Effect of Dry Coffee Residues Fermented with Monascus Ruber on the Metabolism of Apo E mice

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Abstract

Background: Atherosclerosis is a chronic inflammatory disease of multifactorial origin, which occurs in response to endothelial injury. The fungus *Monascus ruber* has hypocholesterolemic activity, and the polyphenols present in coffee residue have an antioxidant activity and can help prevent cardiovascular diseases. Coffee residue has a significant amount of fermentable sugars, being an adequate substrate for growing fungi.

Objective: The objective of this study was to assess the effect of dry coffee residue fermented with *Monascus ruber* on the lipid metabolism of Apo E knockout mice.

Methods: The biological assay was performed with 30 Apo E knockout mice, divided into five groups and undergoing different treatments. The phytochemical prospection and quantification of phenolic compounds of the fermented and non-fermented coffee residues were performed. The sera of the animals were analyzed by using enzyme kits, and the aortic tissue was embedded in paraffin and stained with hematoxylin and eosin to undergo histopathological analysis.

Results: Comparing with the control group, the group receiving 2% non-fermented coffee residue showed a reduction of 42% in the serum levels of triacylglycerols and of approximately 41% in VLDL-c. The groups receiving 10% non-fermented coffee residue and 2% fermented coffee residue showed reductions in the lesion areas of 10.5% and 15.4%, respectively, as compared with the control group. The fermented coffee residue showed a higher content of phenolic compounds as compared with the non-fermented coffee residue.

Conclusion: The present study showed that coffee residue fermentation has a potentially beneficial effect on cardiovascular diseases, especially atherosclerosis. (Arq Bras Cardiol 2012;99(2):747-754)

Keywords: Atherosclerosis; coffee; *monascus*; mice knockout.

Introduction

Brazil has consolidated its position as the world's greatest producer and exporter of coffee beans, accounting for 30% of the world market[1]. However, the by-products of that agricultural activity, which are often not properly treated, generate costs and environmental concern, are also a source of compounds with functional properties[2].

Coffee hull and pulp, residues of de-pulping, have a significant amount of fermentable sugars, being a proper substrate for fungal and yeast growth[3].

Monascus ruber, an *Ascomycetes* fungus, has long been used in the oriental culinary for the production of pigments, and fermented foods and beverages[4]. That fungus called the attention of researchers worldwide due to the hypocholesterolemic activity of one of its metabolites, monacolin K, which inhibits hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA reductase), a key-enzyme in the biosynthesis of cholesterol[5].

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Atherosclerosis is a complex and multifactorial disease, affected by multiple genetic and environmental factors. Several studies have shown that a high-calorie ingestion and consumption of diets rich in saturated fatty acids accelerate atherogenesis; however, the effects of the dietary individual components are still little understood[6].

Of the several classes of antioxidant substances of natural occurrence, phenolic compounds have received a lot of attention in past years, because they inhibit lipid peroxidation and lipoxygenase, acting mainly as antioxidants or pro-oxidants in diseases associated with the lipid metabolism, specially atherosclerosis[7,8].

In that setting, the general purpose of this study was to assess the use of coffee residues as a culture medium for growing *Monascus ruber,* and the effects of a fermented by-product on hyperlipidemic Apo E mice, aiming at controlling and preventing cardiovascular diseases, especially atherosclerosis.

Methods

Collection and preparation of the coffee residue sample

Catuai coffee variety (*Coffea arabica L*) was collected in the Zona da Mata Mineira from May to August, when 90% of

the coffee fruits were in the cherry stage. The residue of pulp extraction, containing the hull, pulp and mucilage adhered to the hull, was collected after wet processing, and was machine dried (Nova Técnica, NT-514 model) at a mean temperature of 55 ºC until humidity below 10% was achieved.

Microorganism and coffee residue fermentation

Monascus ruber CCT 1236 was used in this study. This strain was acquired from the Fundação Tropical de Pesquisas e Tecnologia André Tosello (Campinas, São Paulo, Brazil). The microorganism was stored in potato dextrose agar (PDA) medium under refrigeration.

Ninety-five milliliters of a solution containing 0.1% magnesium sulfate, 0.1% potassium dihydrogen phosphate, and 1% ammonium chloride were added to 50g of the coffee residue to enrich the medium and increase humidity. The residue was then inoculated with a suspension of spores of the fungus cultivated in the PDA medium at 28 ºC for 13 days. Then, the fermented coffee residue was dried and ground to be used in experimental diets.

Phytochemical prospection and quantification of phenolic compounds

The secondary metabolites present in the dried and fermented coffee residue were identified by use of thin-layer chromatography[9]. The total phenol content of the fermented and non-fermented coffee residues was estimated by using the Folin-Ciocalteu reagent[10]. The water/ethanol solution (6:4) was used as a solvent, because it showed greater efficiency in extracting total phenols from residues[11]. The results were expressed as gallic acid equivalents (GAE).

Biological assay and biochemical analyses

The biological assay was performed with 30 Apo E knockout mice of both sexes (mean weight of 22 g). The animals received a Purina® standard food preparation and water *"ad libitum"* for an adaptation period of 15 days. For ten weeks, the animals received the experimental diets and water *"ad libitum"*, and were maintained in collective polyethylene cages, with natural ventilation, 12-hour light cycles and mean temperature of 22 ºC. The experimental planning was approved by the Ethics Committee of the Veterinary Medicine Department of the Universidade Federal de Viçosa (process 11/2008). The mice were divided into five groups containing six animals each as follows: Group 1 (control); Group 2; Group 3; Group 4; and Group 5. The diets of the experimental groups were elaborated according to the Association of Official Analytical Chemistry recommendation[12]. The diets of the Groups 2 and 3 contained 2% and 10% of non-fermented coffee residues, respectively, while the diets of the Groups 4 and 5 contained 2% and 10% of fermented coffee residues, respectively (Table 1).

At the end of the experiment, the animals were weighed, after a 12-hour fasting, anesthetized, and euthanized, blood samples being collected for measuring triacylglycerols, total cholesterol and fractions. Blood was withdrawn by use of exsanguination via abdominal aorta section. Serum was obtained through centrifugation at 5,000 rpm for 15 minutes in a table centrifuge (Marconi, M.A. 860 model).

Serum measurements were performed in the multiparametric measuring apparatus (Bioquímica, Alizé®), using BioMérieux® enzyme kits.

Histopathology

The initial region of the aorta was removed and perfused in phosphate buffered saline (PBS), transferred to a 70% ethanol solution and conserved until histopathological analysis. Aortic fragments were processed in a manual rotary microtome (American Optical®) after routine paraffin inclusion, and 5-μm thick consecutive histological sections were obtained. Two histological slides containing, on average, ten sections per animal were obtained. The slides were stained with hematoxylin and eosin (H/E), encoded and submitted to microscopy.

The initial region of the aorta was used for histological analyses of the arterial constituents. All histological sections obtained were examined aiming at locating the referential anatomical structures of the aortic valve and root. According to the presence of those referential structures, the histological section at the aortic valve anatomic level was selected for the measurements. The sections were obtained at 5-μm intervals, the mean explored extension being 20-30 μm. For morphometric analysis, images of the histologic sections of the five groups were captured, and, for standardizing the group samplings, eight images were randomly selected for measurement.

Images were obtained with the Olympus® Bx50 microscope coupled with a Q-Color 3 Olympus® digital camera, the images being captured by using the Q-Capture Pro software, version 6.0.0.412. The aortic lesion areas were measured by using the Image Pro Plus software, version 4.5.0.29.

Data analyses

The SigmaStat software (version 2.03) was used for the descriptive analysis of the results and the comparison tests of the means of the independent groups. The Kolmogorov-Sminorv test was used to assess the distribution of the variables. Considering the sampling per group of animals and that some variables do not have a normal distribution, non-parametric tests were performed for comparing each two independent groups, using the Mann-Whitney test. Variables with normal distribution underwent parametric analysis, followed by the *t* test. The means were significant for $p < 0.05$.

Results

Phytochemical prospection and quantification of phenolic compounds

The major groups of secondary metabolites identified in the non-fermented coffee residues and in the coffee residues fermented by *Monascus ruber* are shown in Table 2.

Saponins, triterpenes/steroids, coumarins, tannins, flavonoids and alkaloids were detected in both coffee residues. However, there was a difference between the chromatographic profiles of the extracts regarding the secondary metabolite groups.

Table 1 – Composition of the experimental diets (g/100 g)

Component	G1	G2*	G3*	G4**	G5**
Saccharose	48.27	47.94	46.43	47.86	46.24
Casein	20.0	19.7	18.61	19.6	18.0
Cellulose	6.72	5.38	-	5.56	0.87
Soybean oil	1.0	1.0	1.0	1.0	1.0
Cholesterol	1.0	1.0	1.0	1.0	1.0
Hydrogenated vegetable fat	16.0	15.97	15.86	15.97	15.88
Vitamin mixture	1.0	1.0	1.0	1.0	1.0
Mineral mixture	5.0	5.0	5.0	5.0	5.0
Choline	1.0	1.0	1.0	1.0	1.0
Residue	-	2.0	10.0	2.0	10.0
Total	100g	100g	100g	100g	100g

** Non-fermented residue ** fermented residue*
G1 (control), G2 (2% non-fermented residue), G3 (10% non-fermented residue), G4 (2% fermented residue) and G5 (10% fermented residue).

Table 2 – Results of the phytochemical prospection by use of chromatography of fermented and non-fermented coffee residues

Class of secondary metabolites	Fermented residue	Non-fermented residue
Antraquinones	-	-
Saponins	+	+
Triterpenes and steroids	+	+
Flavonoids	+	+
Tannins	+	+
Coumarins	+	+
Alkaloids	+	+

+: present; -: absent.

The fermented coffee residue showed a statistically higher content of total phenols than the non-fermented coffee residue.

Effect of the ingestion of coffee residues on the serum lipid profile of knockout Apo E mice

Table 4 shows the mean levels of triacylglycerols, and total cholesterol and fractions in the different groups of the experiment (Table 4).

The results show a significant reduction in the serum levels of triacylglycerols and VLDL-c.

Aortic histopathology of Apo E knockout mice

Table 5 shows the mean percentage of the aortic lesion area (mm^2) in mice undergoing different treatments with fermented and non-fermented coffee residues.

The results show statistically significant differences between the treatment groups (Groups 2, 3 and 4) and the control group (Group 1).

Figure 1 illustrates the aortic lesions of mice undergoing different treatments with fermented and non-fermented coffee residues.

The aortic lesion was identified as a xanthoma in a progressive stage with no evident deposition of cholesterol crystals, according to the classification by Virmani et al.[13].

Figure 2 shows the morphometric assessment of aortic lesions of mice fed different diets.

The results suggest that the effect of the fermented coffee residue is inversely related to its dietary concentration. The group receiving 2% of fermented residue showed a 15% reduction in the lesion area.

Discussion

The present study assesses, for the first time, the effect of coffee residues fermented and non-fermented with *Monascus ruber* on the lipid metabolism of animals.

Products fermented with fungi of the *Monascus* genus have been used in the treatment and prevention of cardiovascular diseases, especially atherosclerosis[14]. The coffee residue

Table 3 – Content of total phenols of the dry and fermented coffee residues expressed as gallic acid equivalents (GAE)

Residue	Total phenols (mg GAE g residue)	Concentration (%)
Non-fermented	7.772 ± 0.0676	0.7772
Fermented	10.857 ± 0.169*	1.0857

** Statistically different according to the t test, p < 0.001.*

Table 4 – Serum levels of triacylglycerols, total cholesterol and fractions of Apo E knockout mice undergoing different treatments

Parameters (mg/dL)	G1	G2	G3[1]	G4[1]	G5[1]
Cholesterol	1356.0 ± 437.2	1699.6 ± 221.0	1423.7 ± 99.3	1122.8 ± 193.5	1509.9 ± 227.0
TG	118.1 ± 39.6	57.3 ± 6.2**	124.3 ± 79.6	162.3 ± 106.6	213.9 ± 180.9
LDL	1465.1 ± 429.6	1672.3 ± 220.1	-	-	-
VLDL	22.7 ± 8.6	11.4 ± 1.2*	-	-	-
HDL	14.9 ± 5.3	15.7 ± 0.7	-	-	-

TG: triacylglycerols
The results were expressed as mean ± standard deviation for six animals in Group G1 (control) and four animals in Groups G2 (2% non-fermented residue), G3 (10% non-fermented residue), G4 (2% fermented residue) and G5 (10% fermented residue).
1 In groups G3, G4 and G5, the sample was not sufficient to measure cholesterol fractions.
** p = 0.029 (Mann-Whitney test).*
*** p = 0.010 (Mann-Whitney test).*

Table 5 – Mean percentage of aortic lesion areas in Apo E knockout mice undergoing different treatments with fermented and non-fermented coffee residues

Parameters	G1	G2	G3	G4	G5
Mean ± SD	62.28 ± 1.49	72.63 ± 5.86	55.92 ± 1.51	52.18 ± 1.74	64.19 ± 3.26
Median	62.18[b]	71.40[a]	55.62[c]	52.55[c]	64.75[b]
Min – Max	60.46–64.63	64.14–80.26	53.96–58.45	49.27–54.26	56.97–67.70
CV (%)	2.39	8.06	2.70	3.33	5.07

CV: coefficient of variation
G1 (control), G2 (2% non-fermented residue), G3 (10% non-fermented residue), G4 (2% fermented residue) and G5 (10% fermented residue).
Means followed by different letters differ statistically, p < 0.001 (t test)

is rich in polyphenols and bioactive compounds with antioxidant action, which also have a protective action on the cardiovascular system[15-17].

The fermentation process can synthetize or break down compounds that have biological activity. This study evidenced the presence of the same classes of bioactive compounds in fermented and non-fermented coffee residues. However, by use of distinct retention factors, the fermented and non-fermented coffee residues showed different phytochemical compounds in the same classes of secondary metabolites. That difference in compounds might relate to the higher content of polyphenols in the fermented coffee residue.

A difference was observed in the lipid profile of mice receiving non-fermented coffee residue as compared with that of mice receiving only an atherogenic diet (Group 1). Group 2 had a 42% reduction in the serum level of triacylglycerols simultaneously with an approximately 41% reduction in VLDL-c as compared with Group 1, and the differences were statistically significant.

The aorta of the Group 2 animals showed the greatest lesion area as compared with that of Group 1, contrary to that which was expected, because Group 2 had lower levels of triacylglycerols and VLDL-c than those of the control group. Group 3 and Group 4 decreased their lesion areas by 10.5% and 15.4%, respectively, as compared with the control group, and the reductions were statistically significant.

Comparing the groups fed the different residues at the same concentration (Groups 2 and 4), that receiving 2% fermented coffee residue (Group 4) showed a 26.4% decrease in the lesion area as compared with Group 2, showing that fermentation had a beneficial effect, reducing the formation of the atheroma plaque. At the 10% concentration, only the non-fermented residue showed a beneficial effect, reducing the lesion by 14.1% as compared with animals fed a 10% fermented coffee residue (Group 5).

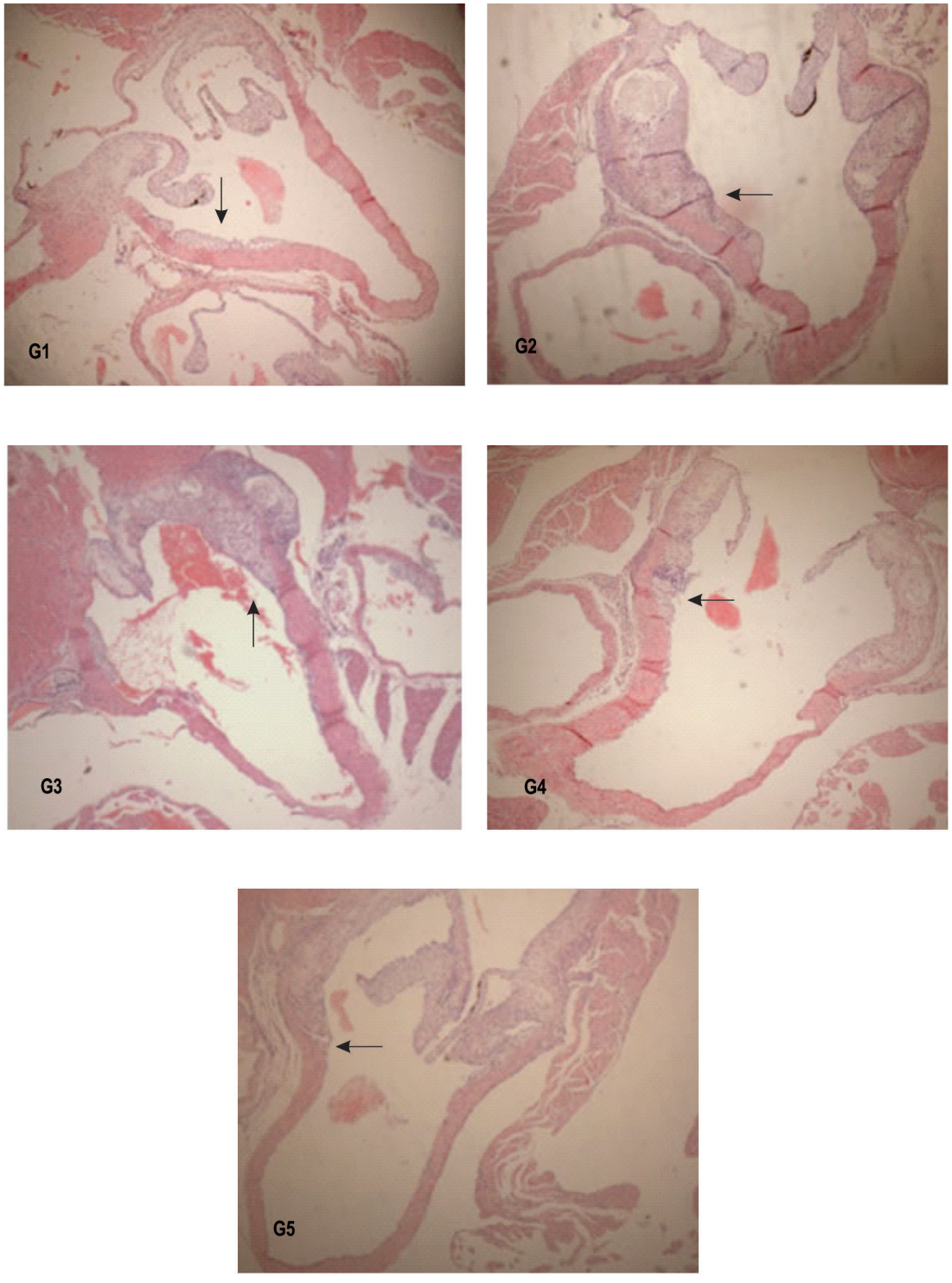

Figure 1 - *Histological sections (hematoxylin/eosin) of the aortic root of mice undergoing different treatments with fermented and non-fermented coffee residues. The arrows (←, ↑ and ↓) indicate the presence of progressive xanthomatous lesion (40x)*

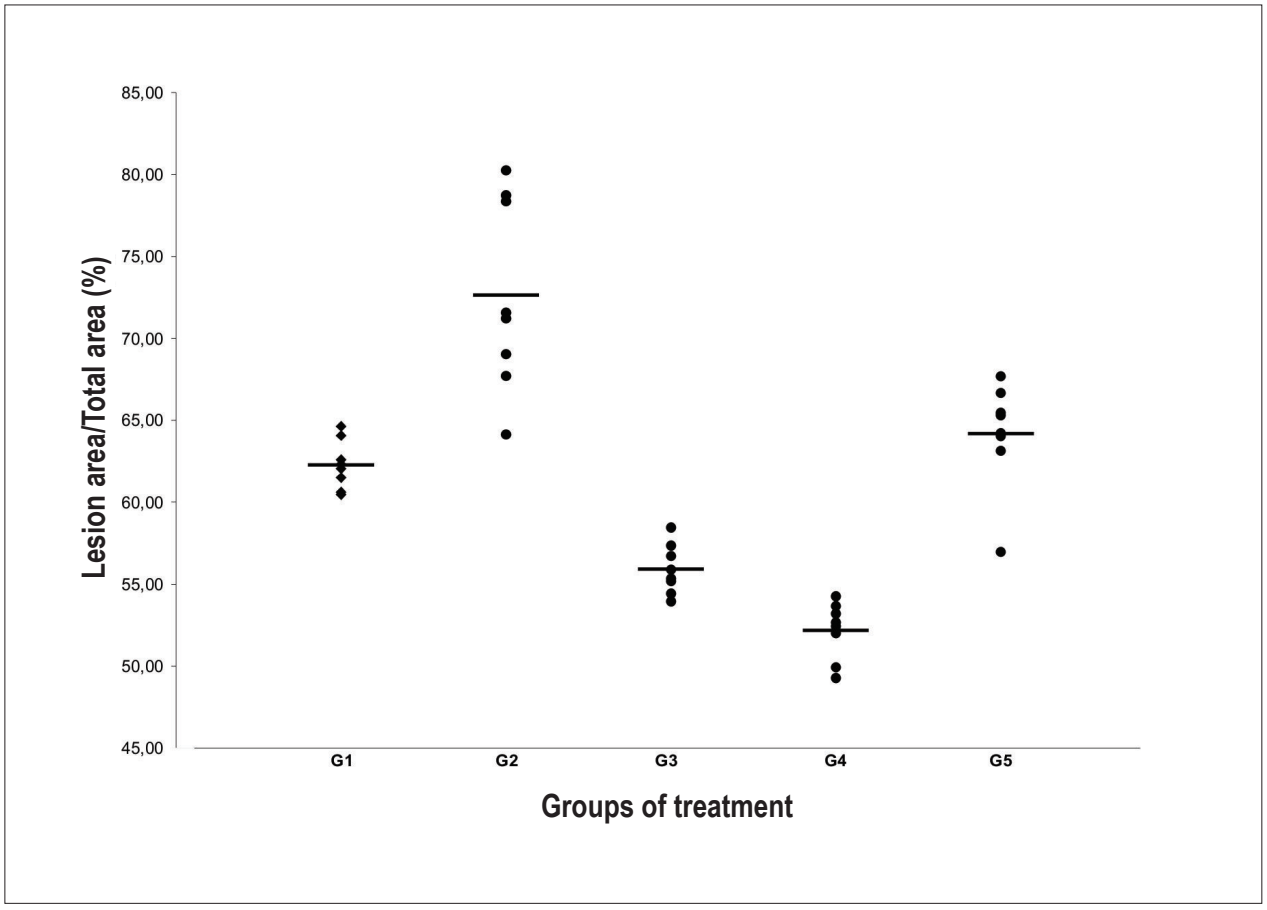

Figure 2 - *Morphometric assessment of aortic lesion areas of Apo E knockout mice undergoing different treatments with fermented and non-fermented coffee residues. Groups: G1 (control), G2 (2% non-fermented residue), G3 (10% non-fermented residue), G4 (2% fermented residue) and G5 (10% fermented residue)*

When comparing equal residues, but at different concentrations, the concentrations influenced the formation of atherosclerotic lesions, evidencing that the higher concentration of the non-fermented residue (Group 3) was efficient in reducing the lesion. On the other hand, the lower concentration of the fermented residue (Group 4) had a better protective effect.

The relationship between the consumption of coffee beverages and the development of cardiovascular diseases has been reported in the literature, mainly involving human beings. If, on the one hand, several studies have shown the noxious effect of coffee on diseases[18,19], on the other, there is evidence of its beneficial effects[20,21]. However, no reports have been found about the effect of products fermented with fungi of the *Monascus* genus and/or coffee residue on animal and human metabolism. The present study showed that the coffee residue is a growth medium for the fungus *Monascus ruber* and can help prevent atherosclerosis.

The antioxidant effect is obtained when the bioactive compounds are used at low concentrations, acting as signaling mediators in mechanisms of cell adjustment, proliferation, defense and death. The concentration increase of those compounds can cause cell injuries related to pathological conditions[22-24]. One possible explanation for our results is that fermentation leads to the formation of specific bioactive compounds, which, at high concentrations (10%), become noxious, but at a 2% concentration decrease those aortic lesions. Regarding the non-fermented residue, the concentration of phenols in the 2% coffee residue might be insufficient to provide a protective effect, but, at the 10% supplementation, the protective effect might be achieved.

The study had limitations, one of them was the small blood volume obtained in this type of animal model, hindering the performance of different analyses.

Conclusions

The results of this study showed that coffee residues reduce the serum levels of triacylglycerols and VLDLc.

Fermentation increased the content of total phenols.

The animals receiving the 2% fermented coffee residue showed the greatest reduction in the aortic lesion area. Those receiving the 10% fermented residue showed no effect on the lesions, evidencing a dose-dependent effect.

Potential conflict of interest

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

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Study Association

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