the oxidation state.[33]

5. Conclusion

The present study shows that the pre-historical South American amaranth grain, consumed in the heat-popped and most palatable form, besides partly mitigating diet-derived hypercholesterolemia, can significantly benefit the vascular system of the rabbit. Such protection was evident independent of whether intake of the hypercholesterolemic diet was discontinued or not. Therefore, heat-expanded amaranth grain could be considered as a food with a new prophylactic property against the unintentional disadvantages of Western diets.

The remarkable reversal of the endothelial dysfunction caused by a diet-induced dyslipidemia may have a different origin from that of the cholesterol-lowering effect. In contrast with the cholesterol-lowering effect, endothelial dysfunction reversal could be related to aortic oxidation, not accomplished by just correcting the diet and less subject to individual variation. Moreover, accumulated aortic tissue cholesterol showed to be non-responsive to the intake of expanded amaranth.

Competing interest

No competing financial interests to be declared.

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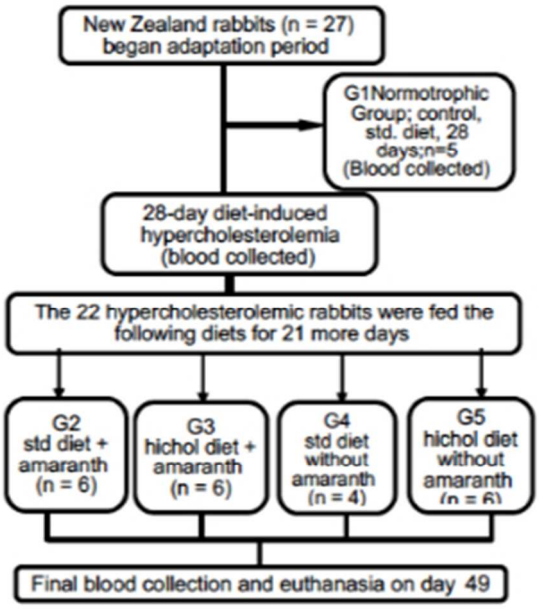
Table 1: Descriptive and comparative analyses of continuous variables for the five groups

Variable	G1		G2		G3		G4		G5	
	Means	± SEM	Means	± SEM	Means	± SEM	Means	± SEM	Means	± SEM
Weight, initial (g)	2270.00	171.76	2325.00	108.40	2292.90	97.59	2362.50	179.70	2375.00	108.40
Weight, final (g) †	3070.00	216.79	3200.00	308.22	3062.90	206.46	3400.00	168.33	3343.30	112.90
TAG (mg/dL) †	92.76[b]	15.62	120.02	20.06	108.33	26.92	102.03	26.61	136.98	9.01
Deoxycholic acid (µg/mg) †	2.70	2.58	6.30[b]	4.12	4.33	3.22	1.14	0.91	0.77	0.15
Lithocholic acid (µg/mg) †	0.15	0.19	0.38	0.27	0.39	0.19	0.21	0.17	0.11	0.08
Chenodeoxycholic acid (µg g/mg) †	0.11	0.10	0.16	0.08	0.14	0.12	0.08	0.03	0.07	0.03
Fecal chol (mg/kg)	100.57	162.94	830.49[ac]	488.12	550.66[ac]	180.01	172.36	116.74	310.99	137.02
Tissue chol (mg/g) †	15.67	2.36	29.57[a]	9.46	23.17[b]	5.98	27.21[a]	5.58	38.78[a]	4.75
Tissue per (nmol/g) †	4.06[b]	0.29	4.15[b]	1.27	3.51[bc]	0.71	4.54	0.19	7.97	0.95
End func (%) †	89.82[bc]	2.50	74.63[bc]	12.89	83.13[bc]	8.86	55.50[a]	15.70	48.33[a]	6.83

† Parameters determined at euthanasia. TAG = serum triacyglycerols. End.func = endothelial function. Tissue per = tissue peroxidation.

[a] $p < 0.05$ in relation to G1; [b] $p < 0.05$ in relation to G5 and [c] $p < 0.05$ in relation to G4.



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GRAPHICAL ABSTRACT

Feeding amaranth grain to hypercholesterolemic rabbits showed the property of recovering the lost endothelial function even without removing the hypercholesterolemia-inducing diet. Results suggest an underlying protective effect.