

## MORPHOMETRY OF AORTIC ARCH OF RABBITS WITH INDUCED ATHEROMA TREATED WITH RESVERATROL AS EXPERIMENTAL MODEL FOR THE PREVENTION OF ATHEROSCLEROSIS

MORFOMETRIA DO ARCO AÓRTICO DE COELHOS COM ATEROMA INDUZIDO TRATADOS COM RESVERATROL COMO MODELO EXPERIMENTAL NA PREVENÇÃO DA ATHEROSCLEROSE

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### SUMMARY

Atherosclerosis is a chronic fiber-proliferative inflammatory condition associated with the production of oxidants. The phenolic compound resveratrol, found mainly in grapes and red wine, seems to have cardioprotective activity by preventing the oxidation of low-density lipoproteins. The effectiveness of resveratrol to prevent induced atheromatosis has been investigated, by the morphometric studies of the tunica intima, media and adventitia of the aortic arch. Twenty rabbits divided into four groups were fed the following diets for 60 days: control group (CT) regular diet; resveratrol group (R) regular diet and 3 mg/kg/day of resveratrol; cholesterol group (CL) 1.5% of cholesterol added to the ration; group cholesterol plus resveratrol (CR) 1.5% of cholesterol added to the ration and simultaneous administration of 3 mg/kg/day resveratrol. The morphometric study showed greater thickening of tunica intima in CL group, thickening of tunica media in CL and CR groups but without differences between them, and when analyzing the thickness of tunica adventitia, CL group differed from CT and R groups but did not differ from CR group. The results obtained in this study using morphometric analysis, suggest that resveratrol acts as a preventive agent in the development of atherosclerotic lesions.

**KEY-WORDS:** Antioxidants. Atherosclerosis. Rabbit. Morphometry. Resveratrol

### RESUMO

Aterosclerose é um condição inflamatória fibro-proliferativa crônica associada à produção de espécies oxidantes. O composto fenólico resveratrol, encontrado principalmente na uva e no vinho tinto, parece ter atividades cardioprotetoras prevenindo a oxidação de lipoproteínas de baixa densidade. Neste estudo investigou-se o efeito do resveratrol na prevenção da aterosclerose induzida, por meio de estudos morfométricos das túnicas íntima, média e adventícia do arco aórtico. Para tanto, foram utilizados 20 coelhos divididos em quatro grupos os quais receberam as seguintes dietas durante 60 dias: grupo controle (CT) ração normal; grupo resveratrol (R) ração normal e resveratrol na dose de 3 mg/kg/dia; grupo colesterol (CL) ração acrescida de 1,5% de colesterol; grupo colesterol mais resveratrol (CR) ração acrescida de 1,5% de colesterol e administração simultânea de resveratrol na dose de 3mg/kg/dia. O estudo morfométrico revelou maior espessamento da túnica íntima no grupo CL, espessamento da túnica média para os grupos CL e CR, porém, sem diferença entre eles, e, quando analisado espessura da túnica adventícia, o grupo CL diferiu dos grupos CT e R, porém, não diferiu do grupo CR. Pelos resultados obtidos neste estudo através de análise morfométrica, segere-se que o resveratrol atua como agente preventivo no desenvolvimento de lesões ateroscleróticas.

**PALAVRAS-CHAVE:** Antioxidante. Aterosclerose. Coelho. Morfometria. Resveratrol.

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## INTRODUCTION

Nowadays, cardiovascular diseases are the main causes of morbidity and mortality, and they are also the most important health problem among the adult population in developed countries. There is wide variation regarding incidence and mortality rates; however, the Mediterranean area has the lowest rates in the world. The World Health Organization predicts that global economic prosperity will spread the problem in the developing countries in case the western habits are acquired (PEREZ et al., 1998).

Atherosclerosis is the pathological condition that precedes the majority of cardiovascular events, such as myocardial infarction and cerebrovascular accidents (CVA)(COTRAN & SCHOEN, 2000).

The atherosclerotic plaques consist mainly of cells, including smooth muscle cells, macrophages and other leukocytes, the extracellular matrix of connective tissue, including collagen, elastic fibers and proteoglycans, as well as intracellular and extracellular lipid deposits. Typical atheromas contain a relatively abundant amount of lipids invading the tunica intima, media and advanced lesions in the adventitia of arteries of medium and large caliber, particularly those located in areas of high blood pressure. The development of lesions is affected by many systemic factors, among them hyperlipidemia, hypertension, obesity, physical inactivity, diabetes, hemodynamic factors, such as smoking among others (COTRAN & SCHOEN, 2000).

Resveratrol is an antioxidant widely found in the Mediterranean diet that has attracted considerable attention as a therapeutic agent.

The objective of this study was to evaluate the effect of resveratrol as a therapeutic agent to prevent induced atherosclerotic lesions in rabbits, by means of morphometric study of the tunica intima, media and adventitia in the region of the aortic arch. The purpose is to provide information to professionals in the fields of human and animal health, since some dog breeds are susceptible to primary hypercholesterolemia and hypertriglyceridemia and may, therefore develop atherosclerosis.

## MATERIAL AND METHODS

Resveratrol and cholesterol were purchased respectively from Pharma Nostra (Rio de Janeiro, RJ) and Vetec (Duque de Caxias, RJ). The feed used was the maintenance ration used to feed adult rabbits, purchased from Purina (Paulínia, SP) and the 1.5% cholesterol was added to the ration in the Animal Nutrition Department. The rabbits were maintained in the Department of Animal Morphology and Physiology of FCAV, Jaboticabal, during 60 days. The experiment was conducted in accordance with the standards established by the Ethics Committee of Universidade Estadual Paulista, UNESP.

Twenty healthy adult male rabbits weighing average 2.5 kg, of New Zealand breed, were kept for 60 days. They were previously fed ration during one

week and then, divided into 4 experimental groups. Each group of 5 animals was fed the following diets: control group (CT), only maintenance diet; group (R), maintenance diet and simultaneous oral administration of resveratrol (3 mg/kg/day); group (CL), 1.5% cholesterol was added to the diet and simultaneous administration of resveratrol capsules orally (3 mg/kg/day). The fourth group (CR) was fed a hypercholesterolemic diet prepared as follows: powdered cholesterol was added to the previously ground maintenance diet, which was mixed, pelleted and kept refrigerated throughout the experimental period. The dose of resveratrol used was based on previous study by Wang et al. (2005). During the experiment water was supplied *ad libitum* and the amount of feed for all animals was 100 g/day.

At the end of the experimental period, the animals were deprived of food for 18 hours, euthanized using intra-venous sodium pentobarbital and the aortic arch was removed. The aortic arch was carefully washed with saline solution and fixed in Bouin solution for 24 hours at room temperature for subsequent inclusion in paraplast. Later, microtomy was performed using an automatic microtome Leica – RM2155 to obtain 5  $\mu\text{m}$ -thick histological cuts at intervals of 10  $\mu\text{m}$ . These histological cuts were stained with Masson's trichrome and hematoxylin-eosin (TOLOSA et al., 2003) observed and photomicrographed by a Leica DM 5000 B photomicroscope used for morphometric analysis with the software *Leica Application Suite (LAS) 2.5.1 R1*. This study was performed by measuring, in micrometers, the thicknesses of the tunica intima, media and adventitia of atherosclerotic areas of the segment of the aortic arch. Five histological slides were prepared for each animal.

Statistical analysis was performed by analysis of variance (ANOVA). Means were compared by Tukey test at  $p < 0.05$  significance level.

## RESULTS

Statistical analysis of the results for the mean  $\pm$  standard deviation of thickness of the tunica intima and media of the aortic arch segment obtained in this study are given in Tables 1, 2, 3 and 4.

Animals fed only maintenance diet, CT group, showed no atherosclerotic lesions, whereas these lesions were evident in the CR and CL groups. The severity of the lesions was significantly reduced in the rabbits of group CR, which received resveratrol, based on the results of the measurements in microns of the inner layer (tunica intima), whose values were  $188.35 \pm 95.36$  and  $64.26 \pm 9.19$ , for CL and CR groups, respectively. Total thickness of the wall values were  $863.47 \pm 105.56$  and  $681.60 \pm 94.27$  for CL and CR groups, respectively. The values of the inner layer in  $\mu\text{m}$  indicated that the CL group differed significantly ( $p < 0.05$ ) from groups CT, R and CR, which did not differ.

Tunica media mean values, in  $\mu\text{m}$ , were higher for the animals of groups CL and CR, indicating a

**Table 1** – Mean thickness ( $\pm$  SD) of wall (intima + media + adventitia) of New Zealand white rabbits submitted to the following treatments: control group (CT), resveratrol (R), cholesterol (CL) and cholesterol + resveratrol (CR).

<b>Group</b>	<b>Mean</b>	<b>Standard deviation</b>
CT	490.47c	52.67
R	498.32c	30.75
CL	863.47a	105.56
CR	681.60b	94.27

Means followed by different letters in the same column differ by Tukey test ( $p < 0.05$ ).

**Table 2** - Mean thickness,  $\mu\text{m}$ , of the inner (intima) layer ( $\pm$  SD) of New Zealand white rabbits submitted to the following treatments: control group (CT), resveratrol (R), cholesterol (CL) and cholesterol + resveratrol (CR).

<b>Group</b>	<b>Mean</b>	<b>Standard deviation</b>
CT	14.83b	1.51
R	11.60b	0.79
CL	188.35a	95.36
CR	64.26b	9.19

Means followed by different letters in the same column differ by Tukey test ( $p < 0.05$ ).

**Table 3** - Mean thickness,  $\mu\text{m}$ , of the middle layer ( $\pm$  SD) of New Zealand white rabbits submitted to the following treatments: control group (CT), resveratrol (R), cholesterol (CL) and cholesterol + resveratrol (CR).

<b>Group</b>	<b>Mean</b>	<b>Standard deviation</b>
CT	333.76b	49.51
R	384.76ba	21.13
CL	472.10a	41.97
CR	443.91a	94.80

Means followed by different letters in the same column differ by Tukey test ( $p < 0.05$ ).

**Table 4** - Mean thickness,  $\mu\text{m}$ , of the adventitia layer ( $\pm$  SD) of New Zealand white rabbits submitted to the following treatments: control group (CT), resveratrol (R), cholesterol (CL) and cholesterol + resveratrol (CR).

<b>Group</b>	<b>Mean</b>	<b>Standard deviation</b>
CT	141.88bc	22.93
R	101.95c	14.18
CL	203.00a	41.33
CR	173.41ba	7.16

Means followed by different letters in the same column differ by Tukey test ( $p < 0.05$ ).

thickening of the layer compared to groups CT and R, which were normal.

As for the values found for tunica adventitia, there was a difference between the groups, CL and CR groups were not different; however, groups CL and CR were significantly ( $p < 0.05$ ) different from groups CT and R.

Total thickness mean values obtained from the measurement of tunicas intima, media and adventitia showed that group CL was significantly ( $p < 0.05$ ) different from groups CT, R and CR.

## DISCUSSION

This work showed the effect of resveratrol as a protective agent in atherosclerosis of hypercholesterolemic rabbits, as noted by decreased wall thickening of the aortic arch. The choice of white New Zealand rabbits in this study as an experimental model for the induction of atherosclerosis was based on descriptions by Wang et al. (2005) who reported that animals of this breed were susceptible to atherogenesis. The dose used in this study (3 mg/kg) was effective as shown in the results. The rabbits fed a maintenance diet and resveratrol did not show any apparent adverse effect, which lead to the conclusion that resveratrol is tolerated well by the species.

The morphometric analysis of the aortic arch showed that the thickness of the tunica intima of the animals of group CR was similar to that of groups CT and R, demonstrating a significant decrease of the atherosclerotic plaque compared to the rabbits of group CL, which agrees with results reported by Wang et al. (2005) that proved the anti-atherogenic effect of resveratrol. On the other hand, Morel et al. (1994) in similar studies using vitamin E and C in rabbits, did not observe any decrease of the atherosclerotic lesions in the aorta.

Tunica media was thicker for rabbits of groups CL and CR compared to groups CT and R, which did not change (CT  $333.76 \pm 49.51$ ; R  $384.76 \pm 21.13$ ; CL  $472.10 \pm 41.97$ ; CR  $443.91 \pm 94.80$   $\mu\text{m}$ ). This result for tunica media differ from those reported by Wang et al. (2005) who found no difference between the control and hypercholesterolemic groups in the thickness of tunica media.

The morphometric analysis results of the tunica adventitia were significantly different between groups, assuming that this was not consequence of the stage of the lesions observed in the morphometric study of the inner layer of the CL and CR groups, one can hypothesize that there was casual interference, considering that the lesions were classified as type III, that is, not advanced. For the animals that without plaques, no alterations were observed. One can conclude that resveratrol decreased the rate at which the atherosclerosis was developing supported by reports in the literature about this polyphenolic compound, which describe a broad spectrum of biological effects, such as antioxidant, anti-platelet, endothelial protective action and its activity in endothelial proliferation of smooth muscle cells

(FRÉMONT, 2000). A study by Wakabayashi (1999) proved that the polyphenols present in red wine were able to reduce plasma oxidation of LDL. Also Fremón et al. (1999) while studying antioxidant compounds *in vitro*, observed that the one found in red wine showed remarkable inhibition of peroxidation of LDL (80-90%) while citric and soy extracts showed 70% and 55% inhibition, respectively.

The antioxidants present in red wine, according to Zhao et al. (2001) were able to inhibit endothelial oxidative stress caused after a meal high in fat, especially in patients with coronary heart disease, which could partly explain the findings for CR group in this study. Moreno et al. (2000) also described an *in vitro* study, where the antioxidant effect provided by phenolic compounds was more pronounced than that promoted by vitamins C and E, a fact that lead us to chose this antioxidant compound. Ascik et al. (1995) while studying *in vitro* the effect of trans-resveratrol, some phenolic compounds derived from the wine and antioxidants, on the plaque formation and eicosanoid synthesis in human cells, observed a blockage of plaque development and a reduction in the eicosanoid synthesis, which according to the authors contributed to the the cardioprotective effect of resveratrol, especially in patients with atherosclerosis and coronary heart disease.

Resveratrol has been shown to improve endothelial function, as observed by Zou et al. (2003) in their work, where the plasma endothelin levels decreased as nitric oxide levels increased, thereby promoting greater vasodilation and cardioprotective activity. Araim et al. (2002) reported that resveratrol inhibits the growth of smooth muscle cells of the aorta in calves.

It is worth noting the beneficial effect of resveratrol in preserving the integrity of the endothelium, as observed in some studies its inhibitory effect on the expression of VCAM-1, ICAM-1 and the transcription factor NF- $\kappa\text{B}$ , the latter being responsible for pro-inflammatory modulating effects (FERRERO et al., 1998; MANNA et al., 2000; WUNG et al., 2005).

## CONCLUSIONS

It was concluded that resveratrol acts as a preventive agent in the development of atherosclerotic lesions in rabbits. Studies in other species, in addition to humans, are needed to verify the effectiveness of this compound with respect to its anti-atherogenic properties in order to completely understand the exact mechanism of its action. However, from the results obtained in this work, it is believed that a dietary supplement for humans and animals is a possible alternative to preventing the development of atherosclerosis.

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