Resveratrol Causes Antiatherogenic Effects in an Animal Model of Atherosclerosis

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Abstract

Background: Resveratrol protects the cardiovascular system by a number of mechanisms, including antioxidant and anti-platelet activities.

Objective: To assess the potential anti-inflammatory and antiatherogenic effects of resveratrol using rabbits fed a hypercholesterolemic diet (1% cholesterol).

Methods: Twenty white male rabbits were selected and divided into two groups: control group (CG), 10 rabbits; and resveratrol group (RG), 10 rabbits. The animals were fed a hypercholesterolemic diet for 56 days. For the RG diet, resveratrol (2mg/kg weight/day) was added from days 33 - 56.

Results: There was no significant difference in the total serum cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides between the groups. Of the CG, 70% had advanced aortic atherosclerotic lesions (types III, IV, V, or VI). All animals from the RG had mild aortic atherosclerotic lesions (types I or II, or no lesions). The intima area and the intima/media layer area ratio was significantly lower in the RG as compared to the CG ($p<0.001$). Positive areas for VCAM-1 molecules were lower in the RG ($p=0.007$). The MCP-1 and IL-6 concentrations were lower in the RG than the CG ($p=0.039$ and $p=0.015$, respectively).

Conclusion: Resveratrol had significant anti-atherogenic and anti-inflammatory effects in an animal model with rabbits fed a hypercholesterolemic diet (1% cholesterol). (Arq Bras Cardiol 2012;98(2):136-142)

Keywords: Revesratrol; antioxidants; atherosclerosis; dyslipidemias.

Introduction

Trans-resveratrol (resveratrol) is a polyphenolic compound found in fresh grapes, grape juice, and wine. Resveratrol is produced by the skin of grapes in response to fungal exposure. Resveratrol has been implicated in many, but not all, studies as a mediator of alcohol-independent cardiovascular protection that is allegedly conferred by drinking red wine[1]. Resveratrol protects the cardiovascular system by a number of mechanisms, including resveratrol-mediated inhibition of low-density lipoprotein oxidation, inhibition of platelet aggregation, synthesis of pro-atherogenic eicosanoids, inhibition of cell proliferation, and increased vasorelaxation. Recent studies have also shown that resveratrol suppresses the induction of procoagulant tissue factor, one of the key components thought to be responsible for high mortality from cardiovascular disease[2-5]. The objective of this study was to assess the potential anti-inflammatory and anti-atherogenic effects of resveratrol using an experimental animal model with rabbits fed a hypercholesterolemic diet (1% cholesterol).

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Methods

Animals

Twenty white adult New Zealand male rabbits at a mean age of 30 days were selected. Animals were handled in compliance with the Guiding Principles in the Care and Use of Animals. Protocol approval was obtained from the Animal Research Committee of the Pontifícia Universidade Católica. The animals were divided into 2 groups, as follows: control group (CG), 10 rabbits; and resveratrol group (RG), 10 rabbits. During the 56 days of the study, the animals were fed a specific diet for the species (Nuvilab®) plus 1% cholesterol from lyophilized eggs. From days 29 – 56, resveratol (Resveratrol®; extracted from the Galena® Laboratory) was added to the RG diet (2mg/kg/day) and administered by oral gavage. On day 56, the animals underwent dissection of the aortic arch and descending aorta. Anesthesia was induced with ketamine (Vetanarcol®, 3.5 mg/kg; König) and intramuscular xylazine (Coopazine®, 5 mg/kg; Coopers). After the procedure, the rabbits were sacrificed by a lethal dose of barbiturate. The sample size was calculated based on the study by Zou et al[6].

Blood chemistry

Blood samples were obtained on the first day of the experiment and immediately before sacrifice by cardiac puncture. Clinical

laboratory assessment included total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TGC). Measurements were taken using an automated system (Abbott Architect ci8200; Abbott Laboratories, Abbott Park, IL, USA).

Histologic analysis

The arteries were removed and washed with 10% formaldehyde buffered with phosphate (ph=7.6) and waxed. For evaluation of this experimental model, qualitative and quantitative histologic measurements were adopted. The hematoxylin and eosin (HE) stained slices were analyzed blindly in a microscopy jellyfish five-head Olympus® BX 40. The atherosclerotic lesions were graded from 0 - VI according to the qualitative criteria proposed by Stary et al[7-9]. The morphometric analysis was performed on stained orcein (elastic) to determine the area of the intima and the medial layers of the aortic arch and descending aorta, as well as to determine the intima/media layer area ratio (IMR - the area of the intimal layer divided by the area of the medial layer). For this assessment, the more injured segment or the segment with more advanced stage of atherosclerosis was previously selected in the HE-stained slice. The analyses were performed microscopically in conjunction with Image Pro-plus® 4.5 software (Media Cybernetics Inc., Silver Spring, MD, USA).

Immunohistochemistry

We evaluated the concentration of the vascular cell adhesion molecule (VCAM-1), monocyte chemotactic protein-1 (MCP-1), and interleucin-6 (IL-6) in the intima of the arteries using primary monoclonal antibodies. The reading was performed with an Olympus® BX50 microscope, with an objective of 20 times.

Statistical analysis

Categorical variables were expressed as percentages and continuous variables were expressed as the mean ± SD and medians. The Shapiro-Wilks test was used for testing sample normality. For symmetry conditions, some variables were submitted to logarithmic transformation. For dichotomic nominal variables, the Fisher exact test was used to compare the groups. For quantitative parameters, Student's *t*-test and Mann-Whitney non-parametric test were used for the comparison between CG and RG. Statistical significance was indicated by $p < 0.05$.

Results

Two animals from the CG died in the middle of the experiment and were removed from the final analysis. The initial mean body weight of animals in the CG (1.78 kg) and RG (1.82 kg) were similar (p=0.274). At the end of the study, CG animals exhibited a mean body weight of 2.58 kg, while RG animals had a mean body weight of 2.39 kg (p=0.537).

Lipid profiles

Baseline TC, HDL-C, LDL-C, and TGC levels were relatively equivalent in all groups before initiation of the diet. At the end of the experiment, an analysis of TC, HDL-C, LDL-C, and serum TG indicated no significant difference between the groups (Table 1).

Table 1 - Lipid profile between control group (CG) and Resveratrol group (RG)

Variable	Group	Mean	Minimum	Maximum	SD (±)	p
TC (basal)	CG	70.30	47.00	115.00	21.79	
	RG	44.50	30.00	62.00	10.98	0.008
TC (euthanasia)	CG	1676.70	347.00	2885.00	768.67	
	RG	2173.75	1626.00	2820.00	353.84	0.112
TGC (basal)	CG	196.40	96.00	295.00	71.94	
	RG	114.00	78.00	181.00	37.11	0.010
TGC (euthanasia)	CG	220.20	60.00	669.00	204.32	
	RG	158.14	114	226.00	75.24	0.762
LDL-C (basal)	CG	11.80	2.00	49.00	15.17	
	RG	2.08	1.37	2.75	0.40	0.346
LDL-C (euthanasia)	CG	1596.12	298.00	2719.20	743.03	
	RG	2084.63	1373.00	2748.00	399.96	0.114
HDL-C (basal)	CG	19.38	12.00	29.00	6.33	
	RG	16.38	11.00	25.00	4.24	0.280
HDL-C (euthanasia)	CG	37.40	29.00	50.00	7.29	
	RG	34.25	25.00	55.00	9.97	0.449

TC means total cholesterol; TGC means triglycerides; LDL-C means low density cholesterol

Histologic analysis

At the end of the experiments, it was shown that there was a statistically significant difference between the animal groups with respect to the probability of type I, II, III, IV, V, and VI lesions or absence of lesions. Of those animals in the CG, 70% had advanced atherosclerotic lesions (types III, IV, and V) in the aortic arch and descending aorta, while 100% of the RG animals had mild atherosclerotic lesions (types I and II, or no lesions) in the aortic arch and descending aorta (Figure 1).

Morphometric analysis

The intimal area was significantly lower in the RG than the CG ($p = 0.001$; Figure 1). There was a significantly greater reduction in the intima/media layer area ratio (IMR) in the RG than in the CG ($p = 0.001$). There was no statistically significant difference between the medial layer area between the groups in the aortic arch and descending aorta (Table 2).

Immunohistochemistry

An analysis of positive areas for VCAM-1 revealed a significant difference between groups, with a higher concentration in CG ($p=0.007$). Analysis of MCP-1 and IL-6 showed significantly higher values in the CG compared with the RG ($p=0.039$ and $p=0.015$, respectively; Table 3; Figure 2).

Discussion

Resveratrol, present in the grape juice and wine, is considered the major polyphenol responsible for cardiovascular benefits because of its antioxidant and anti-platelet activities[10,11]. Resveratrol has anti-inflammatory properties which are manifested as inhibition of ICAM-1 and VCAM-1expression and the attachment of monocytes to endothelial cells, inhibition of lipopolysaccharide (LPS)-induced synthesis of TNF-α and IL-1-β, and release of IL-6 from monocytes[12]. Resveratrol also inhibits the migration and proliferation of vascular smooth muscle cells (SMCs) to the intima layer, which is considered the *sine qua non* of atherogenesis[13,14]. Collectively, these effects are thought to be responsible for decreased incidence of heart disease in the French population, and is thus termed the French Paradox (a very low mortality rate due to cardiovascular disease, despite a high-fat diet), because of the population's

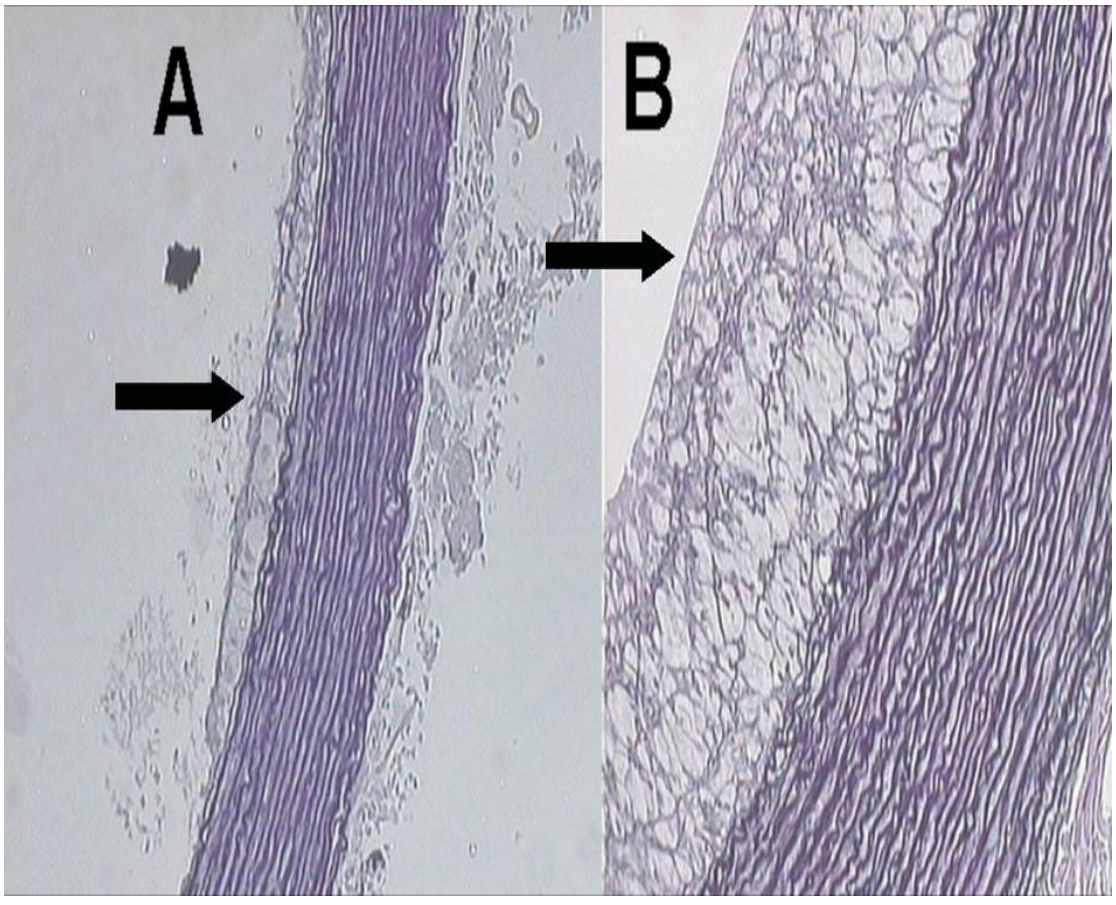

Figure 1 - *Histologic slices from the descending aorta. A – Resveratrol group with atherosclerotic lesion (type I). Black arrow indicates intima layer. B – Control group with atherosclerotic lesion (type III). Black arrow indicates intima layer.*

Table 2 - Quantitative histopathological parameters between control group (CG) and resveratrol group (RG) expressed in square micrometer.

Variable	Group	n	Mean	Median	SD (±)	p*
Intima	CG	10	75.10	68.25	51.66	< 0.001
	RG	8	4.76	1.38	6.75	
Media	CG	10	176.59	196.99	52.69	0.315
	RG	8	209.70	205.49	39.77	
IMR	CG	10	0.41	0.37	0.25	< 0.001
	RG	8	0.03	0.01	0.04	

*IMR represents intima/media area ratio. *Mann- Whitney.*

Table 3 - Immunohistochemical variables in descending aorta between control group (CG) and resveratrol group (RG) expressed in square micrometer

Variable	Group	n	Mean	Median	SD (±)	p*
VCAM-1	CG	10	5339.07	5209.03	3791	0.007
	RG	8	1452.96	1035.59	1137	
MCP-1	CG	10	11772	11003	7947	0.039
	RG	8	5509	4781	2745	
IL-6	CG	10	3852	3623	2694	0.015
	RG	8	1273	1128	580	

*VCAM-1 means vascular cell adhesion molecule; MCP-1 means monocyte chemotactic protein-1; IL-6 means interleucin-6. * Student's t-test.*

preference for red wine[15]. In this study, we induced atherosclerotic lesions with the addition of lyophilized egg to the normal diet of rabbits during 4 weeks. This experimental model of inducing atherosclerosis using lyophilized egg is rarely described in the literature, though it is proven to be efficient and less costly when compared to the use of industrialized cholesterol. Resveratrol was added in the diet of RG after 4 weeks to test its capacity to interfere in established atherosclerotic lesions. We demonstrated that resveratrol inhibits the progression of atherosclerotic lesions by reducing the intima area and the IMR, and showing no advanced lesions in the aortic arch and descending aorta in the RG. In a similar study using rabbits, Castro et al[16] concluded that resveratrol worked as a preventive agent in the development of atherosclerotic lesions as they observed a low-degree of foam cell invasion in the tunica media of animals treated with resveratrol. The mechanisms by which resveratrol inhibits the formation of advanced atherosclerotic lesions appears to be through its inhibitory effect on LDL oxidation, platelet aggregation, and vascular proliferation of smooth muscle cells. LDL oxidation is a main cause of endothelial injury and induction of the expression of pro-inflammatory molecules in endothelial cells. Resveratrol has been shown to protect lipids from peroxidative degradation and to stop the uptake of oxidized LDLs in the vascular wall in a concentration-dependent manner[17-19]. However, there are conflicting results regarding the effects of resveratrol on lipid levels, because some studies have failed to show a reduction in plasma lipids levels induced by such a substance[20]. In this study, we did not uncover any evidence of a significant difference in lipid profiles between groups, which may have reflected the short period of the experiment. Daugherty et al[21], in a similar study in hypercholesterolemic rabbits, showed that the reduction in atherosclerotic lesions secondary to the reduction in LDL-C levels is a process that takes months, or even years. In contrast, in this study, we showed that a hyphercholesterolemic diet causes the development of atherosclerotic lesions associated with an inflammatory process, and resveratrol reduced the concentration of VCAM-1, MCP-1, and IL-6 in the intima layer of hypercholesterolemic rabbits. It is known that inflammation mediates all stages of atherosclerosis from initiation to progression, and eventually plaque rupture[17]. Indeed, VCAM-1, MCP-1, and IL-6 facilitate the adherence of monocytes to the endothelial surface and facilitate leukocyte transendothelial migration[22-25]. This process is exacerbated when blood LDL levels are high from poor dietary habits or an inherited genetic pre-disposition, and signals the endothelium to increase adhesion molecule expression and potentiate the inflammatory response[26]. Even though we did not make the correlation between blood lipid levels and the inflammatory process, we believe that such a possible correlation should be the subject of future studies. Also, we did not analyze the monocyte/macrophage concentration

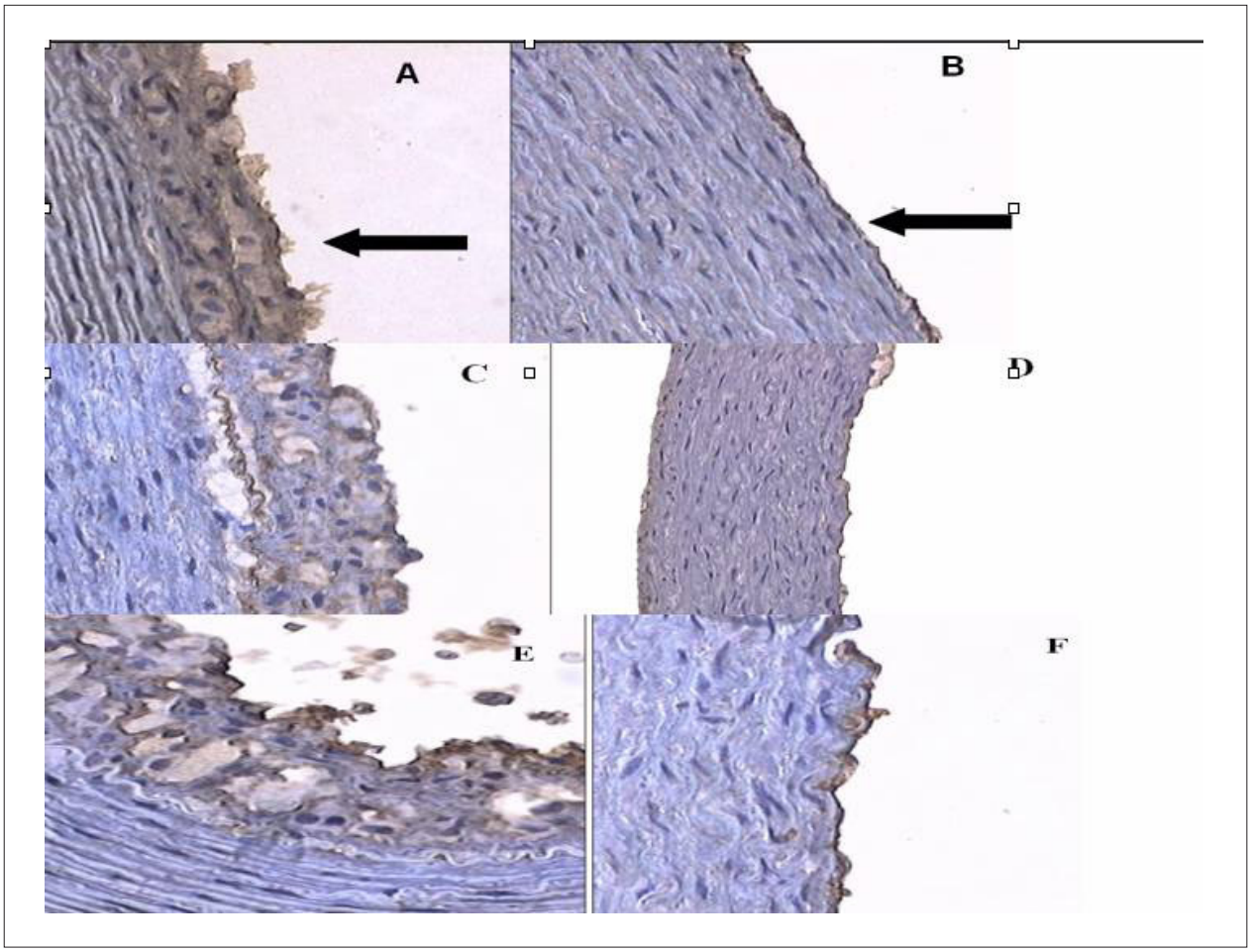

Figure 2 - *Immunohistochemistry showing positive areas for MCP-1, VCAM-1, and IL-6. A – Control group. Black arrow indicates intima layer with positive areas for MCP-1 (brown-speckled areas). B – Resveratrol group. Black arrow indicates intima layer without positive areas for MCP-1. C – Control group. Positive areas for VCAM-1 (brown-speckled areas). D – Resveratrol group. Positive areas for VCAM-1. E- Control group. Positive areas for IL-6 (brown-speckled areas). F- Resveratrol group. Positive areas for IL-6.*

that appears to have a crucial role in the development of atherosclerosis. This cell appears to be involved in all stages of atherosclerotic plaque development and is increasingly regarded as a candidate for therapeutic intervention and as a potential biomarker of disease progression and response to therapy[27-29].

Conclusions

Resveratrol had significant anti-atherogenic and anti-inflammatory effects in an animal model of rabbits fed a hypercholesterolemic diet (1% cholesterol).

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

There were no external funding sources for this study.

Study Association

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