Beneficial Effects of Rosuvastatin in Heart of C57Bl/6 Mice with Diet-Induced Metabolic Syndrome - A Preliminary Study

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Abstract

Background: There is a significant association between the prevalence of cardiovascular disease and metabolic syndrome. Evidence shows that obesity is associated with structural and functional changes in the heart. Estatins drugs can reduce the endogenous synthesis of cholesterol and possess properties that are independent of their effects on lipids, the so-called pleiotropic effects. The present work aims to study the effects of rosuvastatin on the heart of mice with diet-induced metabolic syndrome, such as tissue remodeling and ultrastructural changes.

Methods: In this work we studied the effects of rosuvastatin treatment on the body mass, blood lipids, blood pressure, cardiac remodeling and structure, as well as the ultrastructural changes on the heart of the C57Bl/6 male mice fed with a high-fat diet.

Results: The rosuvastatin treatment reduced levels of blood lipids, blood pressure and body mass of high-fat mice. Furthermore, the cardiac remodeling was attenuated, with a decrease of the interstitial and perivascular fibrosis and the preservation of the integrity of mitochondrial morphology.

Conclusion: Thus, our work concludes that rosuvastatin has a beneficial effect on the heart of C57Bl/6 mice fed high fat diet being an important tool in the treatment and/or on the prevention of heart disease.

Keywords: Rosuvastatin; Heart; Cardiomyocyte; Mitochondria; High-fat diet; Metabolic syndrome

Introduction

Metabolic syndrome encompasses several risk factors of metabolic origin that are highly prevalent in adults [1]. Among the known risk factors for metabolic syndrome, obesity is considered a central and causal risk factor [2,3].

Countless evidences show that obesity is associated with structural and functional changes in the heart in both humans and animal models [4]. Increased body mass index was associated with an increased risk of heart failure in both men and women and that this risk was graded across categories of increasing body mass index [5,6].

Experimental studies in animals have shown that obesity can lead to structural changes in the heart such as ventricular hypertrophy, intracellular lipid accumulation and interstitial fibrosis [4,7]. Some mechanisms that contribute to the structural and functional modifications of the heart in obesity are associated with a change in energy metabolism and mitochondrial dysfunction [4,8].

The mitochondrial oxidative metabolism is a major source of energy in the heart, which normally consumes oxygen and generates ATP to fuel contraction of cardiac muscle cells [9-11]. In addition to producing energy, mitochondria are important sources of reactive oxygen species, which can act as a source of cellular damage, thus compromising the survival of cardiac cells [11-13]. Research performed on the mitochondria of the heart shows that structural and functional changes are not only essential for maintaining normal myocardial function but they also contribute to the pathogenesis of the cardiomyopathies [11,14,15].

Statins are pharmacological agents which impair cholesterol synthesis by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Recent studies show that statins also possess properties that are independent of their effects on lipids and lipoprotein, so-called pleiotropic effects [16-18].

In cardiovascular disease, statin therapy has led to significant reduction in morbidity and mortality from adverse cardiac events. These results are accounted for decrease in serum cholesterol levels as well as the pleiotropic effects, a fact that has drawn the attention of the medical community [19-21].

The present work aims to study the hypolipidemic action and pleiotropic effects of rosuvastatin on the heart of mice fed on a high fat diet, such as tissue remodeling and ultrastructural mitochondrial changes.

Materials and Methods

Animals and diet

The studies were performed according to the guidelines of the animal ethics committee of the State University of Rio de Janeiro (Rio de Janeiro, Brazil). All of the procedures were performed in accordance with the conventional guidelines for experimentation with animals (NIH publication no. 85 – 23, revised 1996), and the experimental protocols were approved by the Local Committee of Use and Care of Experimental Animals of the State University of Rio de Janeiro, Rio de Janeiro, Brazil (protocol number CEAC/256/2008).

Male C57Bl/6 mice were maintained under controlled conditions with free access to food and water until they were 12 weeks old. Then, the mice were randomly assigned to receive one of the following diets for 8 weeks: a standard diet, a normolipidic diet (10 g of lipid/100 g diet), or a high fat diet being an important tool in the treatment and/or on the prevention of heart disease.
very high-fat diet (60 g of lipid/100 g diet), both diets are detailed in Table 1. The mineral and vitamin content in the 2 diets were identical and in accordance with the American Institute of Nutrition’s recommendation (AIN 93G). Diets were produced by PRAGSOLUÇÕES Biociências (Sao Paulo, Brazil). After this period, drug treatment began, and 5 groups of study were formed. One group received only the standard chow during the entire experiment (C group; n=5), whereas the group that received the very-high-fat diet (HF group; n=35) was divided into 4 subgroups. These experimental subgroups were as follows: HF group (untreated group), HF-R10 group (high-fat plus rosuvastatin 10 mg), HF-R20 group (high-fat plus rosuvastatin 20 mg), and HF-R40 (high-fat plus rosuvastatin 40 mg). The dose of 20 mg/kg/day of rosuvastatin was used in the present study is known to produce beneficial effects upon endothelial dysfunction and systemic and regional hemodynamic and has been used previously [22,23]. The doses of 10 mg/kg/day and 40 mg/kg/day of rosuvastatin have to aim to examine whether the beneficial effects of rosuvastatin are dose-dependent. Body Mass (BM) was measured every week during the experiment from age 12 weeks to 25 weeks. Systolic blood pressure was measured in conscious mice (tail-cuff plethysmography, Letica LE 5100, Panlab, Barcelona, Spain) at the beginning and end of the experiment.

On the day before sacrifice, animals were starved overnight (from 1 to 7 A. M.). The animals were deeply anesthetized (intraperitoneal sodium pentobarbital, 150 mg/kg), the thorax was opened, and blood samples were rapidly obtained by cardiac puncture. The heart was removed, and the ventricle was dissected and weighed. Ventricle fragments were obtained by randomly cutting and dividing into groups for analysis by light microscopy, transmission electron microscopy and western blot.

### Analytical assays

After blood collection, the plasma was separated from the blood by centrifuging at room temperature. The blood was centrifuged (120 g for 15 minutes) and stored at -80°C until analysis. The total cholesterol and triglyceride levels were measured by a kinetic-colorimetric assay (Bioclin System II; Quibasa, Bioclin, Belo Horizonte, MG, Brazil).

### Light microscopy

Some ventricular fragments were put into the fixative for 48 h (freshly prepared 4% (w/v) formaldehyde in 0.1 M phosphate buffer pH 7.2. After embedding in Paraplast Plus (Sigma–Aldrich, St. Louis, MO, USA), the blocks were sectioned and stained picrosirius red stains.

### Electron microscopy

The hearts were carefully removed, sectioned and fixed in 2.5% glutaraldehyde (Riedel-deHaen, Germany) in 0.1 M cacodylate buffer (pH 7.2). After fixation (at least 12 hours), the samples were rinsed three times (15 min each) in 0.1 M imidazole buffer (pH 7.5) and post-fixed in 2% osmium tetroxide (Sigma-Aldrich Louis, USA) in imidazole buffer for 30 minutes [24], then rinsed three times (15 min each) in 0.1 M imidazole buffer. After rinsing, the samples were dehydrated through a graded series of acetone (30%, 50%, 70%, 90% and twice in 100%) and then were embedded in Epon (Embed-812). Semithin sections (1 μm) were cut, stained with toluidine blue (Vetec, Rio de Janeiro, Brazil), and observed with a light microscope (Olympus BX 53F, Tokyo, Japan). Ultrathin sections (70 nm) were obtained with an ultramicrotome (Leica Ultracet-UC/UT, Leica Mikrosysteme GmbH, Austria), counterstained with uranyl acetate and lead citrate, and examined with a Zeiss EM 906 transmission electron microscope (Carl Zeiss, OberKœnig, Germany) at 80 kV.

### Stereology

#### Light microscopy

The obtained images were analyzed with a test-system composed of 36 test-points (PT) in a frame with a known area (AT). The following parameters were analyzed: volume density, \( V_s \), \( \psi \), \( \psi_1 \), \( \psi_2 \), \( \psi_3 \); total surface area, \( A \); number of profiles; and cross-sectional area, \( A’ \). The structures estimated were cardiomyocytes (cmy) and cardiac interstitium (focusing on the capillary and Connective Tissue (CT)) of the myocardium [25].

#### Electron microscopy

Three grids per animal were obtained for stereological analysis, and at least five sections per grid were analyzed. Three different fields were assayed in each section, totaling 45 images analyzed per animal. The same stereological parameters used in light microscopy were analyzed in electron microscopy. The structures estimated were lipid droplets, mitochondria and degenerated mitochondria.

### Western blot

The total heart protein was extracted in a homogenizing buffer with protease inhibitors (150 mM sodium chloride, 1.0% NP-40, 50 mM Tris pH 8.0, 1 μg/ml protease inhibitor cocktail, Sigma-Aldrich, MO, USA). The heart protein content was detected according to the previously described method. Then, the homogenates were centrifuged twice for 15 min (8640×g) at 4°C, and the infranatants were collected. Equal quantities of total protein were suspended in SDS-containing sample buffer, heated for 5 min at 100°C and separated by SDS/PAGE. After electrophoresis, aliquots (15 μg) of the proteins were transferred onto PVDF membranes (Hybond-P; GE Healthcare). The membranes were then blocked by incubation in 5% (w/v) bovine serum albumin in TBS-T [Tris-buffered saline [20 mmol/L Tris/HCl (pH 7.4) and 500 mmol/L NaCl]] and incubated with polyclonal antibodies against goat UCP-2 (75 kDa) (SC-6527; Santa Cruz Biotechnology), β-actin (42 kDa) (SC-130301; Santa Cruz Biotechnology). The UCP-2 values were normalized to the numbers of viable mitochondria.

### Data analysis

Data are shown as mean and standard error of the mean. In the

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**Table 1:** Composition and energy content of the control and high fat diet / cal = 4184 J.

| Content (g/kg) | Diet |
|---------------|------|
|               | C    | HF  |
| Casein (≥ 85% of protein) | 140.0 | 190.0 |
| Cornstarch (g/kg) | 620.7 | 250.7 |
| Sucrose (g/kg) | 100.0 | 100.0 |
| Soybean oil (g/kg) | 40.0 | 40.0 |
| Lard (g/kg) | 250.0 | 320.0 |
| Fibre (g/kg) | 50.0 | 50.0 |
| Vitamin mix (g/kg)* | 10.0 | 10.0 |
| Mineral mix (g/kg)* | 35.0 | 35.0 |
| L-cystine (g/kg) | 1.8 | 1.8 |
| Choline (g/kg) | 2.5 | 2.5 |
| Antioxidant (g/kg) | 0.008 | 0.008 |
| Total mass (g) | 1.000 | 1.000 |
| Energy content (Kcal/kg) | 3.573 | 5.404 |
| Carbohydrates (%) | 76 | 26 |
| Protein (%) | 14 | 14 |
| Lipids (%) | 10 | 60 |

**Citation:** Rocha VN, Ferreira RN, Mandarim-de-Lacerda CA, de Carvalho JJ (2014) Beneficial Effects of Rosuvastatin in Heart of C57Bl/6 Mice with Diet-Induced Metabolic Syndrome - A Preliminary Study. Endocrinol Metab Synd 3: 121. doi:10.4172/2161-1017.1000121
cases in which we could confirm homoscedasticity of the variances, comparisons among groups were made by 1-way analysis of variance followed by Tukey post-hoc test, and \( P \leq 0.05 \) was considered to be statistically significant. All of the analyses were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, California).

**Results**

**Analytical assays**

A high-fat diet resulted in the development of hypercholesterolemia and an increase in triglyceride levels in the HF group when compared with the C group (Table 2) \( P \leq 0.05 \). Treatment with rosuvastatin at any dose reduced the cholesterol levels when compared with matched untreated HF group (Table 2) \( P \leq 0.05 \) and was not statistically different from the control group. Rosuvastatin also decreased levels of triglycerides, but only the 20 and 40 mg doses were effective at matching the levels observed in the control group (Table 2) \( P \leq 0.05 \). Treatment with 40 mg of rosuvastatin was the most effective at inducing hypolipidemic effects and displayed the best lipid profile among all of the treated groups.

**Blood pressure, body mass and ventricular mass**

At the end of the experiment, the HF group showed a significant increase in blood pressure when compared with the C group (17% higher). At the same time, the groups treated with rosuvastatin, HF-R10, R20-HF, and HF-R40, showed a significant reduction in blood pressure when compared with the HF group (\( p \leq 0.05 \)) and reached values similar to group C (Table 2).

The body mass was higher in the HF group (30% higher than the C group) and statistically different from the C group (\( p \leq 0.05 \)). The HF groups treated with rosuvastatin had a reduction in body mass, whereas the HF-R10 and HF-R20 groups were significantly different from the HF group (\( p \leq 0.05 \)), but did not reach the normal values; only the HF-R40 group was not different from the C group (Table 2).

The ventricular mass of the C group was 0.0538 ± 0.0022 g. The HF group showed a significant increase (more than 20%, \( p \leq 0.05 \)) in the ventricular mass (0.0648 ± 0.0017 g). The groups treated with different doses of rosuvastatin showed a significant reduction of these values compared with the HF group and were not significantly different from the control group (Table 2). The ventricular mass values were normalized to the length of the tibia.

**Ultrastructural analysis**

An ultrastructural morphological analysis showed mitochondrial degeneration in the cardiomyocytes of the high-fat group, specifically in the destruction of the crests and mitochondrial membranes. Myelin figures were often observed, which are characteristic of degenerating organelles. The groups treated with statins showed a preservation of mitochondrial morphology, which resembled the control group (Figure 1).

**Table 2: Blood pressure, body and heart mass and lipid pr ofile.**

| Parameter     | Groups          | C               | HF               | HF-R10             | HF-R20             | HF-R40             |
|---------------|-----------------|-----------------|------------------|--------------------|--------------------|--------------------|
| Body mass     |                 | 26.9 ± 0.9      | 29.1 ± 0.7       | 27.9 ± 0.6         | 28.4 ± 0.6         | 28.2 ± 0.7         |
| Blood pressure|                 | 125.7 ± 2.6     | 113.0 ± 6.0      | 126.3 ± 1.9        | 118.9 ± 5.4        | 118.0 ± 2.0        |
| Heart mass    |                 | 29.6 ± 0.9      | 39.2 ± 0.6\(^a\) | 36.1 ± 0.7\(^a\)   | 33.9 ± 0.8\(^a\)   | 30.5 ± 0.7\(^a\)   |
| Blood pressure (mmHg) |           | 118.7 ± 6.9     | 148.0 ± 5.0\(^a\) | 126 ± 1.3         | 107.6 ± 5.5        | 120.0 ± 3.0        |
| Blood pressure (mg/dL) |       | 0.070 ± 0.004   | 0.098 ± 0.004\(^a\) | 0.071 ± 0.003     | 0.076 ± 0.001     | 0.077 ± 0.001     |
| Ventricles (mg/dL) |           | 0.054 ± 0.002   | 0.070 ± 0.002\(^a\) | 0.052 ± 0.002     | 0.055 ± 0.001     | 0.057 ± 0.001     |
| TC (mg/dL)    |                 | 103.6 ± 18.1    | 226.2 ± 16.0\(^a\) | 163.0 ± 16.1\(^b\) | 128.8 ± 12.3\(^b\) | 114.6 ± 7.8\(^b\)  |
| Serum TG (mg/dL) |           | 51.0 ± 4.6      | 87.1 ± 4.8\(^a\) | 82.0 ± 7.8\(^a\)   | 43.6 ± 8.4\(^a\)   | 50.0 ± 2.2\(^a\)   |

**Table 3: Stereological analysis.**

| Parameters          | C          |
|---------------------|------------|
|                     | HF-R10     |
| Light Microscopy    | HF-R20     |
| Light Microscopy    | HF-R40     |
| Q\(_c\) (cmy) (3mm\(^2\)) | 1407.42 ± 37.3 |
| V\(_c\) [%]         | 0.900 ± 0.011 |
| A (cmy) (µm\(^2\))  | 320.620 ± 7.2 |
| V\(_c\) [%]         | 0.065 ± 0.008 |
| Eletro Microscopy   |            |
| Q\(_c\) (cmy) (3mm\(^2\)) | 1242328 ± 55680 |
| V\(_c\) [%]         | 0.3823 ± 0.011 |
| A (cmy) (µm\(^2\))  | 61375 ± 331 |
| V\(_c\) [%]         | 0.049 ± 0.002 |

**Abbreviations:** CG: Control Group; HF: High-Fat Diet; R10: 10 Mg/Kg/Day of Rosuvastatin; R20: 20 Mg/Kg/Day of Rosuvastatin; R40: 40 Mg/Kg/Day of Rosuvastatin; TC: Total Cholesterol; TG: Triglyceride. Symbols represent difference with: [a] C group; [b] HF group; [c] HF-R10 group; [d] HF-R20 group.
Stereology analysis

Light microscopy: The density of the cardiomyocytes (QA [cmy]) in the HF group was significantly lower (approximately 20% less) than that of the control group (p ≤ 0.05). Animals treated with rosuvastatin (at different doses) showed no significant difference compared to the C group but were statistically different from the HF group (p ≤ 0.05). The groups showed no significant difference in the volume density of cardiomyocytes (VV [cmy]); however, when analyzing the sectional area of cardiomyocytes (A [cmy]), the HF group showed a significant increase when compared with the C group (p ≤ 0.05), suggesting hypertrophy of the cardiomyocytes. The groups treated with rosuvastatin were not significantly different from the control group (Table 3).

The volume density of the connective tissue (VV [ct]) analysis showed a significant increase of the connective tissue in the HF group (over 30%) when compared with the C group (p ≤ 0.05), suggesting perivascular and interstitial fibrosis. Treatment with rosuvastatin at different doses was effective at reducing this value and was significantly different from the HF group (p ≤ 0.05) and similar to the C group (Table 3 and Figure 2).

Electron microscopy: The stereological study showed no significant difference between the groups regarding the QA [MIT] parameter. However, the Vv [MIT] value showed an increase of more than 20% in the HF group than in C group. The HF groups treated with rosuvastatin showed a reduction of these values, in which the HF-R20 and HF-R40 groups were not significantly different from the C group. The cross-sectional area of mitochondria (A [mit]) showed an increase in the HF group compared with controls (p ≤ 0.05), possibly suggesting mitochondrial hypertrophy. Rosuvastin treatment reduced the values in which the HF-R20 and HF-R40 groups were not statistically different from the C group (Table 3).

The QA [MITdeg] value showed an increase of more than 40% in the HF group than in C group. The HF groups treated with rosuvastatin (HF-R10, R20 and HF-HF-R40) showed a reduction of these values and were not significantly different from group C. An analysis of the QA [mitdeg]/QA [MIT] ratios showed an increase of over 45% in the HF group compared with the C group. The groups treated with rosuvastatin showed a reduction of this value, reaching values similar to the control group, and these values were significantly different HF group (Table 3).

The QA [inclip] value was increased in the HF groups including those treated with rosuvastatin (in 3 doses) compared with the C group, and no significant difference was observed between the HF group and the groups treated with rosuvastatin (Figure 3 and Table 3).

Western blot analysis

The expression levels of UCP2 in the hearts from the group HF was 70% higher than those observed in the hearts of the C group (p ≤ 0.05); these levels were lower in the HF animals treated with different doses of statins, and no statistical difference was observed between the HF-R10, HF-R20, HF-R40 groups and the C group (Figure 4).

Discussion

In the present study, we report that mice that were fed a high-fat diet were overweight and had dyslipidemia and increased blood pressure. These conditions contribute to the development of metabolic syndrome, which is a precursor to the development of cardiovascular diseases. Beneficial effects of rosuvastatin administration were
Figure 2: Photomicrographs of the myocardium. (a) C group, (b) HF group showing increase in perivascular (arrowhead) and interstitial (arrow) connective tissue. Groups (c) HF-R10, (d) HF-R20 and (e) HF-R40. All these groups presented reduction of perivascular connective tissue (arrowhead) and interstitial (arrow). Stain: Picro sirius red.

Figure 3: Electron micrographs of the mice cardiomyocyte showing lipid inclusion (arrows). (a) C group; (b) HF group; (c) HF-ROS10 group; (d) HF-ROS20 group; (e) HF-ROS40 group.

Figure 4: Heart expression of UCP-2. Upper panels, representative Western blots with bands corresponding to the groups. Lower panel, quantification of the protein expression standardized to β-actin expression and normalized to the numbers of viable mitochondria. Symbols represent difference with: [a] C group; [b] HF group; [c] HF-R10 group; [d] HF-R20 group.
observed: reduced gains in body mass improved circulating levels of plasma total cholesterol and triglycerides and reduced blood pressure. Furthermore, rosuvastatin improved cardiac remodeling and reduced mitochondrial degeneration in cardiomyocytes.

Our studies showed that rosuvastatin treatment reduces weight gain and improves the blood pressures of the animals that are fed a high-fat diet. This finding is consistent with other studies that report results that are similar to the effects of statins in experimental models [23,26].

When analyzed with a stereological method, the hearts from the high fat group showed a decrease in the number of cardiomyocytes and an increase in the cardiomyocyte volume, suggesting cardiomyocyte hypertrophy. This phenomenon was later proven by an increase in the sectional area of the cardiomyocytes and may explain the increase in the cardiomyocyte mass. The group treated with rosuvastatin showed neither hypertrophy of the cardiomyocytes nor ventricular hypertrophy, demonstrating that the drug treatment had a cardioprotective effect. Another important finding of our study was the increased amount of connective tissue in the hearts of animals fed a high fat diet, which caused perivascular and interstitial fibrosis. This finding is likely related to the loss of cardiomyocytes and replacement by connective tissue. The animals on a high fat diet and treated with rosuvastatin had less connective tissue when compared with the high fat group, showing the effectiveness of the treatment in preventing cardiac fibrosis.

The literature reports cardiac remodeling in animals with metabolic syndrome, showing cardiomyocyte loss, cardiomyocyte hypertrophy and increased connective tissue, which may generate perivascular and interstitial fibrosis [27-31]. Consistent with our results, treatment with different classes of statins show beneficial effects in cardiac remodeling [32-34].

When the ultrastructure of the heart was analyzed, we observed an accumulation of large lipid inclusions in animals fed a high fat diet, regardless of treatment with different doses of rosuvastatin; this result was later confirmed by stereology. This finding showed that the treatment was not effective at reducing lipid capitation by cardiomyocytes, which may be explained by an increase in receptor triglycerides and fatty acids (CD36) in the membranes of cardiomyocytes in obese animals [4,7,35,36].

The animals fed a high fat diet showed also numerous defects in the mitochondria: a disruption of the inner membrane and a thickest outer membrane; distortion, disruption and loss of the cristae; and lamellar degeneration and vacuolization of the mitochondria. Supakul et al. studied the cardiac mitochondria in obese rats and observed similar results [8]. The animals of the high fat groups treated with different doses of statins had normal mitochondria, showing that rosuvastatin had a mitochondrial protective effect. After a stereological analysis, we found no differences in the number of mitochondria between the groups, but the mitochondrial volume was increased in the high fat group, indicating mitochondrial hypertrophy. These data are confirmed by increased in the cross-sectional area of mitochondria (A [mit]) in the high fat group when compared with other groups. These findings were also observed in recent studies that used a model of metabolic syndrome [37]. The number of degenerated mitochondria was increased in the high fat group when compared with other groups, data confirmed by stereological analyses of the QA[mitdej], supporting our hypothesis of mitochondrial hypertrophy with a compensatory change.

It has been reported that very high free fatty acids concentrations induce the uncoupling of heart muscle mitochondria, thus decreasing the respiration-generated membrane potential [38]. Uncoupling proteins (UCP2) is found in the inner mitochondrial membrane and is upregulated in pathological states such as heart failure. UCP2 is thought to protect cardiomyocytes against oxidative stress by dissipating the mitochondrial proton gradient and membrane potential, thereby reducing the generation of mitochondrial reactive oxygen species [39,40].

Our results showed high levels of UCP 2 in the hearts from animals in the high fat group, which is evidence of a pathological state. The increased oxidative stress produces mitochondrial damage, which leads to further production of reactive oxygen species (ROS) and creates a vicious cycle of oxidative stress and energetic decline [41]. In the control group and high fat groups treated with rosuvastatin, we observed lower levels of UCP2, which showed a lower activation of UCP2. This fact can be explained by the antioxidant properties of the statins and its induction power of mitochondrial biogenesis [13,21,42-44].

We have shown the beneficial effects of rosuvastatin administration in correcting the lipid profile and regulating blood pressure. We have also shown the beneficial effects of rosuvastatin in the heart of the animals subjected to a high fat diet. Furthermore, we showed that beneficial effects of rosuvastatin on cardiac changes, preserving the mitochondrial morphology and thus maintaining the cardiomyocyte integrity. Thus, our work concludes that rosuvastatin has a beneficial effect on the heart of C57BL/6 mice fed a high fat diet and that further research should be conducted to better understand the mechanisms responsible for its benefits.

Acknowledgements

The authors would like to thank Ana Lucia R. Nascimento and Alan C. N. de Moraes for their technical assistance. This work was sponsored by the Brazilian agencies CAPES, CNPq, and FAPERJ.

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