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Effect of rutin on polymorphonuclear leukocytes oxidative metabolism in hypercholesterolemic Golden Syrian hamsters: evaluation by chemiluminescence and flow cytometry

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Atherosclerosis has been described as an inflammatory disease in which polymorphonuclear leukocytes (PMNLs) seem to be involved. These cells may induce atherosclerotic lesions by releasing reactive oxygen species (ROS) and a sort of pro-inflammatory mediators. In this study, the PMNL oxidative metabolic status of Golden Syrian hamsters fed a normal diet (ND), or a high-fat diet (10% coconut oil plus 0.2% cholesterol) supplemented (R-HCD) or not (HCD) with 0.1% (w/w) rutin was evaluated after 120 days of treatment. PMNL oxidative metabolism was assessed by whole blood luminol-enhanced chemiluminescence and 2′,7′-dichlorofluorescein diacetate-dependent flow cytometry. The results obtained by both methods were similar and showed no significant changes in ROS generation by PMNLs in blood samples from HCD or R-HCD animals when compared to ND. Furthermore it was shown that rutin supplementation did not significantly affect plasma lipid and lipoprotein levels in the hypercholesterolemic animals characterized by significantly increased total plasma cholesterol, triglycerides and low- and high-density lipoprotein cholesterol levels. The results suggest that in this model atherosclerosis development is not related to circulating PMNL activation and rutin supplementation has no immunomodulatory or hypocholesterolemic effects.

1. Introduction

Atherosclerosis and its main complications (myocardial infarction and cerebral stroke) are leading causes of death in the USA and Europe (Libby 2002). It is a progressive disease characterized by local thickening of vessel walls caused by accumulating lipids and fibrous elements in the larger arteries. More recently, the concept of atherosclerosis as a chronic inflammatory disorder has been supported by the presence of macrophages and T lymphocytes within the atheroma. However, few studies have investigated the role of polymorphonuclear leukocytes (PMNLs), which are key components of the inflammatory response, in the development of this chronic illness.

PMNLs produce and release antimicrobial reactive oxygen species (ROS) whose excess have been associated with the pathogenesis of chronic inflammatory conditions such as autoimmune diseases, pulmonary emphysema, cancer, and also with the increased levels of oxidized low-density lipoprotein cholesterol (ox-LDL) (Babior 2000). In fact, ox-LDL has been described to increase cellular adhesion molecules expression on the arterial wall, and to act on immune cells by promoting monocytes and lymphocytes recruitment, inhibiting nitric oxide production and increasing pro-inflammatory cytokines secretion (Libby 2002; Alexaki et al. 2004).

Nowadays, the experimental model most used for atherosclerosis and cholesterol metabolism studies has been the Golden Syrian hamster because of its human similarities (Moghadasian 2002). Foam cell accumulations, fatty streaks and plaque formation in the hamster aortic arch are characteristics similar to the development of atherosclerotic lesions in humans (Nistor 1987). This model has been used to study the preventing effects of natural compounds such as flavonoids on atherosclerosis development. This class of compounds has been reported not only to prevent the atherosclerosis development in hamsters at nutritional doses (Auger et al. 2005) but also to *in vitro* inhibit LDL oxidation (de Whalley et al. 1990).

The wide variety of immunomodulatory and antioxidant effects of flavonoids on some atherosclerosis-promoting mechanisms led us to investigate the *in vivo* effect of the flavonoid rutin, widely consumed in dietary foods, on the PMNL oxidative metabolism and plasma lipid and lipoprotein profiles in Golden Syrian hamsters fed a hypercholesterolemic diet.

Table 1: Plasma lipid and lipoprotein levels in Golden Syrian hamsters fed with normal diet (ND), high-fat diet (HCD) and rutin-supplemented high-fat diet (R-HCD)

Biochemical parameters	Groups		
	ND	HCD	R-HCD
TC (mg/dL)	85.78 ± 3.04	352.46 ± 26.00*	344.89 ± 33.05*
TG (mg/dL)	153.96 ± 11.86	379.53 ± 31.93*	343.84 ± 23.03*
HDL (mg/dL)	42.44 ± 1.88	106.76 ± 7.80*	114.51 ± 8.26*
LDL (mg/dL)	42.45 ± 1.56	178.77 ± 22.97*	166.37 ± 15.56*

Values are means ± S.E.M. (n = 7 − 8). *Significantly greater than ND ($p < 0.01$)

2. Investigations and results

2.1. Plasma lipid analysis

As expected the lipid parameters, plasma total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were significantly increased in hamsters receiving high-fat diets when compared to the control group (ND) (Table 1). In addition, rutin supplementation (R-HCD) had no significant effect on the lipid profile in hypercholesterolemic animals (HCD).

2.2. PMNL oxidative burst analysis

In flow cytometric analysis, where FSC vs. SSC was recorded, a single population of granulocytes was easily distinguished from the other leukocytes by their typical location (Fig. 1). The ability of this method to identify different leukocyte populations was particularly important to the present study, since it allowed specific determination of ROS production by hamster blood PMNLs. To measure median fluorescence intensity (MFI), PMNLs were selected by gating. The FL-1 peaks indicated that blood granulocytes consisted of a single population.

The MFI peak values from ND (16.86 ± 0.50), HCD (16.65 ± 0.75) and R-HCD (16.80 ± 0.69) PMNLs were not significantly different to each other, which suggests that high-fat diet, supplemented or not with rutin, did not change the hamster PMNL activation state under the assessed conditions (Fig. 2).

In agreement with the above described results, ROS generation in whole blood samples from the ND (8.83 ± 2.56 × 10^6 cpm), HCD (7.50 ± 1.46 × 10^6 cpm) and R-HCD (7.19 ± 1.98 × 10^6 cpm) groups were not significantly different, as measured by the luminol chemiluminescence assay (Fig. 3).

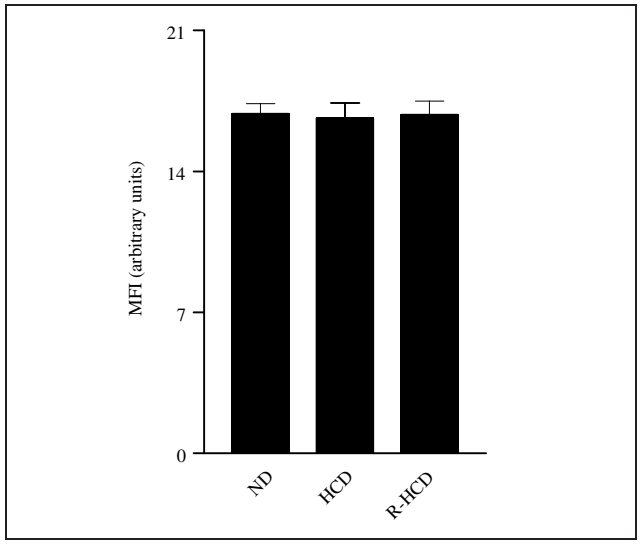

Fig. 2: Evaluation of PMNL oxidative burst in normal diet (ND), high-fat diet (HCD) and rutin-supplemented high-fat diet (R-HCD) treated hamsters by DCFH-DA-dependent flow cytometry. Values (mean ± S.E.M.; n = 7 − 8) are expressed as median fluorescence intensity (MFI) in arbitrary units

3. Discussion

Atherosclerosis has been classified as an inflammatory disorder, since a sort of immune system cells are found within the atheroma (Libby 2002). Recently, ROS overproduction has been considered as a mechanism by which phagocytes (especially macrophages) amplify oxidative processes found in the early stages of atherosclerosis by oxidizing LDL and enhancing inflammation (Steinberg and Witztum 1990). There is evidence demonstrating that ROS may act as intracellular signaling molecules to external stimuli for redox-sensitive gene expression or induction of pro-inflammatory molecules (Griendling et al. 2000).

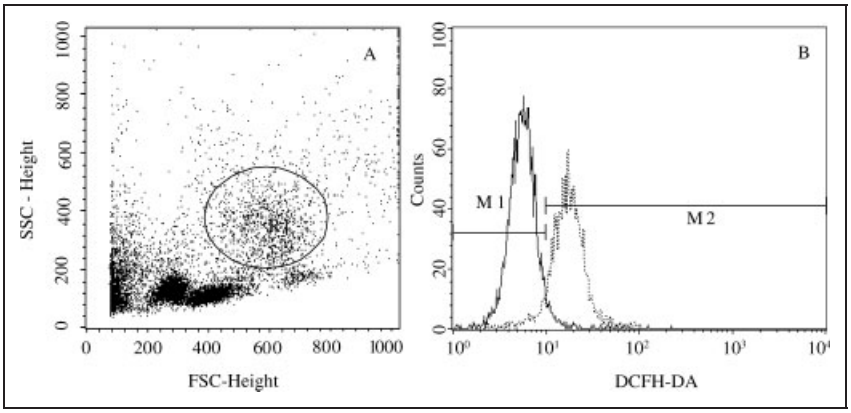

Fig. 1:
Flow cytometry analysis. (A) Representative cytogram of leukocytes from hamster lysed blood. PMNLs were easily distinguished on FSC vs. SSC dot-plot and a gate was drawn round the PMNLs. (B) Representative histogram of fluorescence signal obtained from the gated region. M1: PMNLs without DCFH-DA probe (thin lines); M2: PMNLs incubated with DCFH-DA (dotted lines)

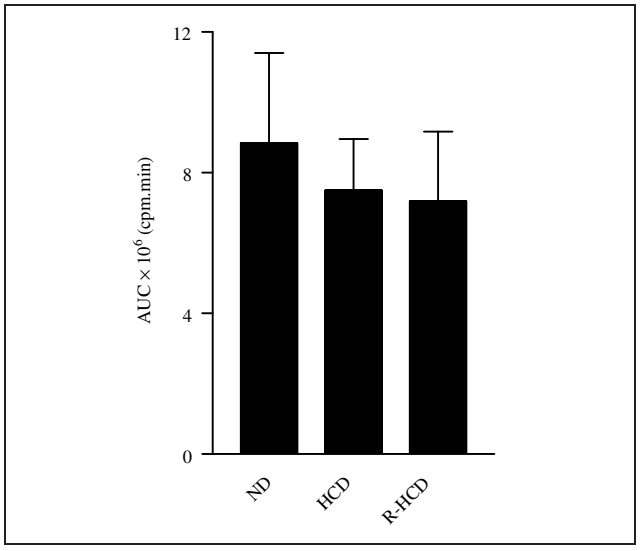

Fig. 3: Evaluation of whole blood ROS generation in normal diet (ND), high-fat diet (HCD) and rutin-supplemented high-fat diet (R-HCD) treated hamsters by luminol-dependent chemiluminescence. Values (mean ± S.E.M.; n = 7 − 8) are expressed as area under the time-course curves (AUC)

Among the wide variety of ROS sources in mammalian organisms, three enzymes have been implicated in cardiovascular diseases and LDL oxidation: xanthine oxidase, endothelial nitric oxide synthase and endothelial NADPH oxidase (Cai and Harrison 2000). However, few studies have investigated the role of PMNL-derived ROS in atherosclerosis development. Increased levels of circulating ox-LDL in atherosclerotic patients suggest activation of circulating PMNLs and a highly active oxidative metabolism (Tertov et al. 1996). In addition, Maeba et al. (1995) observed that ox-LDL, but not native LDL, enhanced ROS generation by PMNLs.

In the present study, the PMNL oxidative metabolism status in normal and hypercholesterolemic hamsters was evaluated by measuring leukocyte ROS production in whole blood samples, through luminol-enhanced CL and DCFH-DA dependent FC techniques. The use of diluted whole blood instead of isolated cells does not require cell separation (Richardson et al. 1998; Pavelkova and Kubala 2004), thus reducing sample manipulation and unspecific cell activation that could distort the final results.

Luminol-enhanced CL may reflect both extra- and intra-cellular metabolic events because luminol is a small lipophilic molecule able to penetrate the cell (Calfedie-Chezed et al. 2002). On the other hand, DCFH-DA flow cytometry measures only intracellular ROS generation. DCFH-DA, a stable non-fluorescent compound, diffuses through the cell membrane and is deacetylated by cytosolic enzymes to polar DCFH, which is trapped within the cell. The non-fluorescent DCFH is the substrate for oxidation by different generated ROS, with the resultant production of a highly fluorescent intracellular product (DCF) (van Eeden et al. 1999). Luminol-enhanced CL and FC have different technical principles, but a correlation was found between their results, as previously reported by Calfedie-Chezed et al. (2002).

In the present study, there were no significant differences between PMNL basal oxidative metabolism in hamsters fed normal or hypercholesterolemic diets, as measured by both techniques. In agreement with our results, Egger et al. (2001) reported no significant differences between PMNL ROS release from atherosclerotic patients and healthy volunteers. On the other hand, Maeda et al. (2005) observed enhanced PMNL oxidative burst in hyperlipemic guinea pigs by FC assay. These findings suggest that alteration of PMNL oxidative metabolism status induced by hypercholesterolemic diets may be dependent on the animal species and/or the source of PMNLs.

In addition, the hypolipemic and antioxidant/immunomodulatory effects of rutin on hypercholesterolemic hamsters were also investigated. Rutin, a glycosilated flavonoid widely distributed in various plants, has been suggested to have sparing effects on the cardiovascular system (Morton et al. 2000; Middleton et al. 2000). On the other hand, various studies in isolated PMNLs described rutin as strong immunomodulatory compound in several cellular systems, including in PMNL functions, such as ROS generation and lysosomal enzyme release (Limasset et al. 1993; Middleton Junior et al. 2000; Ostrakhovitch and Afanas'ev 2001). However, we found that rutin supplementation had no effect on plasma lipid and lipoprotein profiles of hypercholesterolemic animals, and did not show any inhibitory effect on PMNL oxidative metabolism.

Taking into account the *in vitro* modulatory effects of rutin on mechanisms underlying atherosclerosis development, the lack of effect on Golden Syrian hamsters suggests a low bioavailability or transformation into metabolite(s) without immunomodulatory and hypolipemic activities. These possible mechanisms will be evaluated in a further work.

In conclusion, the findings in this study suggest that although the atherogenesis process is largely dependent on cellular and humoral immune system components, the development of atherosclerosis in hypercholesterolemic Golden Syrian hamsters is independent of PMNL oxidative burst activation. Moreover, the flavonoid rutin had no effect on the plasma lipid profile and oxidative metabolism of PMNLs in hypercholesterolemic Golden Syrian hamsters, after a 120-day period of treatment.

4. Experimental

4.1. Chemicals

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DCFH-DA (2′,7′-Dichlorofluorescein diacetate) was purchased from Molecular Probes Co. (Eugene, Oregon, USA). Rutin (purity ≥ 95%, determined by HPLC analysis) was supplied by Produtos Vegetais do Piauí S.A. (Piauí State, Brazil).

4.2. Animals and diets

Twenty four male Golden Syrian hamsters (120–150 g, 8 weeks old) were divided in three groups of seven or eight animals each and three or four animals per cage with equal mean group body weights. Group ND: animals received normal rodent diet. Group HCD: animals received high-fat diet (10% coconut oil and 0.2% cholesterol) (w/w). Group R-HCD: animals received high-fat diet mixed with 0.1% (w/w) rutin. The animals were housed in environmentally controlled conditions with alternating 12 h light/12 h dark cycles with free access to both food and water.

After 120 days of feeding and 16 h of food deprivation, the animals were anesthetized with xylazine (25 mg/kg) plus ketamine (50 mg/kg) and blood collected by cardiac puncture into EDTA containers. Plasma was separated by centrifugation (1500 g for 15 min) and stored at −70 °C until analysis.

Animal Care and Use Ethics Committee from Universidade de São Paulo, campus of Ribeirão Preto, approved this project (protocol number: 05.1.1049.53.0).

4.3. Plasma lipid profile analysis

Plasma total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were assayed by colorimetric and enzymatic methods (Labtest Diagnostica, Lagoa Santa, Brazil).

4.4. Flow cytometry (FC) analysis

The PMNL oxidative burst was evaluated by FC according to Richardson et al. (1998) with some modifications. Briefly, 0.1 mL of whole blood samples from ND, HCD and R-HCD groups was mixed with 0.2 mL of DCFH-

DA (0.3 mmol/L, prepared in Hank's Balanced Salt Solution (HBSS)) in polypropylene tubes. After 20 min incubation at 37 °C, 2.0 mL of lysis solution (NH_4Cl 0.16% w/v) were added to all tubes. Samples were left at room temperature in the dark for 10 min and then centrifuged at 1000 g for 10 min. Supernatants were discarded and cells were suspended in 0.5 mL of HBSS. Samples were kept on ice to stop reactions until FC analysis, which was performed using a FACSscan (Becton & Dickinson, USA) flow cytometer. DCFH-DA green signal fluorescence was measured at 530 nm (FL1 detector). Data from 50.000 events was collected and analyzed by Cell Quest software (Becton & Dickinson, USA). PMNLs were recognized on the basis of forward-angle (characteristic size) and side-angle (characteristic granularity) light scattering (FSC/SSC), which also allowed exclusion of other cell types (lymphocytes and erythrocytes), debris and aggregates.

4.5. Chemiluminescence analysis

The PMNL oxidative burst was evaluated by chemiluminescence (CL) assays as described previously with some adaptations (Pavelkova and Kubala 2004). Briefly, luminol (5 mg/mL, prepared in DMSO) was added to 0.5 mL of 1 : 10 diluted whole blood (in HBSS) samples from ND, HCD and R-HCD groups. After 1 min incubation at room temperature, 0.5 mL of HBSS were added to all sample tubes.
Light emission or chemiluminescence (CL) was measured in a luminometer (Auto Lumat LB953, EG&G Berthold, Germany) and recorded in photon counts per minute (cpm) for 60 min at 37 °C. The area under the time-course curves (AUC) was calculated.

4.6. Statistical analysis

Data are reported as mean ± standard error of the mean (S.E.M.) of 7–8 measurements per group. Significance of differences between the diet treatment groups was determined using Student's t-test. $p < 0.05$ was accepted as a significant difference.

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