Original Article

Effect of Pitavastatin on Vascular Reactivity in Hypercholesterolemic Rabbits

Eros Antonio de Almeida and Michiko Regina Ozaki
Universidade Estadual de Campinas, Campinas, SP - Brazil

Abstract

Background: **Pitavastatin is the newest statin available in Brazil and likely the one with fewer side effects. Thus, pitavastatin was evaluated in hypercholesterolemic rabbits in relation to its action on vascular reactivity.**

Objective: **To assess the lowest dose of pitavastatin necessary to reduce plasma lipids, cholesterol and tissue lipid peroxidation, as well as endothelial function in hypercholesterolemic rabbits.**

Methods: **Thirty rabbits divided into six groups (n = 5): G1 - standard chow diet; G2 - hypercholesterolemic diet for 30 days; G3 - hypercholesterolemic diet and after the 16th day, diet supplemented with pitavastatin (0.1 mg); G4 - hypercholesterolemic diet supplemented with pitavastatin (0.25 mg); G5 - hypercholesterolemic diet supplemented with pitavastatin (0.5 mg); G6 - hypercholesterolemic diet supplemented with pitavastatin (1.0 mg). After 30 days, total cholesterol, HDL, triglycerides, glucose, creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT) were measured and LDL was calculated. In-depth anesthesia was performed with sodium thiopental and aortic segments were removed to study endothelial function, cholesterol and tissue lipid peroxidation. The significance level for statistical tests was 5%.**

Results: **Total cholesterol and LDL were significantly elevated in relation to G1. HDL was significantly reduced in G4, G5 and G6 when compared to G2. Triglycerides, CK, AST, ALT, cholesterol and tissue lipid peroxidation showed no statistical difference between G2 and G3-G6. Significantly endothelial dysfunction reversion was observed in G5 and G6 when compared to G2.**

Conclusion: **Pitavastatin starting at a 0.5 mg dose was effective in reverting endothelial dysfunction in hypercholesterolemic rabbits. (Arq Bras Cardiol. 2014; 103(1):4-12)**

Keywords: **Hydroxymethylglutaryl - CoA Reductase Inhibitors; Endothelial Dysfunction; Rabbits; Hypercholesterolemia.**

Introduction

Inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA), the statins, are potent inhibitors of cholesterol biosynthesis in the liver by blocking the conversion to mevalonate[1]. Clinical studies with simvastatin (4S) and pravastatin (WOSCOPS) demonstrated that statins act by decreasing the concentration of cholesterol in the blood, decreasing the incidence of myocardial infarction and its mortality[2,3].

Aiming at correlating decreases in mortality with the atherosclerotic plaque size, studies were carried out with different commercial products of statins. Aware of changes in lipid profile, the studies MARS[4] and REGRESS[5] showed that there were stabilization and regression of atherosclerosis as assessed by coronary angiography.

Mailing Address: Eros Antonio de Almeida •
Rua Professor Jorge Nogueira Ferraz, XX Jardim Chapadão.
Postal Code 13070-120, Campinas, SP – Brazil
E-mail: erosaa@cardiol.br; eros@fcm.unicamp.br
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Ribeiro Jorge et al[6] in 1994 suggested that statins might have an antioxidant action when they observed that hypercholesterolemic rabbits showed improvement of endothelial dysfunction that was disproportionate to lipid reduction, when treated with pravastatin. This observation was once again seen in 1997[7], when the rapid reversal of hypercholesterolemia with statins was studied in the same animal model. These actions, in addition to lowering cholesterol known as pleiotropic effects, refer to endothelial function protection, anti-inflammatory and anti-thrombotic action and stabilization of atherosclerotic plaque, among others[8-14].

Pitavastatin is the latest available statin in the market, also known as nisvastatin and itavastatin. It was developed in 2003 in Japan, and approved in 2009 by the U.S. Food and Drug Administration of the United States of America, being the seventh statin to be developed and commercialized[15,16].

It is a synthetic and lipophilic statin, of which pharmacokinetics and pharmacodynamics have distinct properties compared with other statins and can offer greater pleiotropic effects in relation to endothelial function, inflammation, oxidative stress and antithrombosis. It is minimally metabolized in the liver and primarily metabolized by enzymes CYP2C9 and CYP2C8, showing bioavailability of 80% of the administered dose[17,18]. The low affinity of

pitavastatin to CYP3A4 reduces interactions with other drugs metabolized by this enzyme and can decrease toxic manifestations[19-21].

To date, no clinical or experimental study on the pitavastatin was found in the Brazilian literature and thus, the aim of this study was to verify the action of this statin, particularly in decreasing endothelial dysfunction in experimental hypercholesterolemia, as well as defining its lowest effective dose for this purpose.

Methods

The experimental protocol was approved by the Ethics Committee for Animal Experimentation (EAEC)-IB-UNICAMP, under n. 2528-1.

The animals were fed 100g a day of standard chow with the following composition (g/100): Proteins, 16.00; Carbohydrates, 45.00; Fibers, 20.00; Fat, 5.00 and Ash, 14.00. We studied 30 New Zealand male rabbits initially weighing from 2.0 to 2.5 kg, which were divided into six groups. In the group considered as control in relation to hypercholesterolemic ones (G1), the rabbits were sacrificed after one month of standard diet and adaptation to the biothery. The other 25 rabbits received a hypercholesterolemic diet, containing standard chow supplemented with 0.5% cholesterol and 10% coconut oil. With the exception of the hypercholesterolemic control group (G2), the other groups were treated with pitavastatin (Kowa Company, Nagoya, Japan) during the last 15 days, by gavage, at doses of 0.1 mg/animal/day (G3), 0 25 mg/animal/day (G4), 0.5 mg/animal/day (G5) and 1 mg/animal/day (G6).

Biochemical analysis

Total plasma cholesterol, HDL-cholesterol (high-density lipoproteins), triglycerides, glucose, AST (aspartate aminotransferase), ALT (alanine aminotransferase) and creatine kinase were measured using enzymatic kits (Laborclin, Bioliquid, Pinhais, PR, Brazil), and the reading was performed by spectrophotometry (Thermo Spectronic, Genesys 10 uv, Rochester, NY, USA) with a wavelength of 500 nm. LDL was calculated using Friedewald formula.

Tissue Cholesterol

At the end of the experiment, the animals were sacrificed and the thoracic aorta was removed. Tissue cholesterol was measured in segments according to the method of Naito and David[22]. In brief, the specimens were dried and homogenized at 4° C in 5 mL of Tris HCl buffer, pH 7.4, plus 0.01 $NaNO_3$. Total lipids were extracted and homogenized in 10 vol of chloroform-methanol. The extracted total cholesterol was measured by enzymatic kits.

Tissue lipid peroxidation

One segment of the thoracic aorta was homogenized with trichloroacetic acid (1 g tissue + 10 vol 20% TCA). After centrifugation, 0.67% thiobarbituric acid volume was added and the mixture was heated at 100 ° C for 20 minutes. The concentration of malondialdehyde was calculated from the absorbance of 532 nm using extinction coefficient of 1.49 x 10-5 expressed as nmoL/mg tissue x 10^{-7} [23].

Endothelial function

Endothelial function was measured in the thoracic aorta segments of approximately 5 mm, with intact endothelium, suspended in a recipient with a capacity of 10 mL in Krebs-Henseleit solution at 37° and pH 7.4 and heated to 37º C. The solution was continuously aerated with a carbogen mixture containing 95% oxygen and 5% carbonic gas. The segments were mounted on two metal hooks attached to a support in the container and the force transducer (Narco, 40 Narcotrace, Texas, USA). Then they were left to equilibrate for 60 minutes with replacement of the Krebs Henseleit solution every 20 minutes. The segments were stretched to a basal tension of 1 g. All segments of the aorta were contracted with NE (10^{-7}M) and, after stabilization, ACh was added cumulatively (10^{-8} to 10^{-5} M)[6,24] and relaxation was verified.

Statistical Analysis

The SAS for Windows (Statistical Analysis System) software, version 9.2 (SAS Institute Inc., 2002-2008, Cary, NC, USA) was used in the statistical analysis.

ANOVA with rank transformation was used to compare treatment groups through the collected variables, followed by Tukey test, to locate the differences. When comparing endothelial function to locate the differences in concentrations between the groups, the contrast profile test was used. The significance level for statistical tests was 5%.

Results

The results of means and standard deviations of the different parameters studied are shown in Table 1.

Figure 1, depicting total cholesterol in the end of the experiment, showed that there was a decrease in total cholesterol in groups G5 (25.8% reduction) and G6 (25.7%), where the rabbits were treated with 0.5 and 1.0 mg of pitavastatin, when compared to the hypercholesterolemic group G2, with statistically significant difference.

Figure 2, depicting LDL, showed that there was a decrease in G5 (20.07%) and G6 (26.62%), with no statistically significant difference when compared to G2.

Figure 3, depicting HDL, showed that a decrease occurred in G3 (40.88%), G5 (56.68%) and G6 (56.53%) when compared to G2, with a statistically significant difference.

Figure 4, depicting triglycerides, showed that a decrease occurred in G3 (44.62%), G4 (33.53%), G5 (52.05%) and G6 (45.56%), but with no statistically significant difference when compared to G2.

Figure 5, depicting tissue cholesterol, showed a decrease in groups G5 (28.2%) and G6 (20.09%), but with no statistically significant difference when compared to G2.

Figure 6, depicting lipid peroxidation, showed a decrease in G5 (26.25%) and G6 (31.25%), with no statistically significant difference compared to G2.

Table 1 – Results of groups G1 to G6 with means and standard deviations

	G1	G2	G3	G4	G5	G6
Col (mg/dL)	63.6 ± 4.3	753.9 ± 32.0	650.5 ± 212.5	705.3 ± 164.0	559.6 ± 203.6	527.6 ± 100.9
HDL (mg/dL)	16.5 ± 3.7	61.4 ± 8.8	*36.3 ± 15.0	*25.2 ± 10.9	*26.6 ± 12.5	*26.7 ± 5.7
LDL (mg/dL)	26.6 ± 3.9	650.0 ± 33.3	599.7 ± 196.3	663.0 ± 161.1	519.6 ± 202.3	477.7 ± 103.4
Trig (mg/dL)	104.2 ± 16.2	212.5 ± 99.9	117.7 ± 32.9	141.2 ± 36.5	101.9 ± 22.5	115.7 ± 15.0
Gli (mg/dL)	115.8 ± 19.2	127.3 ± 28.9	114.6 ± 25.5	123.5 ± 21.6	119.8 ± 23.7	94.7 ± 26.2
Col tec (mg/g)	21.6 ± 4.9	28.7 ± 4.8	22.8 ± 2.0	29.1 ± 7.8	20.4 ± 5.6	22.7 ± 4.5
Perox (ng/mg de prot)	5.1 ± 0.6	8.0 ± 1.9	5.7 ± 1.6	5.3 ± 1.4	5.9 ± 1.2	5.5 ± 1.4
Rel Máx (%)	93.2 ± 6.7	60.2 ± 12.64	62.3 ± 12.1	61.3 ± 11.7	*80.40 ± 5.1	*79.8 ± 12.0
Cknac (U/l)	236.1 ± 79.9	354.0 ± 62.3	243.8 ± 89.0	200.5 ± 88.7	336.1 ± 135.2	298.3 ± 118.6
AST (U/l)	35.7 ± 15.4	25.9 ± 8.3	50.0 ± 24.5	22.7 ± 8.3	30.0 ± 9.1	34.7 ± 10.8
ALT (U/l)	20.3 ± 11.2	25.1 ± 6.7	37.2 ± 18.6	36.1 ± 13.8	*18.3 ± 6.1	30.1 ± 6.8

*Col: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: triglycerides; Glu: glucose; Tissue Col: tissue cholesterol; Perox: peroxidation tissue (ng / mg protein); Rel Max (%): endothelial function; Cknac: creatine phosphokinase; AST: aspartate aminotransferase; ALT: alanine aminotransferase. * p < 0.05 in relation to G2.*

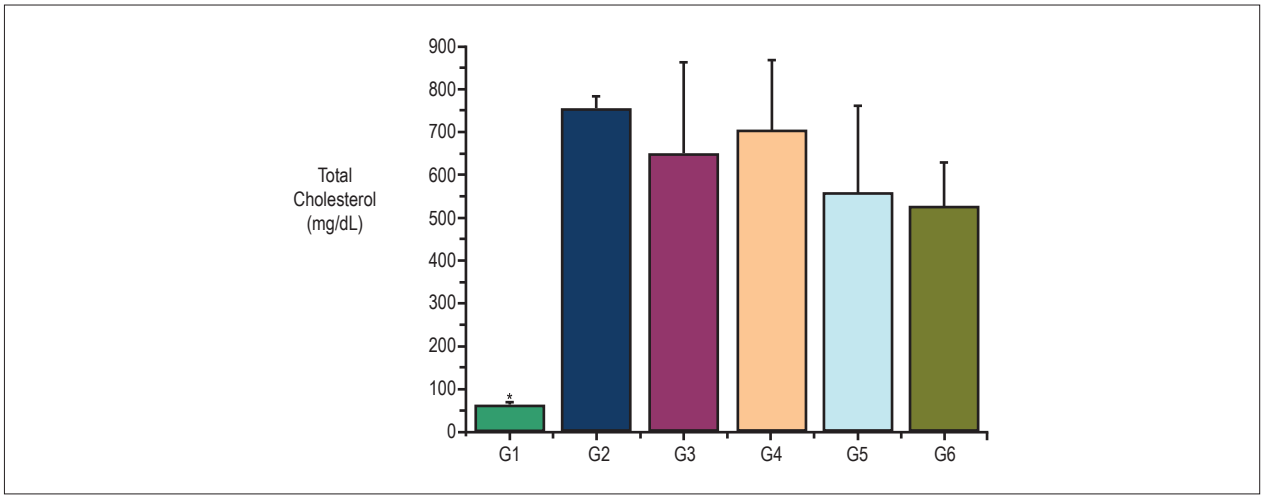

Figure 1 – *Total cholesterol in all groups expressed as mean and standard deviation. * p < 0.05 in relation to G2.*

Figure 7 shows an improvement in endothelial function in G5 and G6 in relation to group G2, which was statistically significant.

Regarding glucose, creatine kinase, AST and ALT, there were no statistically significant alterations between the groups (Table 1).

Discussion

Several different statins are available in the pharmaceutical market, acting through the inhibition of 3-Hydroxy-3-Methylglutaryl Coenzyme A (HMG-CoA) reductase, which makes them members of a group of a specific class of drugs, all with the precise indication of hypercholesterolemia reduction. Molecular pharmacokinetic and pharmacodynamic modifications have been performed to differentiate the statins, almost always seeking the more effective blocking of HMGCoA reductase and thus, better control of plasma lipids, but also for the purpose of drug individualization, in addition its generic quality.

Pitavastatin is the newest statin in the market, starting in 2003 in Japan and currently available in Brazil[25]. To the best of our knowledge, this constitutes the first experimental study available in the Brazilian literature, to date, which evaluated pitavastatin action on plasma and tissue lipids, lipid peroxidation and vascular reactivity, seeking to identify the lowest dose at which it can be effective in controlling these parameters. Moreover, clinical studies have not been published in the national literature addressing the different aspects involving pitavastatin.

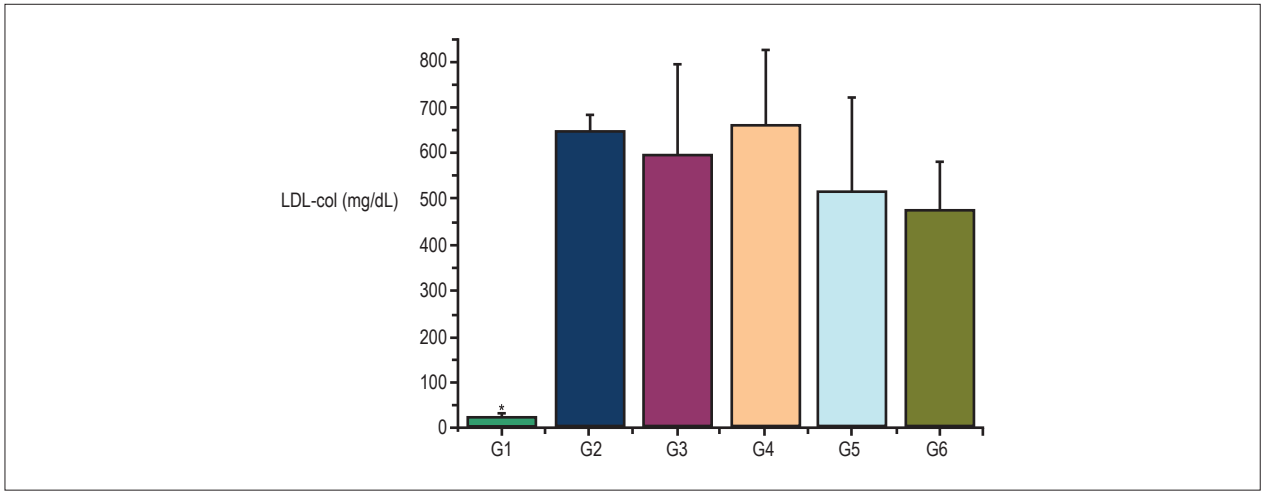

Figure 2 – *LDL-col in all groups expressed as mean and standard deviation.*
** p < 0.05 in relation to G2.*

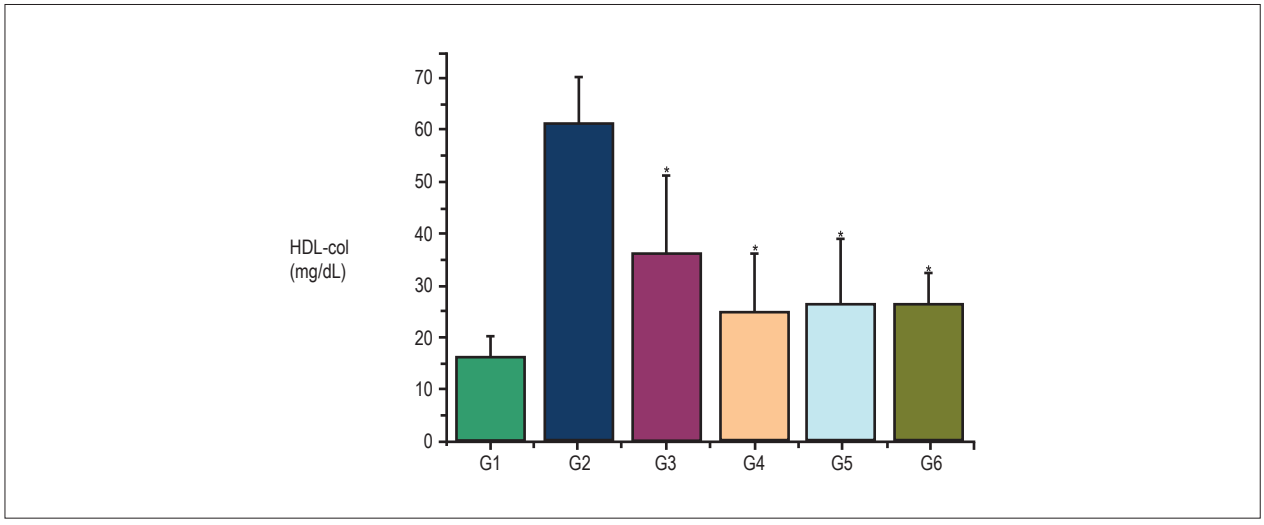

Figure 3 – *HDL-col in all groups expressed as mean and standard deviation.*
** p < 0.05 in relation to G2.*

The addition of fat to the standard chow fed to the rabbits has been the most widely used model for induction of experimental hypercholesterolemia and it was effective in this study. The results shown in Table 1 showed that group G2 showed significantly higher elevations in serum lipids than those in G1, fed the standard chow. The same occurred with the tissue parameters, with elevations in total cholesterol, lipid peroxidation and reduced endothelial function in aortic segments.

The pitavastatin dose proposed for human use ranges from 1 to 2 mg/day, with a maximum dose of 4 mg/day, whereas in experimental studies, a dose < 1 mg/kg/day has been used, with no reports of groups of animals receiving increasingly higher doses, as in the present study[19,26,27]. The pitavastatin doses used in this study, although lower than the lowest used in humans, are high for rabbits, considering the differences between the species, especially weight. However, they are necessary to achieve the effect of cholesterol reduction in these animals, which was observed only with a minimum dose 0.5 mg/animal. Differences in metabolism between species may probably explain why high doses are not toxic or lethal to some of them. Not only were the doses different regarding their action on lipids, but also their time of use. In the present study, only 15 days of drug use were sufficient for the lipid-lowering action of pitavastatin to occur, while other studies used at least 12 weeks[19].

These data are similar to those observed in studies using other statins, when doses are optimized for a same total cholesterol percentage reduction[28]. A clinical study has demonstrated that pitavastatin improves peripheral microvasculature function verified through reactive hyperemia measured by arterial tonometry

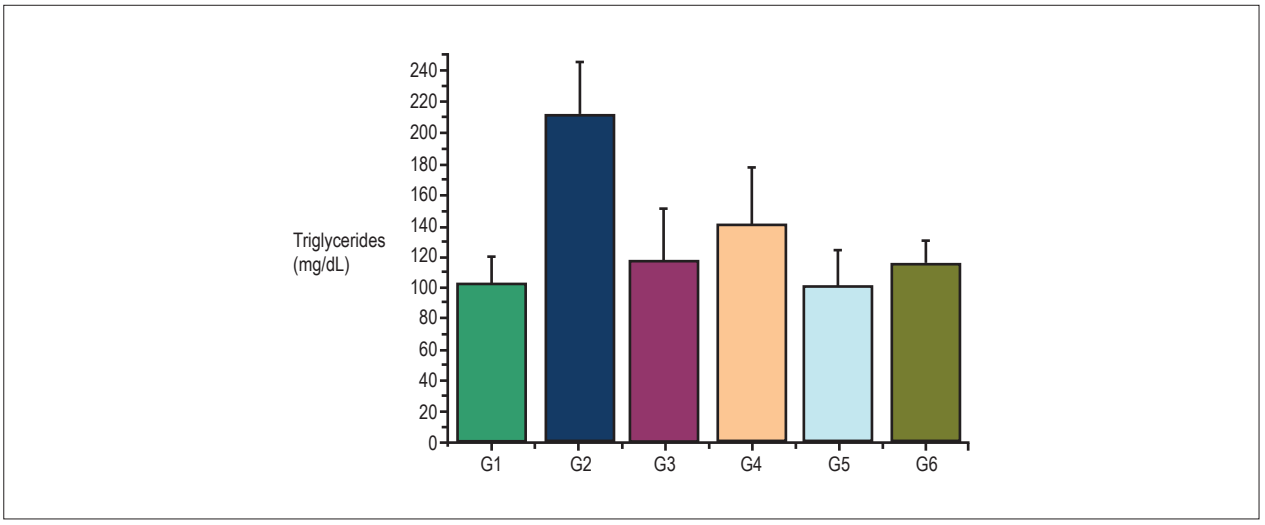

Figure 4 – *Triglycerides in all groups expressed as mean and standard deviation.*

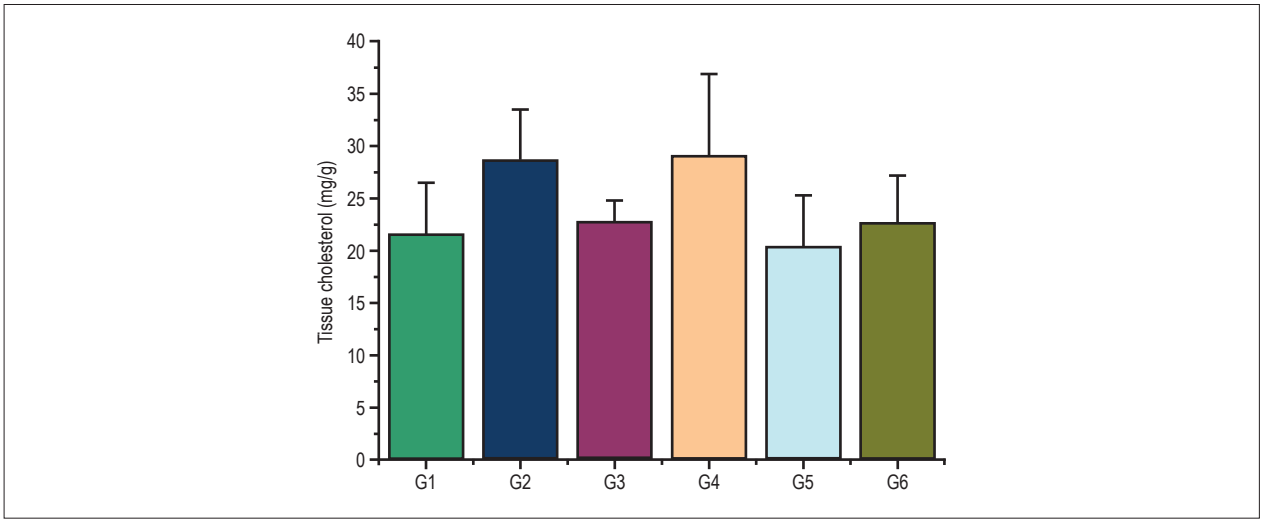

Figure 5 – *Tissue cholesterol in all groups expressed as mean and standard deviation.*

in hypercholesterolemic individuals with coronary artery disease, only two hours after oral administration of the drug, demonstrating the promptness of drug action in improving endothelial function, regardless of its action on plasma cholesterol[29].

The results shown in Table 1 and Figure 7 demonstrated that pitavastatin was effective in improving vascular reactivity, as there was a significant improvement in endothelial dysfunction in the treated groups when compared to the hypercholesterolemic group. However, this effect occurred only with doses ≥ 0.5 mg, meaning that lower doses are unable to exercise the same effect during the time period of the experiment. Similar results in endothelial dysfunction reversal by pitavastatin have been reported in other experimental studies[19] and in humans[29] without great differences from those observed when other statins were assessed[28,30-32].

The improvement in endothelial dysfunction cannot be determined only by LDL reduction, although this reduction has occurred, as shown in Table 1 and Figure 2, because the absolute values still remained much higher than in the G1 group, not hypercholesterolemic. However, the percentage of relaxation was very close to that of G1. Oxidative stress involving LDL in hypercholesterolemia has been held responsible for the endothelial dysfunction observed in these situations and was one of the goals of this study. Even without full control of hypercholesterolemia, one can achieve reversal of endothelial dysfunction by reducing oxidative stress. This effect has been produced by statins and occurs with pitavastatin, as observed in the results, as there was a decrease in tissue lipid peroxidation in relation to G2, in the treated groups.

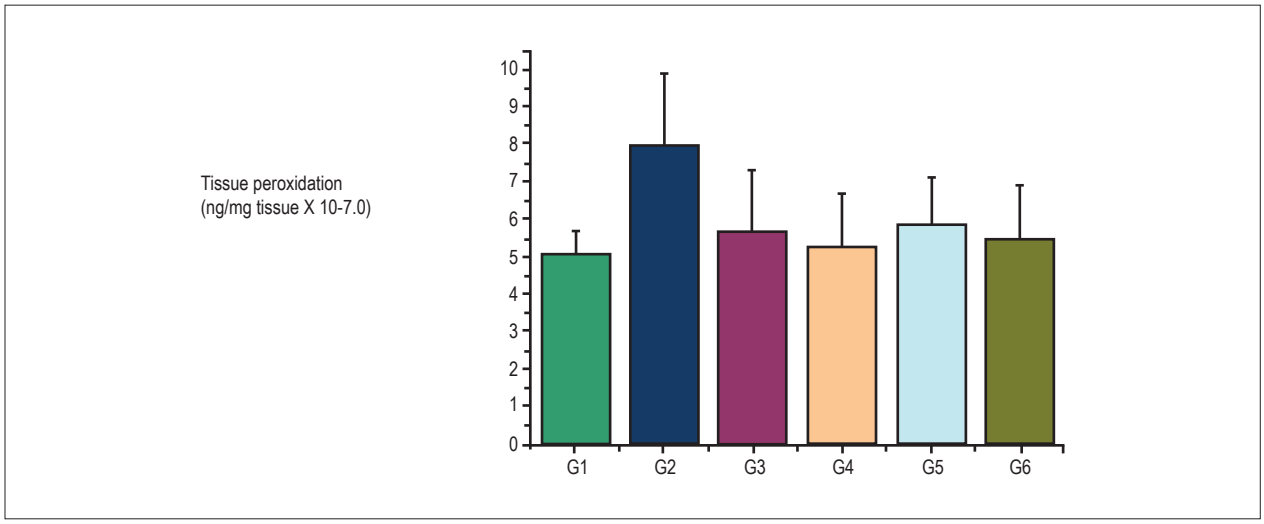

Figure 6 – *Tissue peroxidation in all groups expressed as mean and standard deviation.*

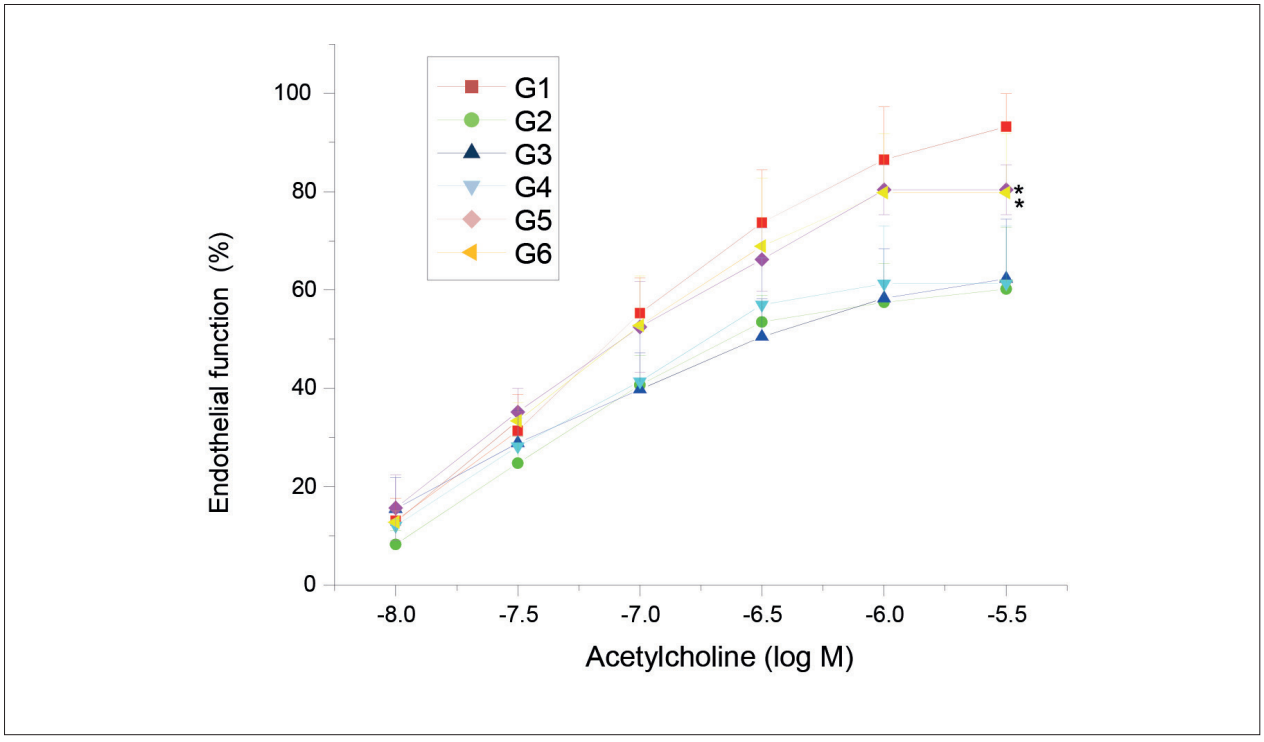

Figure 7 – *Endothelial function in all groups expressed as mean and standard deviation.*
**p < 0.05 in relation to G2.*

Closely related to both situations, an improvement in endothelial dysfunction and decreased lipid oxidation is the reduction in tissue cholesterol, as reported in Table 1 and Figure 5, as well as in previous studies[28,30-32]. However the reduction in tissue cholesterol and lipid peroxidation occur similarly in all treated groups, unlike endothelial dysfunction reversal, which was observed only in the groups where animals received higher doses of pitavastatin (G5 and G6).

A literature study that used the same experimental model[26] aimed to evaluate the effect of pitavastatin and probucol on atherosclerosis progression by studying oxidative stress. For that purpose, superoxide dismutase and the expression of peroxisome proliferator-activated receptors (PPARs) were used as parameters. The authors observed that, at a dose of 0.05 mg / kg / day, pitavastatin was effective in reducing oxidative stress without any action on serum

cholesterol levels. Thus, it appears that pitavastatin acts on other mechanisms, which could explain the results obtained in this study, justifying the reduction in lipid peroxidation, without altering plasma cholesterol levels.

Thus, endothelial dysfunction reversal may depend on other factors in addition to oxidative ones, requiring larger doses of pitavastatin for it to occur.

One of the remarkable features of pitavastatin, observed from the clinical point of view, is its action in increasing HDL, especially in individuals in which it is reduced[33,34]. The main mechanism by which this statin is better than others in increasing HDL levels is the capacity to increase the expression of ApoA-1 gene, through the activation of PPARs[35], the largest intra- and extracellular regulator of fatty acid metabolism, increasing its secretion.

In the present study, there was a reduction of HDL, accompanying a decrease in total cholesterol and LDL levels. Pitavastatin did not determine the increase or prevented the decrease in HDL, as observed in clinical studies. In another experimental study in ovariectomized hypercholesterolemic rabbits[19], the authors found no significant changes in HDL and triglycerides. These findings regarding HDL have been observed with other statins in the literature[30,32], and they were not aimed at specifying the mechanisms by which these animals exhibit such behavior. Differences in lipid metabolism between species that justify such results should exist and should be the objectives of future researches in order to better understand this phenomenon.

The results observed in relation to triglycerides (Table 1 and Figure 4), showing significant decrease in the treated groups, demonstrate the efficacy of pitavastatin in this sense, as observed in other experimental studies[19,27], which would make this statin the choice to treat individuals with hypercholesterolemia, as well as those with hypertriglyceridemia, especially diabetic ones.

The actions of statins, in addition to those dependent on the reduction in LDL and cholesterol, are known in the literature[12,14]. Such effects, known as pleiotropic ones, have been generally beneficial by reducing lipid oxidation and reversing endothelial dysfunction, as demonstrated in the present study, in addition to blocking inflammatory processes, among others, resulting in the interruption of atherosclerosis progression and, consequently, of clinical events. However, lately it has been observed that these pleiotropic effects may also be deleterious to the body, especially in relation to glucose metabolism[35].

Although clinical studies have shown controversial results regarding the adverse events of statins in inducing diabetes as, in the WOSCOPS[3] study, pravastatin prevented diabetes onset, and in the JUPITER study[36], rosuvastatin induced it, experimental evidence consistently demonstrate that statins may impair glucose homeostasis[37]. In the present study, there was no change in blood glucose levels of treated groups when compared to controls.

Although experimental studies involving pitavastatin and adverse events related to glucose metabolism have not been performed, clinical studies comparing it to other statins have shown that the onset of diabetes in users has been significantly lower, especially regarding atorvastatin and rosuvastatin, and comparable to pravastatin[38]. Furthermore, this event has occurred when higher doses of statins were used, perhaps justifying the results of this study, in which lower doses were used. The same result occurred in relation to liver and creatine kinase enzymes, which showed no change in the groups treated with pitavastatin when compared to controls, demonstrating that the doses used were safe (Table 1). This fact has been reported in the literature[39].

Conclusion

Pitavastatin was effective in reducing plasma lipids, lipid peroxidation and tissue cholesterol, reversing endothelial dysfunction in hypercholesterolemic rabbits, starting with a 0.5 mg dose.

Author contributions

Conception and design of the research and Analysis and interpretation of the data: Almeida EA, Ozaki MR; Acquisition of data and Writing of the manuscript: Ozaki MR; Obtaining financing and Critical revision of the manuscript for intellectual content: Almeida EA.

Potential Conflict of Interest

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

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Study Association

This study is not associated with any thesis or dissertation work.

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