Original Article

Effects of Atorvastatin, Fluvastatin, Pravastatin, and Simvastatin on Endothelial Function, Lipid Peroxidation, and Aortic Atherosclerosis in Hypercholesterolemic Rabbits

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Objective

To compare the effects of atorvastatin, fluvastatin, pravastatin, and simvastatin on endothelial function, aortic atherosclerosis, and the content of malondialdehyde (MDA) in native and oxidized LDL and in the arterial wall of hypercholesterolemic rabbits after adjusting the dosages of those statins to reduce total serum cholesterol levels to similar values.

Methods

Male rabbits were divided into the following 6 groups of 10 animals (n=10): 1) GH (control) - hypercholesterolemic animals; 2) GA - atorvastatin; 3) GF - fluvastatin; 4) GP - pravastatin; 5) GS - simvastatin; and 6) GN - normal. The animals were fed a standard food preparation enriched with 0.5% cholesterol and 2% coconut oil for 45 days. Fifteen days after beginning the experiment, atorvastatin, fluvastatin, pravastatin and simvastatin were administered for 15 days through gavage and the dosages were adjusted to obtain similar cholesterol values in each group. At the end of the experiment, a blood sample was withdrawn for determining total cholesterol and separating the lipoproteins, and a segment of the thoracic aorta was removed to be used for studying endothelial function and lipid peroxidation, and for measuring aortic atherosclerosis in histological sections.

Results

The statins significantly reduced total serum cholesterol levels, LDL-cholesterol levels, and aortic atherosclerosis. The MDA content was also significantly reduced in native and oxidized LDL, as well as in the arterial wall. Endothelium-dependent relaxation was significantly greater in the treated group compared with that in the hypercholesterolemic group.

Conclusion

The statins, at dosages adjusted, had a significant and similar effect in reducing lipid peroxidation in native and oxidized LDL-C and in arterial walls, in decreasing aortic atherosclerosis, and in reverting endothelial dysfunction.

Key words

HMG-CoA reductase inhibitors, aortic atherosclerosis, lipid peroxidation

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The HMG-CoA reductase inhibitors effectively reduce mortality and coronary events [1-6], representing a powerful instrument in preventing and controlling atherosclerosis. One of the major actions of these drugs is reducing serum LDL-cholesterol, interfering with the biosynthesis of cholesterol and increasing the number of hepatic receptors for ApoB100 [7].

More recently, additional effects of these drugs have been demonstrated and considered important for stabilizing the atherosclerotic plaque. Such effects, called pleiotropic, refer to endothelial protection, lipid peroxidation reduction, and control of inflammatory reactions and hemostasia [8-11].

Some adverse effects of the statins have been reported and include cephalea, myalgia, pharyngitis, and interaction with other drugs metabolized in cytochrome P-450 [12-14].

Although statins have a similar mechanism of action, they differ in their potency for reducing serum cholesterol, their solubility and metabolism [15,16].

Previous studies [17,18] have assessed the effect of simvastatin and pravastatin on endothelial function, tissue cholesterol, and the number of foam cells in aortic rings of hypercholesterolemic rabbits. Those studies showed that pravastatin was more effective in reversing endothelial dysfunction caused by hypercholesterolemia, in addition to more intensely reducing the number of foam cells in the histological sections obtained from the thoracic aorta. Those findings were carefully interpreted because they derived from an experimental study on animals, and the differences between the effects of those statins encompassed the controversial discussion about the pharmacological difference of the HMG-CoA reductase inhibitors.

The present study aimed at comparing the effects of 4 statins (atorvastatin, fluvastatin, pravastatin, and simvastatin) on endothelial function, lipid peroxidation, and aortic atherosclerosis in hypercholesterolemic rabbits. The dosages of statins were adjusted to reduce total serum cholesterol levels to similar values.

Methods

The experiments were conducted according to the recommendations of the US National Institutes of Health for the care and use of laboratory animals (NIH publication N 85-23 revision).

Sixty male rabbits of the New Zealand breed, aged approximately 12 weeks and weighing from 1.6 to 2.4 kg, were divided into the following 6 groups (n=10): 1) normal group (GN); 2) hypercholesterolemic (GH); 3) atorvastatin (GA); 4) fluvastatin (GF); 5) pravastatin (GP); and 6) simvastatin (GS). The animals were separated in individual cages and fed a standard preparation of Purina brand food, enriched with 0.5% cholesterol and 2% coconut oil for 45 days. They received 100 g of food preparation per day and unlimited water. After 15 days, a blood sample was obtained through cardiac puncture for determining serum cholesterol. Then atorvastatin (10 mg/day), fluvastatin (20 mg/day), pravastatin (20 mg/day), and simvastatin (15 mg/day) were administered to the respective groups. The dosages were based on previous studies [17,18] and the drugs administered once a day through gavage. After 15 days, another blood sample was obtained for determining serum cholesterol levels, and the dosages of the statins were adjusted to obtain similar values of serum cholesterol in each group. The final dosages were 10 mg/day, 15 mg/day, 15 mg/day, and 10 mg/day for atorvastatin, fluvastatin, pravastatin, and simvastatin, respectively.

At the end of the experiment, another blood sample was obtained for determining serum cholesterol levels and for separating the lipoproteins. The animals were euthanized by having their necks severed. Median thoracotomy was then performed, and the aorta was removed to obtain the rings for assessing endothelial function and MDA content, and for measuring aortic atherosclerosis on histological sections. Liver and muscle fragments were also obtained for histological examination.

The thoracic aorta, free from connective tissue, was sectioned into rings of approximately 5 mm, with special care not to damage the endothelium. In some rings, however, the endothelium was mechanically damaged by a small forceps. The aortic rings were suspended in 10 mL of the Krebs-Henseleit solution at pH 7.4 (composition in mmol/L: NaCl, 113; $CaCl_2$, 2.19; $NaHCO_3$, 25.0; $MgSO_4$, 0.44; KH_2PO_4, 1.18; EDTA, 0.03; glucose, 11.0). The solution was maintained at 37°C and continuously aerated with a gas mixture containing 95% O_2 and 5% CO_2. The rings were mounted on 2 metallic supports coupled to a force transducer (Narco Byosystem) and distended at a baseline tension of 1g. The aortic rings were allowed to stabilize for 60 minutes, with Krebs Henseleit solution exchange every 20 minutes. To prevent the synthesis of prostaglandins, the experiments were performed in the presence of 10 μM of indomethacin.

The rings of the thoracic aorta with and without endothelium were contracted with noradrenaline (NA, 10^{-7} M). When the contraction was kept stable, acetylcholine (ACH) was added to the solution in a cumulative form (10^{-8}-$10^{-5.5}$ M) to obtain the concentration-effect curves. Then, the solution was replaced for the Krebs-Henseleit solution, and the tension was stabilized at baseline values with frequent renewal of the solution. After 30 minutes, the aortic rings were contracted with NA (10^{-7} M), and other concentration-effect curves were obtained with sodium nitroprusside (SNP, 10^{-8} - $10^{-5.5}$ M).

The 5-mm-thick aortic rings were obtained in a standardized form from defined areas of the superior part of the thoracic aorta. Fragments from the liver and thigh muscle were also obtained. The tissues were fixed in 10% formalin for 24 hours, processed, and embedded in paraffin. Serial sections were mounted on slides and stained with hematoxylin-eosin.

Total serum cholesterol level was measured with commercial enzymatic kits in a spectrophotometer (Genesys 10, Spectronics) and the results were expressed in mg/dL. The GOT and GPT levels were also measured with the commercial enzymatic kit, and the results were expressed in U/L.

Lipid peroxidation was assessed by use of MDA measurement, one of the end-products of peroxidation. The aortic samples were homogenized in cold trichloroacetic acid (TCA) (1 mg of tissue per mL of 10% TCA). After centrifugation, a portion of the supernatant was added to an equal volume of thiobarbituric acid (0.6% v/v), and the mixture was heated at 100°C for 20 minutes. The MDA concentration was calculated by use of a spectrophotometer, with absorption of 532 nm and a molar extinction coefficient of $1.49x10^{-5}$, and the results were expressed in nmol/mg of dry tissue x 10^{-7} [19]. The lipoproteins were separated by plasma sequential ultracentrifugation according to the method by Havel et al [20]. The plasma was centrifuged at 40000 rpm for 18 hours at 4°C in a Ti50 rotor (Beckman), and the VLDL layer (d < 1,006 g/mL) was removed. The supernatant was adjusted to a density of 1063 g/mL by using potassium bromide, being then centrifuged at 40,000 rpm for 20 hours. The isolated LDL-C was dialyzed in 0.01 M of phosphate-buffered saline (PBS), pH 7.4 at 8°C for 24 hours, with frequent buffer washing. The LDL protein concentration was determined by use of the method of Lowry et al [21] with bovine albumin as the standard. The LDL oxidation was performed by use of incubation with 5 μM copper sulfate (100 μg of protein/mL, 1 M PBS) for 24 hours at 37°C [22]. The concentration of peroxide lipids of the native and oxidized LDL was measured by the reaction of the thiobarbituric acid (TBARS), as reported by Buege and Aust [23]. The TBARS values were expressed as MDA equivalents (nmol/protein LDL) using the standard solution of 1,1,3,3 tetramethoxypropane.

Images of the histological sections of the aortic rings were obtained with a Leica DMLD microscope and imported to a computer. By use of the Scion program [24], areas of the arterial wall and of the extracellular liposomes were manually selected. The number of pixels was determined and used as a measurement. The results were expressed as % of extracellular liposomes in relation to the arterial wall [25].

The acetylcholine chloride, noradrenaline bitartrate, sodium nitroprusside, trichloroacetic acid, thiobarbituric acid, and indomethacin were obtained from Sigma Chemical Co (St. Louis, MO, USA). The reagents of the Krebs-Henseleit solution were obtained from Merck Chemicals.

The descriptive analyses were performed by use of the position and dispersion measurements for the continuous variables. For comparing the groups of treatment through the variables collected, the simple analysis of variance (one-way ANOVA) was used, with transformation of the variables according to ranks. For identifying the differences, the Tukey test, a multiple comparisons procedure, was used.

The repeated measures ANOVA was used for comparing the dose-response curves. The multiple comparisons Tukey test and the contrast profile test were used for identifying the differences. The significance level adopted was 5% [26,27].

Results

At the beginning of the study, the mean weights of the rabbits in the different groups were as follows: 1) GN: 1.80±0.2 kg; 2) GH: 2.0±0.18 kg; 3) GA: 1.75± 0.12 kg; 4) GF: 1.85±0.14 kg; 5) GP: 2.09±0.23 kg; and 6) GS: 1.79±0.10 kg. All animals gained weight during the study, but no significant difference was observed between the groups.

Figure 1 shows the occurrence of marked elevation in total serum cholesterol levels in the hypercholesterolemic group (GH) when compared with the total serum cholesterol levels in the normal group (GN). The treatment with statins significantly reduced serum cholesterol levels. No difference was observed between the groups, and the same occurred in regard to LDL-cholesterol levels (fig. 2).

The content of malondialdehyde (MDA) (nmol/mg x 10^{-7}M) in the oxidized LDL particles and aortic wall is shown in figures 3 and 4. The MDA content of the aortic wall and oxidized LDL particles was significantly reduced in the treated group when compared with that in the hypercholesterolemic group ($P < 0.05$). No significant difference was observed between the treated groups. A similar response was obtained for the MDA content of native LDL particles.

Figure 5 expresses the percentage of relaxation of the aortic rings of hypercholesterolemic rabbits in response to acetylcholine. It was significantly smaller than that of the animals in the normal group. All statins reversed the relaxation until close to normal in varied degrees, pravastatin being the most effective. Maximum relaxation with nitroprusside was 100% in all groups. No relaxation was observed with ACH in the aortic segments whose endothelium had been mechanically removed. The intensity of contraction with NE was similar between the groups.

The histological examination showed the presence of extracellular liposomes in the intimal layer of the aorta of hypercholesterolemic rabbits and no inflammatory cells. The percentages of extracellular liposomes in the vessel wall in the hypercholesterolemic, atorvastatin, fluvastatin, simvastatin, and pravastatin groups were as follows, respectively: 21.0±0.15, 2.58±1.35, 3.83± 0.89, 2.95±0.91, and 3.84±1.08. When comparing the hypercholesterolemic group with the treated groups, statins significantly ($P<0.05$) reduced aortic atherosclerosis, and no difference was observed between the treated groups.

Figure 6, in its left lower portion, illustrates the evolution of atherosclerosis in animals treated with a 2% diet. A greater concentration of extracellular liposomes is observed in the intimal layer.

The histological study of liver fragments revealed moderate steatosis in all groups studied, while the muscle tissue appeared normal on microscopic examination.

The GOT and GPT levels were within the normal range in all treated groups.

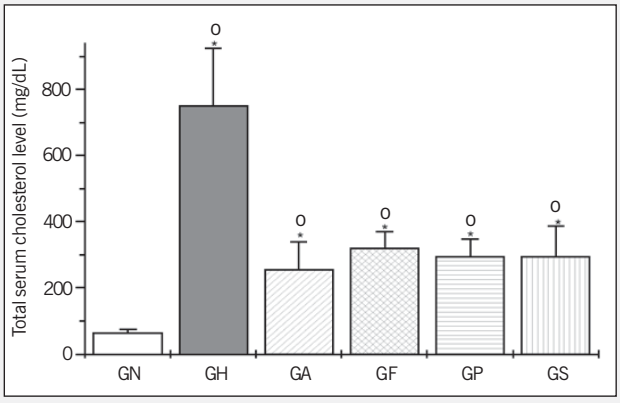

Fig. 1 - Total serum cholesterol levels. Values expressed as mean and SD. The groups were as follows: GN = normal, GH = hypercholesterolemic, GA = atorvastatin, GF = fluvastatin, GP = pravastatin, GS = simvastatin. * $P < 0.05$ for GH; $P < 0.05$ for GN.

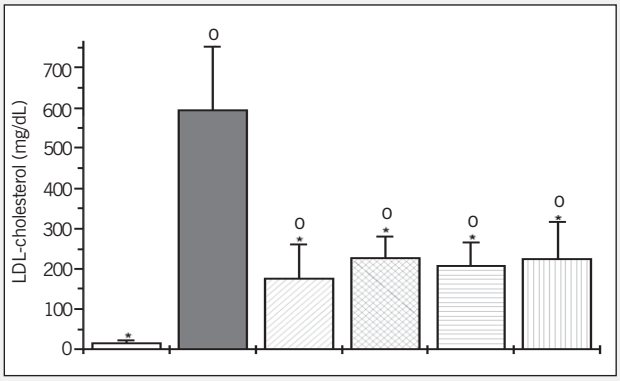

Fig. 2 - Serum LDL-cholesterol levels (mg/dL). Values expressed as mean and SD. The groups were as follows: GN = normal, GH = hypercholesterolemic, GA = atorvastatin, GF = fluvastatin, GP = pravastatin, GS = simvastatin. * $P < 0.05$ for GH; $P < 0.05$ for GN.

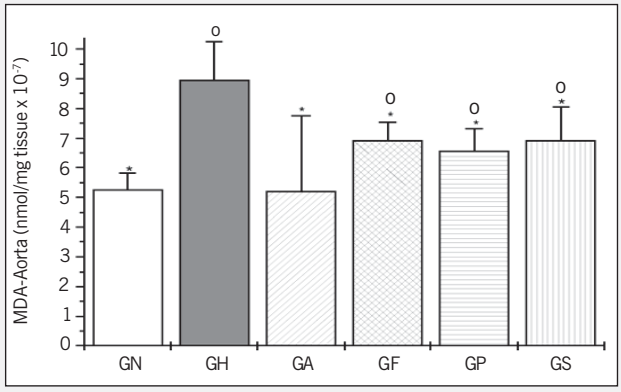

Fig. 3 - Concentration of malondialdehyde (MDA) in the aortic wall (x 10^{-7} nmol/mg/tissue). Values expressed as mean and SD. The groups were as follows: GN = normal, GH = hypercholesterolemic, GA = atorvastatin, GF = fluvastatin, GP = pravastatin, GS = simvastatin. * $P < 0.05$ for GH; $P < 0.05$ for GN.

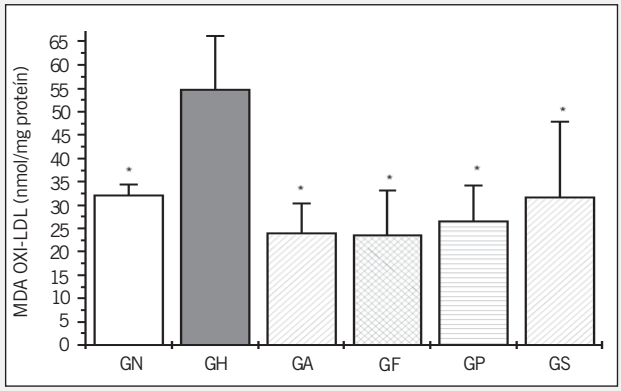

Fig. 4 - Malondialdehyde in the oxidized LDL particles (nmol/mg protein). Values expressed as mean and SD. The groups were as follows: GN = normal, GH = hypercholesterolemic, GA = atorvastatin, GF = fluvastatin, GP = pravastatin, GS = simvastatin. * $P < 0.05$ for GH.

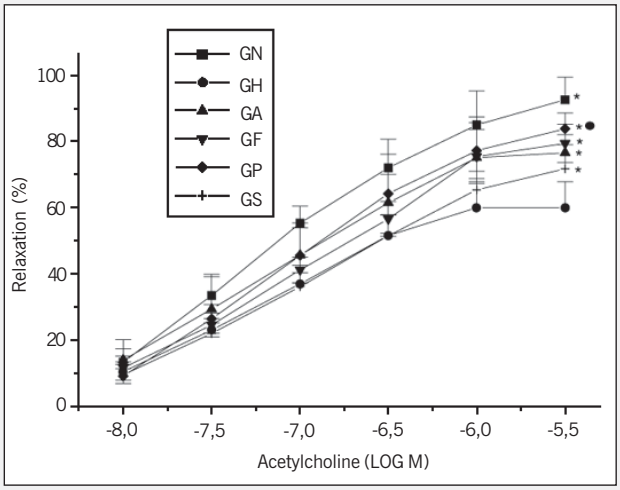

Fig. 5 - Endothelium-dependent relaxation of the aortic rings precontracted with norepinephrine (NE) in response to acetylcholine (ACH). Relaxation expressed as percentage of NE-induced contraction (10^{-7}). The points represent the mean and SD of 10 rabbits per group. * $P < 0.05$ comparing the hypercholesterolemic and normal groups. $P < 0.05$ between the groups.

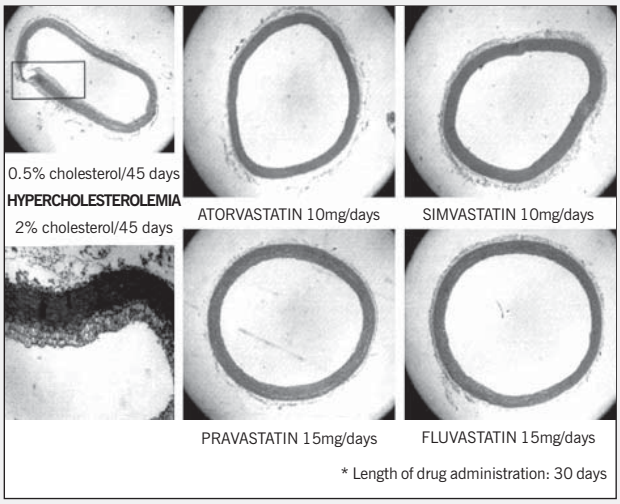

Fig. 6 - Histological section of the aorta of hypercholesterolemic animals and those treated with atorvastatin, fluvastatin, pravastatin, and simvastatin. Note the significant regression of extracellular liposomes in the animals treated.

Discussion

Statins vary considerably in their pharmacological and lipophilic characteristics, their mean life, metabolism, and other properties[28]. Pravastatin is hydrophilic, while the other statins are lipophilic. Atorvastatin, simvastatin, and fluvastatin circulate in the blood bound to protein in 95 to 98%, while pravastatin circulates bound to protein in 50% [29]. Except for pravastatin, the statins are metabolized in cytochrome P-450-3A4 (fluvastatin is metabolized in cytochrome P-450-2C9). Atorvastatin, fluvastatin, and simvastatin, but not pravastatin, decrease the migration and proliferation of smooth muscle cells in the arterial wall [30], probably by blocking RhoA- and Rac1-, which are cell signalers [31]. Evident alterations in inflammatory response have been reported with the use of statins, including the inhibition of cytokines, C-reactive protein, expression of the metalloproteinases of the matrix, and decrease in the adhesion of monocytes to the endothelial cell [32]. Recent studies about the pleiotropic properties of statins have revealed their capacity to inhibit the synthesis of important intermediates of the isoprenoids, which serve as bindings to a variety of proteins implicated in intracellular signaling [33,34]. On the other hand, well-conducted studies have revealed a difference in the potential of statins to reduce cholesterol and serum LDL particles [15,16]; differences in their pleiotropic effects and supposed tissular selectivity have been discussed.

The major objective of this study was to assess the pleiotropic effects of different statins, considering the controversial results reported in the literature. Pentikainem et al [35] reported that pravastatin is an HMG-CoA reductase inhibitor with greater affinity to peripheral tissues, which was confirmed by Germershausen et al [36]. The latter authors attributed that finding to the active species derived from pravastatin, which bind to proteins in approximately 50%, while the other statins are intensely bound to proteins (>95%); thus, drugs that bind to proteins in smaller amounts have greater penetration in tissues. The hydrophilic HMG-CoA reductase inhibitors, on the other hand, may reach a greater concentration in endothelial cells, therefore increasing the NO synthase activity. However, the tissular selectivity of HMG-CoA reductase inhibitors is controversial [14,37]. Thiery et al [38], investigating the extension of atherogenesis in hypercholesterolemic rabbits treated with lovastatin, simvastatin, and pravastatin (15 mg/day), reported a smaller concentration of tissular cholesterol in animals treated with lovastatin and simvastatin than that in animals treated with pravastatin. De Vries et al [39] stated that the synthesis of cholesterol in the lens is 100 times more effectively inhibited by pravastatin than by simvastatin. Tesfamarian et al [40] reported different effects of pravastatin, simvastatin, and atorvastatin in the release of cytoplasmic Ca^{2+} and in vascular reactivity, while Joukhadar et al [41] reported a similar effect for atorvastatin, simvastatin, and pravastatin in some parameters relating to hemostasia and inflammation in the plasma of hypercholesterolemic patients.

However, the supposed differences between statins apparently do not have a major clinical significance, because the great randomized studies have shown a significant reduction in mortality and coronary events with the use of the different statins studied [1,3,4,6].

Our results showed that all statins were effective in reducing lipid peroxidation in native or oxidized LDL and also in the arterial wall. No significant difference was observed in the intensity of the effect between the different drugs. The antioxidizing effect of simvastatin [42], fluvastatin [43], pravastatin [44], and atorvastatin [45] has been reported in the literature and represents an important step in preserving endothelial function [34].

In the group of hypercholesterolemic animals, all statins were effective in partially reversing endothelial dysfunction. The effect was similar in the different drugs, except for pravastatin, whose effect on endothelium-dependent relaxation was more significant. The mild difference in the intensity of endothelium-dependent relaxation observed with pravastatin was interpreted as due to its hydrophilic characteristic, but this finding should be reassessed in further studies.

The increase in endothelium-dependent relaxation observed in the aortic rings of rabbits treated with statins should be related to the reduction in tissular cholesterol and oxidative stress in the arterial wall [46], in addition to the action on the mediators of cellular transcription, which was not studied in this experimental model. In a previous study [18], we observed that the reversion of endothelial dysfunction in hypercholesterolemic rabbits treated

with pravastatin and simvastatin occurred rapidly, which is beneficial to the treatment of acute coronary syndrome.

Another point that deserves to be stressed in the present study due to clinical implications is the regression of intimal thickening, represented by the build up of extracellular liposomes. The LDL particles enter the endothelial cell due to the mechanism of pinocytosis, forming vesicles of pinocytosis, which are incorporated as endosomes and liposomes in the cell cytoplasm. In hypercholesterolemia, the lipid material carried by the endosomes accumulates in extracellular liposomes in the intimal layer of the artery. Considering that the presence of extracellular liposomes are the earliest morphological expression of atherosclerosis, their regression may represent a potential mechanism of control of the atherosclerotic disease. The regression of the atherosclerotic plaque caused by statins has been demonstrated in several experimental studies [47-49].

Studies with magnetic resonance imaging and spectroscopy have reported mobilization of the lipid content in the atherosclerotic plaques of animals treated with different drugs [50-52].

More recently, the REVERSE study [53] has confirmed the interruption of progression, and even a small regression, of the atherosclerotic plaque (0.4% of median reduction) evidenced on coronary ultrasound in patients with stable coronary disease. Those results open an exceptional perspective in the management of atherosclerosis.

The present study showed no significant difference in atherosclerosis regression between the different statins studied. However, it is worth emphasizing that the dosages of statins were adjusted for obtaining similar values of serum cholesterol in the different groups considering the different potencies of the drugs. Atorvastatin was the most potent, because a lower dose of atorvastatin was required for a similar reduction in serum cholesterol in the groups; an eventual difference in the effect of regression could not be assessed considering the intensity of the reduction in serum cholesterol and LDL particles. More recently, a more intense reduction was observed in serum LDL in patients with acute coronary syndromes leading to a greater benefit regarding a reduction in mortality rate and other coronary events [54]. This initiated the discussion about the best LDL serum level for controlling atherosclerosis, stressing the significance of the lipid-lowering potency of statins.

The most intense reduction in serum lipids is significant when one considers that the major causal factor in atherosclerosis is the afflux of LDL particles and their epitopes to the intimal region of the vessel through the endothelial cell. This causes an immune response mediated by several factors related to inflammation, with mobilization of monocytes, neutrophils, and lymphocytes. This restricts the lipid core through a predominantly proliferative reaction or makes the plaque unstable due to a greater cellular and enzymatic response [55]. Probably the magnitude of the reduction in cholesterol and LDL serum levels, in addition to the other factors already mentioned, has a beneficial effect on reversing endothelial dysfunction and mobilizing the lipid core of the atherosclerotic plaque, mainly in the earliest phase. The safe limit to which serum LDL can be lowered has yet to be established.

The authors of the REVERSE [53] and PROVE-IT [54] study have also emphasized the existence of a pharmacological difference between the statins studied, atorvastatin and pravastatin. A well-conducted experimental study [55] using fluvastatin has also considered the existence of an indirect effect of the drug, independently of the reduction in serum cholesterol. This reinitiates the discussion about a possible pharmacological difference and tissular specificity between these drugs, independently of the reduction in lipids.

In this study, the statins did not alter the enzymatic activity of the liver. The histological examinations showed moderate hepatic steatosis and a normal appearance of the muscle tissue, probably consequent to the elevated serum concentration of cholesterol. Areas neither of necrosis nor of inflammation were observed. These findings suggest that the drugs had no toxic effect at the dosages administered.

The lack of inflammatory reaction in the intimal layer of the vascular wall, despite the presence of extracellular liposomes in hypercholesterolemic rabbits, reinforces the idea that the inflammatory response occurs later to repair the inadequate presence of LDL and its epitopes in the intimal layer of the artery [56]. This, once more, reinforces the impression that a more intense reduction in tissue LDL will have a more favorable effect on disease control.

The results of this study showed that all statins were effective in reducing serum total cholesterol, the MDA content in the arterial wall and in native and oxidized LDL, and in reversing the endothelial dysfunction in hypercholesterolemic rabbits.

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