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Modulatory effects of rutin on biochemical and hematological parameters in hypercholesterolemic Golden Syrian hamsters

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ABSTRACT

Flavonoids have been reported to exhibit several pharmacological properties, mainly in cardiovascular and inflammatory diseases. In the present study, we observed that rutin, a known glycosylated flavonoid isolated from *Dimorphandra mollis*, had a lowering effect on plasma triglyceride levels of diet-induced hypercholesterolemic Golden Syrian hamsters, but did not change total cholesterol and high-density lipoprotein cholesterol levels. Moreover, high-fat or rutin-supplemented diets showed no immunotoxic effects, since no significant changes were observed on total white blood cells, granulocytes and mononuclear cells, as well as on the neutrophil apoptosis degree, when compared to untreated animals. Therefore, rutin seems to be a selective and non-toxic modulator of hypercholesterolemia, which can be promising for the development of new drugs.

Key words: rutin, Golden Syrian hamster, hypercholesterolemia, atherosclerosis, apoptosis, *Dimorphandra mollis*.

INTRODUCTION

Nowadays, atherosclerosis is the leading cause of death in modern societies (Ross 1999). Epidemiological studies have established a straight relationship between the development of this disease and high serum cholesterol levels, which has guided the investigation of many new classes of hypolipidemic agents for the treatment of atherosclerosis-associated hyperlipidemia during the past decade (Stocker and Keaney-Junior 2004). Recently, a large number of reports have highlighted the role of inflammation and oxidative stress in atherosclerosis, since leukocyte infiltration has been found within the atheroma. Thus, therapeutic strategies with anti-inflammatory and antioxidant compounds can exert beneficial effects in the prevention of atherosclerosis progression (Parker et al. 1995, Kourounakis et al. 2002).

Dimorphandra mollis (Leguminosae - Mimosoideae) is a common tree found in the cerrado (savannah-like) ecosystem in central Brazil, where it is called "Fava d'Anta" (Bizerril et al. 2005). Biological screening studies on isolated compounds of *D. mollis* have shown antitryptic (Macedo et al. 2000) and insecticidal activities (Batista-Pereira et al. 2002). This plant is also known as a plentiful source of rutin, whose pharmacological properties as anti-inflammatory (Selloum et al. 2003), antioxidant (Rice-Evans et al. 1996) and immunomodulator (Middleton-Junior et al. 2000) have been largely explored.

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Quercetin, the main rutin metabolite in the human, has shown *in vitro* inhibitory effect on hepatic cholesterol biosynthesis and *in vivo* hypocholesterolemic effect (Glässer et al. 2002, Auger et al. 2005). However, as glycosylated flavonoids are more common in nature, evaluation of their biological activities is also relevant. Therefore, the objective of the present study was to investigate the effects of rutin, the parent glycosylated form of quercetin, isolated from *D. mollis*, on (i) biochemical parameters (serum lipid profile and total protein) and (ii) hematological parameters of diet-induced hypercholesterolemic Golden Syrian hamsters, which could contribute to hyperlipidemia and atherosclerosis modulation.

MATERIALS AND METHODS

Plant Material

Rutin (purity $\geq$ 95%, determined by HPLC analysis) extracted from *Dimorphandra mollis* was a kind gift from PVP S.A. (Produtos Vegetais do Piauí; Parnaíba, Piauí State, Brazil).

Animals and Diets

Male adult Golden Syrian hamsters, weighing 100 $\pm$ 20 g, were housed under standard conditions of temperature, relative humidity and 12h light-dark cycles, with free access to both food and water. Animals were randomly separated in four groups (n = 4/cage). Group 1 (control): animals received normal rodent diet (ND); Group 2: animals received high-fat diet (10% coconut oil and 0.2% cholesterol) (w/w) (HCD); Group 3: animals received high-fat diet supplemented with 0.8% (w/w) rutin (R-HCD); Group 4: animals received normal diet supplemented with 0.8% (w/w) rutin (R-ND). After 45 days of feeding and 12 hour of food deprivation, animals were anesthetized with xylazine (25 mg/kg) plus ketamine (50 mg/kg) and blood was collected by cardiac puncture into EDTA containers. Plasma was separated by centrifugation (1500 g for 15 min) and stored at –70°C until analysis. This project was approved by Animal Care and Use Ethics Committee from University of São Paulo, campus of Ribeirão Preto (protocol number 05.1.1049.53.0).

Plasma Analysis

Plasma total protein, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) levels were determined by colorimetric and enzymatic assays (Labtest Diagnostica, Lagoa Santa, MG, Brazil) using an ABBOTT automated analyzer (model ABBA VP) (Martinello et al. 2006).

Hematological Determinations

Total white blood cells (WBC) were counted by using a Neubauer chamber. Briefly, a blood sample (diluted 20-fold in Türck solution) was placed on the counting area of the lamina and cover slipped. The blood sample was therefore monolayered in a space of 0.1 mm height. The WBC in a 5 mm^2 area was counted and expressed as leukocyte number from 1 mm^3 (μL) of whole blood. Differential leukocyte count (granulocytes and mononuclear cells) was determined from blood smears stained with Leishman and expressed as percentage.

Assessment of Neutrophil Apoptosis

An Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (R&D Systems, MN, EUA) was used to detect late apoptotic process. This assay allows the detection of phosphatidyl serine translocation from the inner to the outer leaflet of the cytoplasmic membrane as an early event in the apoptotic pathway. This event precedes the loss of membrane integrity [propidium iodide (PI) was used as an indicator] which accompanies the final stages of cell death resulting from late apoptotic processes. Briefly, a stock solution (0.16 mol/L NH_4Cl) was diluted (l:9) in 0.17 mol/L Tris buffer to obtain the lysing solution. Then, 2.0 mL of this solution were added to 100μL of whole blood samples, gently mixed and incubated for 10 min at 37°C. The sample tubes were centrifuged at 300 g for 10 min at room temperature, and supernatants discarded. The pellet was suspended in 1.0 mL of phosphate buffered solution (PBS) and centrifuged at the same conditions. Afterwards, cells were suspended in isotonic binding buffer, had Annexin V-FITC and PI added, and incubated in the dark for 15 min. Samples were kept on ice to stop reactions until analysis, which was performed using a FACSscan (Becton & Dickinson, USA) flow cytometer. Data from 10.000

events was collected and analyzed in Cell Quest software (Becton & Dickinson, USA). The forward- and side-angle light scatter (FSC/SSC) allowed neutrophil recognition and exclusion of mononuclear cells, debris and aggregates from the analysis. Annexin V-FITC binding was determined by FITC signal detector (FL1) and PI staining by emission signal detector (FL2). The amount of late apoptotic neutrophils was expressed as percentage of the total number of gated neutrophils. All samples were assayed at optimal concentrations and according to the manufacturer's instructions.

Statistical Analysis

Data are reported as mean ± standard error of mean (S.E.M.). Significance of the differences among diet treatment groups was determined using analysis of variance (ANOVA) followed by Tukey´s *post-hoc* test. $P < 0.05$ was accepted as a statistically significant difference.

RESULTS

Biochemical Parameters

In the present study, we observed that the high-fat diet (HCD) was effective in promoting hypercholesterolemia in Golden Syrian hamsters, since plasma lipids and lipoprotein levels and final weight of hamsters fed this diet were significantly increased when compared to those fed normal diet (ND) (Table I). Rutin supplementation significantly lowered plasma triglycerides (TG) but had no effect on high-density lipoprotein cholesterol (HDL-C) and total cholesterol (TC) levels of hypercholesterolemic animals. Moreover, rutin induced a slight but not significant decrease on final weight and plasma total protein concentration of hypercholesterolemic animals (R-HCD *vs.* HCD) and had no significant effect on final weight and all tested biochemical parameters of animals treated with normal diet (R-ND *vs.* ND) (Table I).

Hematological Parameters

We also observed that the total and specific number of white blood cells (WBC) were not significantly different among normal and hypercholesterolemic diet fed animals, supplemented or not with rutin (Table II). In addition, neutrophil late apoptosis was assessed by flow cytometry in annexin V and propidium iodide (PI) double labeled cells in order to evaluate immunotoxicity of high-fat diet and rutin supplementation on hamsters (Fig. 1). There was no significant alteration on this parameter among the four tested groups (Table II).

DISCUSSION

Accumulating results from epidemiological and animal studies have identified elevated LDL cholesterol levels as risk factors for coronary artery disease (Stocker and Keaney-Junior 2004). Many types of cholesterol-lowering drugs have been developed to treat hypercholesterolemia and prevent the incidence and progression of atherosclerosis. Nowadays, many studies have demonstrated that medicinal plants have been used in folk medicine as alternative treatments for a wide range of diseases, including inflammatory processes of several origins, and have produced relief of symptoms comparable to that obtained for allopathic medicines (Verpoorte 1999, Clardy and Walsh 2004). Several plant-derived secondary metabolites, mainly flavonoids, have been described to interfere directly with molecular mechanisms involved in several chronic conditions.

The present model, diet-induced hypercholesterolemia in Golden Syrian hamsters, has been used for atherosclerosis and cholesterol metabolism studies due to its similarities to humans (Moghadasian 2002). Foam cell accumulation, fatty streaks and plaque formation in the hamster aortic arch and coronary arteries are characteristics similar to the development of atherosclerotic lesions in humans (Nistor et al. 1987). This model has been used to study drug preventing effects on atherosclerosis development using natural compounds, including polyphenols and flavonoids.

Rutin, a glycosylated flavonoid widely distributed in various plants, has been suggested to have effects on the cardiovascular system, possibly due to its antioxidant and anti-inflammatory activities (Middleton-Junior et al. 2000). Park et al (2002) demonstrated in rats that rutin promoted the excretion of fecal sterols, thereby decreasing absorption of dietary cholesterol as well as lowering plasma and hepatic cholesterol concentration. Nonetheless, in our experimental conditions, we observed that rutin had no significant effect on total cholesterol or high-density lipoprotein cholesterol levels in this hypercholesterolemia model. Many studies

TABLE I
Biochemical parameters and final body weight of Golden Syrian hamsters fed normal diet (ND), high-fat diet (HCD), and rutin-supplemented normal (R-ND) and high-fat (R-HCD) diets[a].

Parameter	Groups			
	ND	R-ND	HCD	R-HCD
TC (mg/dL)[b]	101.35 ± 1.77	106.10 ± 9.15	298.76 ± 12.56**	324.04 ± 3.97**
HDL-C (mg/dL)[b]	47.98 ± 0.60	52.22 ± 4.11	135.35 ± 7.82**	133.24 ± 4.48**
TG (mg/dL)[b]	195.89 ± 11.39	166.94 ± 7.83	290.53 ± 23.87*	221.71 ± 14.33*#
Total protein (mg/dL)	6.28 ± 0.13	6.47 ± 0.13	7.14 ± 0.13**	6.83 ± 0.20
Final weight (g)	123.88 ± 4.88	127.18 ± 4.28	145.63 ± 4.78**	132.75 ± 3.16

[a]Values are mean ± S.E.M. (n = 8). [b]Abbreviations: TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides. * $P < 0.05$, ** $P < 0.01$ *vs.* ND group. # $P < 0.05$ *vs.* HCD group.

TABLE II
Hematological parameters and neutrophil late apoptosis of Golden Syrian hamsters fed normal diet (ND), high-fat diet (HCD), and rutin-supplemented normal (R-ND) and high-fat (R-HCD) diets[a].

Parameter	Groups			
	ND	R-ND	HCD	R-HCD
WBC[b] (10^3cells/mm^3) (n= 8)	7.30 ± 1.28	8.95 ± 0.65	7.96 ± 0.56	6.90 ± 1.15
Granulocytes (%) (n= 8)	31.29 ± 4.10	29.13 ± 2.32	33.00 ± 1.21	30.57 ± 1.84
Mononuclear cells (%) (n= 8)	69.43 ± 4.22	71.13 ± 2.30	67.13 ± 1.17	68.29 ± 2.26
Neutrophil apoptosis (%) (n= 4)	7.23 ± 0.55	6.64 ± 0.73	7.27 ± 1.28	5.01 ± 0.77

[a]Values are mean ± S.E.M. [b]WBC: white blood cells.

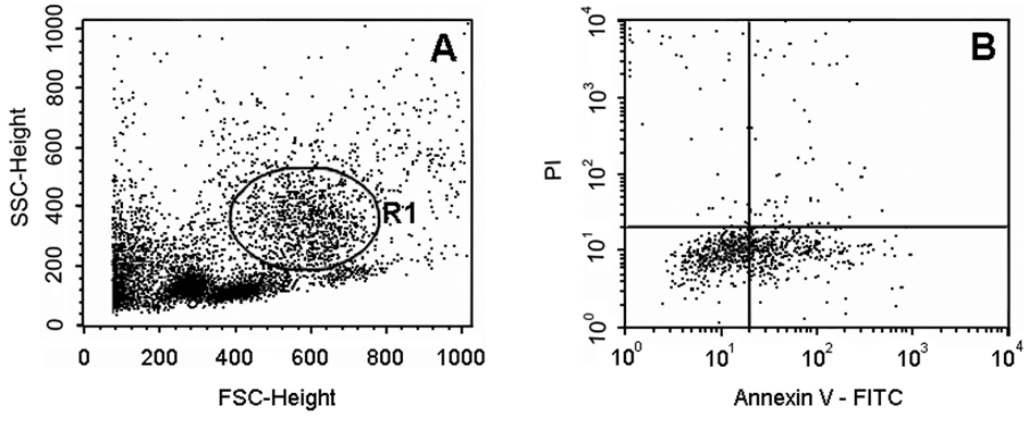

Fig. 1 – Flow cytometry analysis. (a) Typical side- (SSC) and forward- angle light scatter (FSC) cytogram of a lysed whole blood sample showing the R1 gate that corresponds to neutrophils. The ordinate and the abscissa represent cell size and granularity, respectively. (b) Representative analysis of neutrophil double staining with annexin V-FITC and propidium iodide (PI). Late apoptotic cells are shown in upper right quadrant.

have demonstrated that rutin need to be hydrolyzed and converted to quercetin by the cecal microflora and enterocytes before intestinal absorption in humans and rats (Walle 2004, Manach et al. 1997). Auger et al. (2005) reported that quercetin prevented atherosclerosis development in hamsters by hypolipidemic effects. On the other hand, Lauridsen and Mortensen (1999) and Enkhmaa et al. (2005) observed no hypocholesterolemic effect in quercetin-treated Watanabe heritable hyperlipidemic rabbits and low-density lipoprotein receptor-deficient mice, respectively. Together, these findings suggest that the hypolipidemic effect of rutin and derivatives may be dependent on the animal species or/and type of diet.

On the other hand, the high serum triglycerides level is also associated with abnormal lipoprotein metabolism, as well as with other Coronary Heart Disease risk factors including obesity, insulin resistance, and diabetes mellitus (Yuan et al. 2007). Interestingly, the results of the present study demonstrated a lowering effect in the plasma TG concentration in hypercholesterolemic Golden Syrian hamsters after 45 days-treatment with rutin-enriched diet (1%). Supporting our result, Santos et al. (1999) demonstrated that rutin was the most effective compound to reduce TGC levels in hyperlipidemic rats when compared with naringenin and nicotinic acid. However, in contrast with the present work, we have already observed that low doses of rutin (0.1%) did not modify plasma TG levels in the same animal model (Kanashiro et al. 2007).

Finally, no signs of cellular immunotoxicity, which was investigated by levels of late apoptotic neutrophils as well as total and differential leukocytes counts, were observed on animals fed normal or high-fat diet, supplemented or not with rutin for 45 days. In agreement with our results, Hasumura et al. (2004) reported no clinical signs of toxicity or hematological alterations in rats treated with a rutin analogue for thirteen weeks. Together, both studies suggest that rutin and its derivatives may be investigated on treatment or prevention of chronic diseases due to its non-toxic effects.

In summary, our results suggest that short-term treatment of hypercholesterolemic Golden Syrian hamsters with a relatively high concentration of rutin (1%) led to a selective negative modulation of plasma TG levels, but did not interfere in the other biochemical parameters evaluated and showed no toxic effect. Thus, together with previous studies, the present work suggests the use of rutin, which can be promising for the development of new drugs, in high plasma triglycerides level-associated chronic diseases.

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RESUMO

Os flavonóides possuem diversas propriedades farmacológicas, principalmente nas doenças cardiovasculares e inflamatórias. No presente estudo, observamos que a rutina, um conhecido flavonóide glicosilado isolado da *Dimorphandra mollis*, diminuiu o nível de triglicerídeos plasmáticos em hamsters Golden Syrian hipercolesterolêmicos sem alterar os níveis de colesterol total e colesterol HDL. Além disso, observamos que dietas hipercolesterolêmicas ou suplementadas com rutina não apresentaram efeito imunotóxico, uma vez que nenhuma alteração significativa foi observada nos leucócitos totais, granulócitos e células mononucleares, bem como no grau de neutrófilos em apoptose, quando comparado com animais não tratados. Portanto, a rutina parece ser um modulador seletivo e não tóxico da hipercolesterolemia, o que pode ser promissor para o desenvolvimento de novos fármacos.

Palavras-chave: rutina, hamster Golden Syrian, hipercolesterolemia, aterosclerose, apoptose, *Dimorphandra mollis*.

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