

A Comparison of the Effects of Lovastatin and Pravastatin on Ubiquinone Tissue Levels in Rats

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ABSTRACT

Treatment with HMG-CoA reductase inhibitors could theoretically reduce ubiquinone tissue levels, a condition which could decrease resistance to oxidative stress. Further, effects of various HMG-CoA inhibitors may vary because of different lipophilicity and tissue distribution. To clarify this question, the effect after a 4 week treatment with lovastatin or pravastatin (0, 20, 40 or 80 mg/kg/day) on ubiquinone levels was evaluated in mature male rats (n=7 per dose). Ubiquinone-9 (Q₉) and ubiquinone-10 (Q₁₀) levels in blood, myocardium, skeletal muscle and liver were measured by HPLC, at each dose level, and the groups were compared statistically by non parametric tests with a significance level of p<0.05. In blood, decreases in Q₉ levels were observed with both lovastatin and pravastatin (32 and 29% respectively, p<0.05 vs. control) and were not dose-related. No significant effects were observed in Q₁₀ levels. In the myocardium, pravastatin showed a 20% decrease in Q₉ levels which plateaued at the lowest dose (20 mg/kg/day) (p<0.05 vs. control). A dose-related decrease in Q₉ levels, which reached 40% at the highest dose (p<0.05 vs. control), was observed with lovastatin. Similar results were obtained in Q₁₀ levels. In skeletal muscle, a trend towards lowered Q₉ levels was observed with both lovastatin and pravastatin. No significant difference was observed in Q₁₀ levels. In the liver there was a trend toward increased ubiquinone levels which was not significant. It is concluded that lovastatin and pravastatin tend to lower concentration of ubiquinones in myocardium, muscle, and blood. The greater effect of lovastatin, in the myocardium, can probably be attributed to its higher tissular concentrations owing to its greater lipid solubility. Because of the proposed role of ubiquinone, both as a liposoluble antioxidant and in mitochondrial bioenergetics, these findings may indicate that the use of some HMG-CoA reductase inhibitors may not be appropriate in patients with preexisting pathological conditions associated with decreased levels of ubiquinone. Further studies in other animal species are indicated to confirm the potential pharmacological and therapeutic importance of our findings.

INTRODUCTION

HMG-CoA reductase inhibitors are considered as relatively safe and effective cholesterol-lowering agents. In a few patients acute myolysis has been reported during treatment with lovastatin [1-5]. These cases were mainly observed in heart transplant recipients treated concomitantly with cyclosporine. Reports suggest that cyclosporine administration leads to altered clearance of lovastatin and increased tissue exposure [1]. However, the reasons for acute myolysis in these patients still remain to be elucidated.

Decreased formation of mevalonate, due to HMG-CoA reductase inhibition, could theoretically reduce ubiquinone levels [6] - a condition which could lead to a decrease in the resistance to oxidative stress. It has been reported that HMG-CoA reductase inhibitors decrease the blood and tissue levels of ubiquinone in the rat [7-10], as well as blood levels in humans [11].

Ubiquinone is present in all cellular membranes. The major form in rat contains 9 isoprene residues (Q_9); but a relatively high level of the lipid with a side chain of 10 isoprene units (Q_{10}) is also present in rat tissues [12]. In humans, it is the opposite, the major form of ubiquinone being Q_{10} . Besides its role in mitochondrial bioenergetics (as an obligatory member of the electron transfer chain), there is also evidence, both *in vitro* and *in vivo* [13-15], of another very important function of ubiquinone: a role as an antioxidant in the inner mitochondrial membrane where its concentrations are much greater than those of the other components of the electron transfer chain. Ubiquinone would then be the only lipid soluble antioxidant of the body synthesized endogenously and, in contrast to all other antioxidants, its level is independent of dietary supply [16]. The ubiquinone antioxidant mechanism is still unclear and the enzymatic mechanism of this process outside the mitochondria also remains to be elucidated.

In view of the roles of ubiquinone in mitochondrial bioenergetics and as a liposoluble antioxidant, further investigations on the documented decrease of ubiquinone by HMG-CoA reductase inhibitors is warranted. This is even more relevant for patients with ischemic heart disease since they have lower levels of plasma ubiquinone/ low density lipoprotein cholesterol (LDL-C) ratios and of myocardial ubiquinone [17-18].

Therefore, the primary objective of our study was to compare the effects of pravastatin and lovastatin on ubiquinone levels, and to determine whether these are dose-related. The secondary objective was to establish a correlation between the effects observed in blood and those observed in the myocardium and/or the muscle. This experiment was performed further to previous findings indicating that lovastatin decreases tissue ubiquinone levels in the rat at doses of 20 mg/kg/day [8].

MATERIALS AND METHODS

Materials

Solvents of HPLC grade and all other chemicals of analytical grade were purchased from Anachemia (Montreal, Canada). The internal standard ubiquinone-11 (Q_{11}), was kindly provided by the Nisshin Chemicals Company, Tokyo, Japan. Ubiquinone-9 (Q_9) and ubiquinone-10 (Q_{10}), BHT (2,6-Di-tert.-butyl-p-cresol) and ascorbic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium dodecylsulfate (SDS) was obtained from BDH Biochemicals (Toronto, Canada).

The HPLC system consisted of two Constametric III pumps (LDC Milton Roy, LDC Division, Riviera Beach, Fla., USA), a model 7125 injector (Rheodyne, Cotati, CA), a UV detector (Waters, Milford, MA) set at a wavelength of 275 nm and an integrator (Shimadzu Chromatopac CR 601, Kyoto, Japan). The octadecyl silica (ODS) column was packed with 5 mm Spherisorb C8. A Potter-Elvehjem homogenizer (Polytron, Kinematica, GMBH, Luzern, Switzerland), a Speed-vac plus concentrator (Model SC-210A, SAVANT Instruments, Farmingdale, NY, USA), and a Speed Mate 10 vacuum manifold (Applied Separations, Lehigh Valley, PA, USA) were used.

Analysis of Ubiquinones

Blood and tissue ubiquinone levels were determined using a procedure which is further described elsewhere [19]. One hundred (100) mg freeze clamped tissue was accurately weighed in the frozen state and subsequently homogenized with 1 mL of a 1mM buffer solution (consisting of $\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ adjusted to pH 7.4) in a homogenizer with a motor-driven pestle. A volume of 50 μL of a solution of BHT in reagent alcohol (10 mg/mL) was added to each sample to prevent autooxidation. The internal standard (Q_{11}) was then added. Also, since the potential photodegradation of ubiquinone cannot be completely ruled out, all extraction steps were performed in the absence of incandescent light or direct sunlight. After addition of 1 mL of 0.1 M SDS and brief mixing by homogenization, the sample was transferred to a 10 mL test tube fitted with a PTFE-lined screw cap, 2 mL of reagent alcohol was added, and the mixture was vortex-mixed for 30 s. Then 2 mL of hexane was added and the tightly capped test tube was vigorously vortex-mixed for 2 min. It was then centrifuged for 5 min at 1000 g and 1 mL of the hexane layer was transferred to a small vial. The solvent was evaporated under nitrogen and the residue redissolved in methanol:ethanol (70:30 v/v). Final volumes vary from 0.2 to 1.0 mL, depending on the expected concentrations in the tissue. Samples were injected into the HPLC shortly after preparation. The mobile phase consisted of a mixture of methanol:ethanol (70:30). The solvent flow rate was 1.2 mL/min and the procedure was performed at room temperature. The retention times (expressed in minutes) for ubiquinone standards were as follows: Q_{11} : 9.5, Q_{10} : 7.2, Q_9 : 5.5. The UV detection was performed at their maximum absorbance (275 nm).

The assay proved to be sensitive (0.0625 $\mu\text{g}/\text{mL}$), reproducible (the coefficients of variation ranging from 3 to 7%) and linear (up to 400 $\mu\text{g}/\text{g}$ of tissue for Q_9 and up to 100 $\mu\text{g}/\text{g}$ for Q_{10}). Reference calibration curves, for each tissue were used to derive the

tissular concentrations corresponding to the peak height ratio of Q_9 or Q_{10}/Q_{11} obtained for each sample. The ubiquinone levels in control tissues were within the ranges of those previously reported. The recovery of Q_9 and Q_{10} from different tissues ranged from 90 to 112%. The accuracy (expressed in %), for Q_9 and Q_{10} respectively, was as follows: 99.7 ± 6.4 and 103.9 ± 5.8 for the myocardium, 90.4 ± 7.8 and 88.5 ± 3.2 for the skeletal muscle, 106.1 ± 13.7 and 109.2 ± 11.8 for the liver, and 82.8 ± 3.5 and 117.3 ± 15.4 for the blood.

Analysis of Lovastatin and Pravastatin

Two HPLC methods of analysis were developed for lovastatin and pravastatin, respectively [20-22]. The same HPLC system as described for the ubiquinone analyses was utilized at a wavelength of 238 nm. For lovastatin, the mobile phase consisted of a 50:50 mixture of acetonitrile:buffer ($\text{NH}_4\text{H}_2\text{PO}_4$ 0.05M and H_3PO_4 14.7M) at an apparent pH of 3.7. The internal standard used was the simvastatin β -hydroxyacid form. C_8 cartridges were conditioned with 2 mL of methanol and 2 mL of buffer (Na_2HPO_4 0.1M). For the extraction 0.3 mL of plasma mixed with 0.3 mL of buffer and 20 ng of internal standard were passed through the C_8 cartridge which was then washed twice with 1 mL of buffer and twice with a 80:20 mixture of acetonitrile:buffer. The acid form of lovastatin was then eluted with 1 mL of acetonitrile:water (75:25) and centrifuged at 1000 g for 4 minutes. A volume of 30 μL was then injected into the HPLC system.

For pravastatin, the mobile phase consisted of a mixture of $\text{NH}_4\text{H}_2\text{PO}_4$ 0.025M (pH: 3.5) and of acetonitrile (72.5 : 27.5) to which a solution of DBA (dibutylamine) was added to obtain a final concentration of 1%. The pH was adjusted at 4.0. For this analysis, propranolol was used as an external standard. C_8 cartridges were conditioned with 2 mL of methanol followed by 2 mL of water. A mixture of 0.5 mL of plasma and of 0.5 mL of Na_2HPO_4 (0.1 M) was then added to the cartridge. The sample was washed with water (2 x 1 mL) and eluted with 500 μL of acetonitrile: H_2O (75:25). The eluate was then evaporated to dryness and redissolved in 200 μL of methanol: buffer (50:50). The buffer consisted of KH_2PO_4 0.1M (pH=5.0) containing 1 $\mu\text{g}/\text{mL}$ of propranolol (the external standard). After vortexing for 40 seconds, 30 μL were injected into the HPLC system. The assay was sensitive (lower limit of detection of 1 ng/mL for pravastatin and lovastatin) and linear from 20 to 500 ng/mL for pravastatin and 5 to 200 ng/mL for lovastatin.

Analysis of Lipids

The lipid analyses were performed on plasma samples at the clinical laboratory of Ste. Justine Hospital (Montreal, Canada) using standard methods. The Friedwald formula was used to calculate the LDL-C fraction ($LDL-C = [tot. C] - [HDL-C] - [TG / 2.22]$) where HDL-C represents the high density lipoprotein cholesterol and TG, the triglycerides.

Study Design

Seven groups of 7 adult male Sprague Dawley rats, weighing approximately 250 g and of the same age [23] were used. The study animals were housed in colony cages and subjected daily to 12h of light and 12h of dark during the acclimatization period (one week) and throughout the experiment (four weeks). Standard laboratory diet was fed ad lib and the HMG-CoA reductase inhibitors were administered during the mid-dark phase of the light cycle. Two treatment groups and one control group were used in this study.

One treatment group was administered lovastatin and the other, pravastatin; there were 3 subgroups per group, each corresponding to one of the following dosage regimen: 20, 40 or 80 mg/kg/day (O.D.). The HMG-CoA reductase inhibitors were administered by oral gavage as a dosing suspension prepared weekly by homogenizing crushed tablets with an appropriate volume of a 10% ethanol aqueous solution. The suspension was then filtered under vacuum. The control animals received an equal volume (approximately 0.4 mL/100g) of the vehicle by gavage. The weight of the rats was recorded at the beginning of the experiment and biweekly thereafter.

At the end of the treatment period, the animals were euthanized and samples of heart, muscles and liver were "freeze clamped" immediately at -196°C (liquid nitrogen). These tissues and the blood were stored at -80°C .

Statistical Analysis

The results are expressed as mean \pm SD. Due to lack of normality (as indicated by the Shapiro-Wilk Test), non parametric tests were used. Differences between pairs of treatments (ubiquinone means) were compared using the Wilcoxon Rank Sums test and dose responses were compared using the Kruskal-Wallis test ($p < 0.05$). For the lipids an Anova was performed to test for differences between treatments where the assumption of normality was reasonable (HDL-C, cholesterol). For HDL-C levels, a Tukey's honestly significant difference (HSD) test was performed to test for differences between pairs of treatments. For triglycerides and LDL-C a Wilcoxon Rank Sums test was used. For the analyses described above all dose groups within a given treatment were pooled for comparison to placebo and between treatments; if there was a significant difference versus placebo, the dose groups within a treatment were compared. Only those comparisons that reached significance are reported in the figures. The absolute weight changes, from week 0 to 4 were compared by Anova ($p < 0.05$). The lovastatin and pravastatin levels were also compared by Anova ($p < 0.05$).

RESULTS

There was no significant difference in weight changes in rats treated with either the vehicle, lovastatin or pravastatin for four weeks. Further, the effects of lovastatin and pravastatin treatment on the plasma lipid levels and profile were marginal. It appears that after 4 weeks of treatment, neither of the HMG-CoA reductase inhibitors significantly influenced the plasma lipid levels (cholesterol, LDL-C and TG) as described in Table I. Only the HDL-C increase observed with lovastatin was statistically significant when compared to the control group. However, this effect was not dose-related. These plasma lipids results are consistent with what has been previously reported and described as a species-specific effect [24-25].

TABLE 1: Lipid plasma levels after a four week treatment with vehicle, pravastatin or lovastatin (20, 40, and 80 mg/kg/day). L=lovastatin, P=pravastatin. For HDL-C, *p<0.05 all lovastatin pooled groups vs. control.

Group (mg/kg/day)	Cholesterol (mM)	Triglycerides (mM)	HDL-C (mM)	LDL-C (mM)
Control	1.44 ± 0.16	0.83 ± 0.06	0.75 ± 0.12	0.27 ± 0.19
P20	1.37 ± 0.14	0.46 ± 0.13	0.83 ± 0.11	0.34 ± 0.08
P40	1.83 ± 0.23	1.02 ± 0.31	0.85 ± 0.09	0.37 ± 0.22
P80	1.53 ± 0.18	0.83 ± 0.25	0.80 ± 0.1	0.37 ± 0.22
L20	1.65 ± 0.32	0.48 ± 0.11	1.01 ± 0.22	0.44 ± 0.11
L40	1.73 ± 0.24	0.82 ± 0.23	0.86 ± 0.16	0.45 ± 0.23
L80	1.58 ± 2.80	0.54 ± 0.08	0.90 ± 0.16	0.33 ± 0.19

HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; * p<0.05 for lovastatin-pooled groups versus control

Ubiquinone levels

In the present study, four tissues (blood, myocardium, skeletal muscle and liver) were analyzed for their content in ubiquinone and the results were as follows:

Blood (figure 1): Similar significant decreases in Q₉ levels i.e. 32% (from 0.41 to 0.28 µg/mL) for pravastatin pooled groups and 29% (from 0.41 to 0.29 µg/mL) for lovastatin pooled groups were observed (p<0.05 vs. vehicle) with both drugs and were not dose-related. There was no statistical difference between lovastatin and pravastatin. No statistically significant difference were observed in Q₁₀ levels.

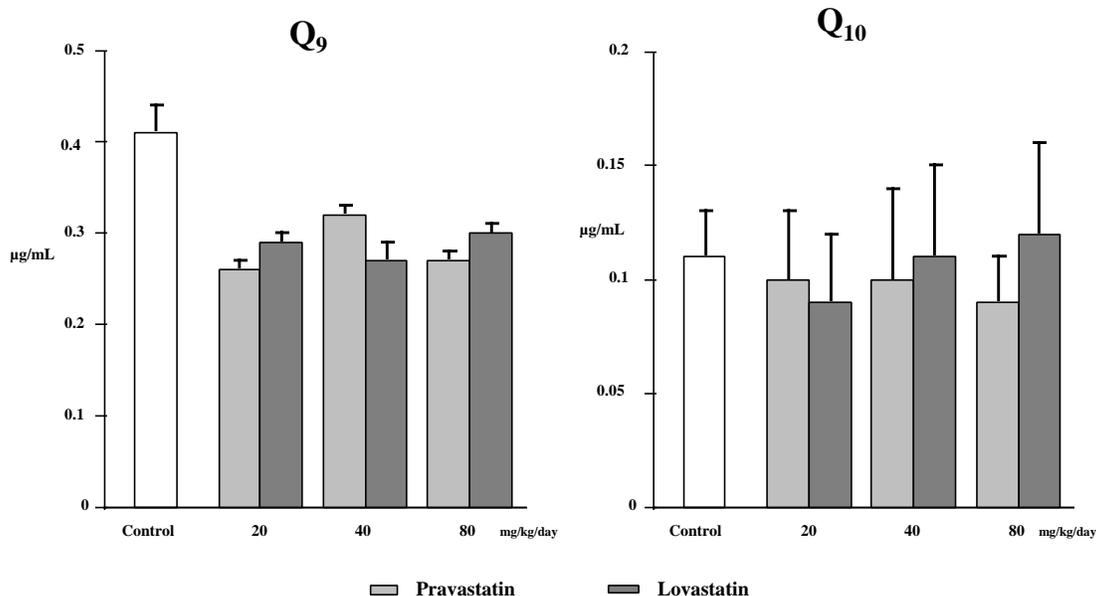


Figure 1: Q-9 and-10 blood levels (mean \pm SD) after a four week treatment with either pravastatin, lovastatin or vehicle. For Q₉, $p < 0.05$ for pravastatin pooled groups vs. control, and for lovastatin pooled groups vs. control.

Myocardium (figure 2): For pravastatin, a maximum 20% decrease in Q₉ levels (from 272 $\mu\text{g/g}$ to 220 $\mu\text{g/g}$) reached a plateau at the lowest dose. However, with lovastatin, a dose-related decrease in Q₉ levels was observed and reached 40% (from 272.8 to 162.0 $\mu\text{g/g}$) at the highest dose. For Q₁₀, a significant decrease was observed with pravastatin pooled groups (maximum of 22% from 27.8 to 21.8 $\mu\text{g/g}$) which was not dose-related while with lovastatin pooled groups, a greater significant decrease (40% from 27.8 to 16.6 $\mu\text{g/g}$) was observed and was dose-related. For Q₉ and Q₁₀, the following comparisons reached statistical significance ($p < 0.05$): pravastatin pooled groups vs. control, lovastatin pooled groups vs. control; pravastatin pooled groups vs. lovastatin pooled groups; lovastatin 20 mg vs. 40 mg vs. 80 mg.

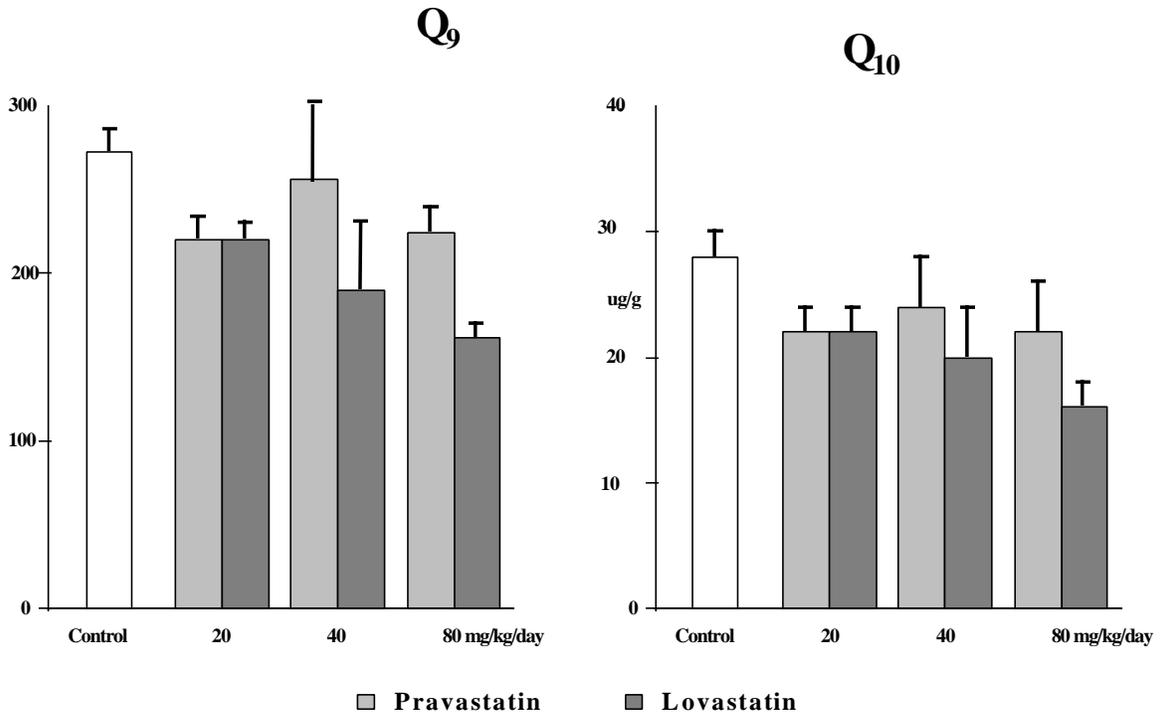
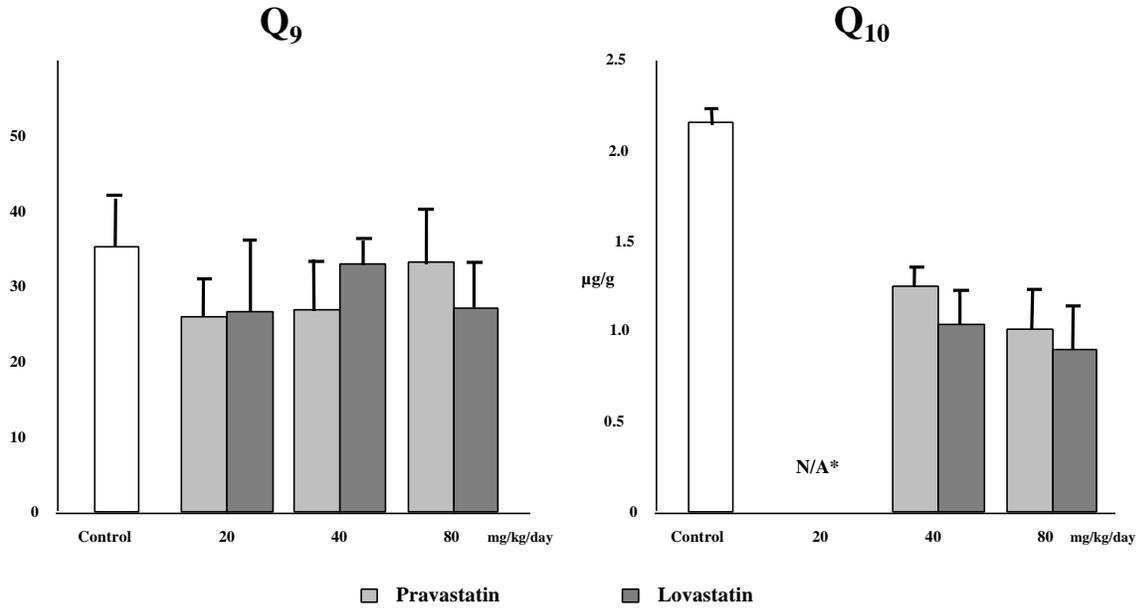


Figure 2: Q-9 and-10 myocardium levels (mean \pm SD) after a four week treatment with either pravastatin, lovastatin or vehicle. For Q_9 and Q_{10} , $p < 0.05$ for the following comparisons: pravastatin pooled groups vs. control, lovastatin pooled groups vs. control; pravastatin pooled groups vs. lovastatin pooled groups; lovastatin 20 mg vs. 40 mg vs. 80 mg.

Skeletal Muscle (figure 3): For Q_9 , similar decreases were observed with pravastatin and lovastatin but the reductions were not significant nor dose-related. For Q_{10} levels, no change reached statistical significance. Unfortunately, the results for the groups at 20 mg/kg/day are not available further to a laboratory mishap.



* N/A: not available

Figure 3: Q-9 and-10 muscle levels (mean ± SD) after a four week treatment with either pravastatin, lovastatin or vehicle. No difference was statistically significant. N/A = not available because of a laboratory mishap.

Liver (figure 4): There was a trend toward increased levels of Q₉ and Q₁₀, however these increases did not reach statistical significance.

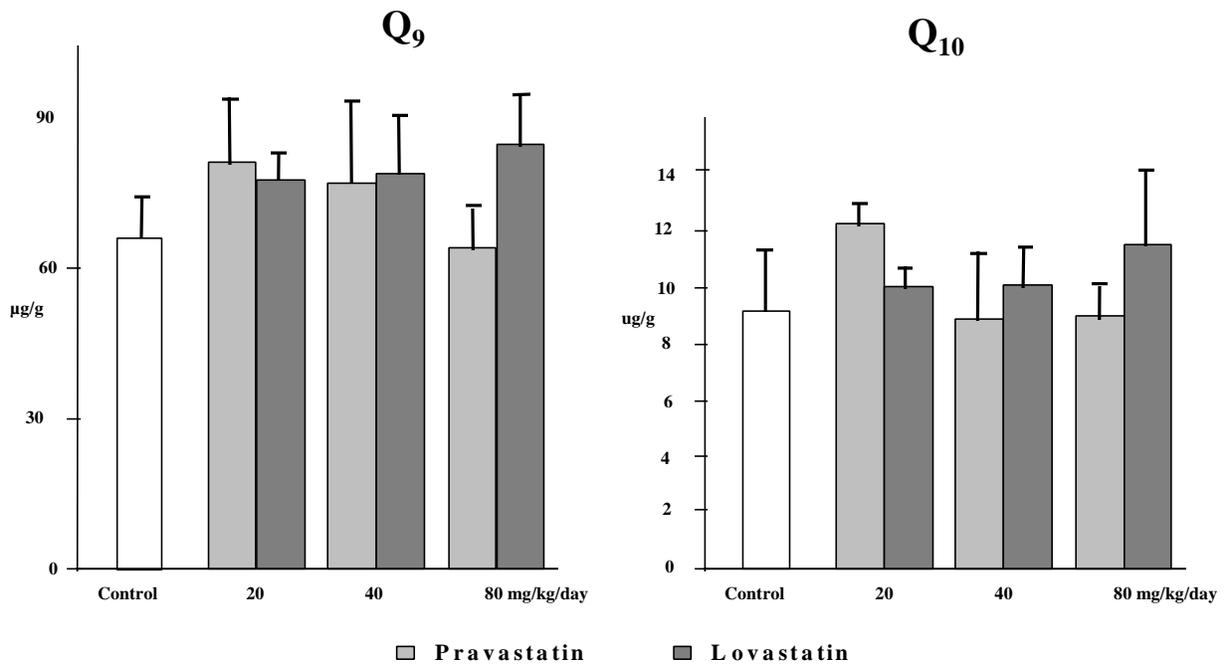


Figure 4: Q-9 and-10 liver levels (mean ± SD) after a four week treatment with either pravastatin, lovastatin or vehicle. No difference was statistically significant

Lovastatin and Pravastatin Levels

The plasma levels of lovastatin and pravastatin (for the 20, 40 and 80 mg/kg/day group) were as follows: 26.3 ± 4.7 ng/mL, 27.9 ± 7.1 ng/mL and 44.1 ± 8.2 ng/mL for pravastatin, and 1.7 ± 0.4 ng/mL, 4.6 ± 2.2 ng/mL and 6.2 ± 1.5 ng/mL for lovastatin (N.B. The plasma samples were obtained more than 24 hours after the last dose administered). For pravastatin, the differences were statistically significant between the 80 mg/kg/day group and those of 40 or 20 mg/kg/day. However, the difference between the last two groups was not. For lovastatin, the differences between the groups were not statistically significant and might be explained by lower levels due to a longer delay since the plasma sampling was performed several hours after the sampling for the placebo and pravastatin groups.

DISCUSSION

Our results show that lovastatin and pravastatin decrease the ubiquinone levels in the blood, myocardium and skeletal muscle. In addition, it is suggested that the dose-related decrease observed with lovastatin was more pronounced than that with pravastatin. This is consistent with a previous report [26]. We were unable to establish a correlation between the effects observed in the blood and those observed in the myocardium. Therefore, the possibility of detecting a muscular or myocardial ubiquinone deficit through determination of ubiquinone levels in the peripheral blood does not appear to be possible.

Measurements of Ubiquinone Levels: Our objective was to measure the long-term effect of HMG-CoA reductase inhibitors on ubiquinone levels in basal condition, and in order to achieve this, we measured the oxidized form (Qox), i.e. not including ubiquinol (the reduced form). This decision was based on the fact that the main proportion of ubiquinones (in the tissues of interest) exists in the oxidized form: 90% in the rat myocardium [27], 90% in the rat muscle [28]. A specific analysis of the levels of ubiquinone *versus* those of ubiquinol in different tissues [29] also reported high levels of ubiquinone (above 90%) in the myocardium and in the blood cells (erythrocyte, leukocytes and platelets). Because Qred/Qox ratio should not vary following a lipid lowering treatment we are confident that the ubiquinone levels measurements adequately addresses our needs [30]. Treatments used should affect the biosynthesis of ubiquinones, but not the enzymatic system responsible for their reduction. In addition, BHT is added immediately to the homogenate in order to prevent it from any autooxidation and to keep the ubiquinones in their initial form [28].

Since the measurements of ubiquinone were performed under conditions which kept the oxidative changes to a strict minimum, it is very unlikely that the differences observed would have resulted from changes in the Qred/Qox ratio. Therefore it appears that the decrease in the ubiquinone levels observed in the blood, muscle and myocardium of the rat following treatment with HMG-CoA reductase inhibitors represents a potential adverse effect of these drugs. The trend toward an increase observed in the liver is probably related to the over-response in cholesterol biosynthesis in this species further to a cholesterol lowering treatment [24].

Differential Effect of Lovastatin and Pravastatin: With respect to our first objective, since there are known differences in tissue distribution [31-33] between lipophilic lovastatin and hydrophilic pravastatin, their respective effects on selected tissue ubiquinone levels was investigated. The results obtained indicate that lovastatin and pravastatin differentially lowered tissue concentrations of ubiquinones. The greater effect observed with lovastatin, in the myocardium and muscle, can probably be attributed to the differences between the tissular uptake of lovastatin and pravastatin owing to lovastatin's greater lipid solubility and tissue affinity. Previous investigations have shown that lovastatin is present at higher concentrations than pravastatin in the majority of organs with the exception of the liver and ileum [25]. It is noteworthy that, in a recent experiment in human serum, lovastatin led to a greater decrease of ubiquinone levels than pravastatin and that the decreases were dose-dependent [26]. However, this is the first time that the comparison between lovastatin and pravastatin is reported in myocardium, muscle and liver.

Lovastatin and pravastatin did not induce any significant changes in plasma lipid levels. These results are consistent with what has been previously reported [24-25] and are also linked to the over-response with regard to cholesterol biosynthesis in the rats (further to treatment by HMG-CoA reductase inhibitors) [24].

We elected to determine the plasma concentrations of lovastatin and pravastatin for the following reasons: the gavage solutions used in this experiment were prepared from crushed commercial tablets, both drugs are poorly absorbed and no apparent effect can be seen on the plasma lipid levels in rats. The presence of pravastatin and lovastatin in the systemic circulation was confirmed. However, since sampling was performed more than 24 hours after the last dose, low levels were observed and the sensitivity of the assay did not allow an appropriate dose-response evaluation for lovastatin.

Physiological considerations: The tissue levels of ubiquinones after treatment with drugs and chemicals are often changed considerably [34-36]. A number of human pathological conditions such as cardiomyopathy, muscle diseases and ischemia are also associated with decreases in ubiquinone levels [37-40]. The ratio ubiquinone/LDL -C is significantly lower in patients with ischemic heart disease [17]. In the inner mitochondrial membranes, a normal respiratory rate requires the maintenance of a high ubiquinone concentration and even a limited decrease can be deleterious [15]. Also, because of its presence in cellular structure, ubiquinones may play an important role relative to their unique antioxidant properties (ubiquinones being the only antioxidant lipid endogenously synthesized), it would be of interest to evaluate if the decrease in ubiquinone tissue levels after a treatment with the HMG-CoA reductase inhibitors are associated to a decreased capacity of the tissue to withstand an oxidative stress.

CONCLUSIONS

Our results suggest that, in the rat, the decrease in ubiquinone myocardium tissue levels observed with lovastatin is greater than that observed with pravastatin. Thus, in patients with preexisting pathological conditions associated with a decrease in ubiquinone levels, pravastatin may have potential advantages in that it could lower the endogenous antioxidant activity to a lesser extent than lovastatin. However, before such a conclusion is reached, it is necessary to perform additional experiments in *in vivo* animal models where, unlike in the rat model, the cholesterol regulatory pathways mimic those in man.

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