Original Article

Protective effects of coenzyme Q_{10} and L-carnitine against statin-induced pancreatic mitochondrial toxicity in rats

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Abstract

Statins are widely used in patients with hyperlipidemia and whom with high risk of cardiovascular diseases. Unfortunately, statins also exert some adverse effects on the liver and pancreas and enhance the risk of type 2 diabetes mellitus. The objective of the present research was to investigate the protective effects of coenzyme Q_{10} (Co-Q_{10}) and L-carnitine (LC) on statins induced toxicity on pancreatic mitochondria *in vivo*. Seven groups of male Wistar rats received atorvastatin (20 mg/kg, p.o.), atorvastatin + Co-Q_{10} (10 mg/kg, i.p.), atorvastatin + LC (500 mg/kg, i.p.), lovastatin (80 mg/kg, p.o), lovastatin + Co-Q_{10} (10 mg/kg, i.p.), and lovastatin + LC (500 mg/kg, i.p.). Serum glucose and insulin levels were measured before and after two weeks of treatment, while the pancreas was removed and toxic effects of statins, as well as the protective effects of Co-Q10 and LC were assessed. The results showed that atorvastatin and lovastatin significantly increased glucose level and decreased insulin secretion. The glucose level in Co-Q_{10} and LC groups was significantly lower than statins alone groups. The findings also showed that statin groups had higher rate of pancreatic toxicity including higher level of reactive oxygen species production, decreased cytochrome c oxidase activity, collapse of mitochondrial membrane potential and swelling in comparison to controls. These factors were significantly diminished by co-administration of Co-Q_{10} or LC compared to statin groups alone. Additionally, supplements caused a significant increase in serum insulin and succinate dehydrogenase activity. Our study provided new evidence supporting beneficial effects of Co-Q_{10} and LC on statin-induced pancreatic toxicity.

Keywords: Statins; Diabetes mellitus; Pancreatic mitochondria; Coenzyme Q_{10}; L-carnitine

INTRODUCTION

In recent years, the prevalence of diabetes, a major lifestyle disorder, becomes a global burden, and it is rising steeply in developing countries (1). A number of drugs used to reduce cardiovascular risk also predispose to the development of diabetes. These include the thiazide diuretics, beta-blockers, and statins (2). Statins are first choice treatment for hypercholesterolemic patients and can decrease low-density lipoprotein (LDL) cholesterol concentrations and induce atherosclerosis regression (3).

Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) an enzyme involved in the synthesis of cholesterol, especially within the liver. Statins are also used to treat hyperlipidemia in patients with chronic liver diseases and are the most effective drugs for lowering LDL cholesterol (4).

Recent studies suggested that statins are associated with an enhanced risk of developing type 2 diabetes mellitus (5,6). Although precise mechanism (s) of diabetogenesis with statins are under active investigation, there are

several hypotheses including impaired insulin sensitivity and compromised β-cell function via enhanced intracellular cholesterol, decreased insulin secretion, insulin resistance, and hepatotoxicity (7,8).

The statins decrease glucose transporter-2 and -4 expressions in pancreas, muscle, and adipocytes, and lead to decrease insulin secretion and increase insulin resistance. Statins are metabolized to reactive metabolites and increase formation of reactive oxygen species (ROS) in the liver that causes hepatotoxicity and interfere with lipid peroxidation and induce mitochondrial injury (9,10). Other studies showed that statins promote cell death mediated by mitochondrial dysfunction, interaction in calcium homeostasis, inhibition of β-oxidation, inhibition of complex I of the electron transport chain and mitochondrial oxidative stress (11). Recently studies suggest the possible protective mechanism of L-carnitine (LC) and coenzyme Q_{10} (Co-Q_{10}) on statin's toxicity (12,13). LC, an essential mitochondrial respiratory cofactor, plays an important role in the transmission of long chain fatty acids from cytosol to mitochondria. In addition, LC can also improve the antioxidant status and free radical scavenging activity. LC was also disclosed to protect lipid peroxidation by reducing the formation of hydrogen peroxide (12). Co-Q_{10} is one of the most important lipid antioxidants, which prevents the production of free radicals and changes in proteins, lipids, and DNA. Under many disease conditions that are related to increased production and action of ROS, the concentration of Co-Q_{10} in the human body decreases and the deficiency of Co-Q_{10} leads to respiratory chain dysfunction.

The statin medications reduce the production of mevalonate which is the precursor of isoprenoids and cholesterol. Moreover, this reduction in cholesterol, isoprenoids, and prenylated proteins results in reduced dolichol and Co-Q10 concentration which in turn affect the hepatic cell structure and function (14). An important side effect of statins is to decrease the production of Co-Q10, which in turn disturbs cell respiratory chain and ATP generation which results in

pancreatic beta-cells dysfunction and subsequently impairs insulin secretion (15). Statins-mediated Co-Q_{10} depletion decreases the mitochondrial activity of muscle that can induce insulin resistance (16). Moreover, both LC and Co-Q_{10} can directly act as free radical scavenger which protect against statin-induced oxidative damage in skeletal muscle mitochondria (12).

There are physicochemical and pharmacokinetic differences as well as adverse effect profiles between the members of statins which encouraged us to delineate this differences between two statin prototypes; lovastatin and atorvastatin in the current study (17,18). Due to this intra-group diversity and lack of detailed mechanistic information about the toxicity of statins and approved protective effects of LC and Co-Q10 on drugs toxicity in some organs e.g. skeletal muscle, kidney, liver, and heart, we decided to determine the *in vivo* toxic mechanisms of atorvastatin and lovastatin in rat pancreatic mitochondria (13,19,20,21). Besides we examined the *in vivo* protective effects of LC and Co-Q10 on selected statin toxicity on pancreatic mitochondria.

MATERIALS AND METHODS

Materials

D-mannitol, 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFH-DA), Tris-HCl, sodium succinate, sucrose, KCl, Na_2HPO_4, $MgCl_2$, potassium phosphate, Rhodamine 123 (Rh 123), coomassie blue, ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All chemicals were of analytical grades.

Atorvastatin, lovastatin, and L-carnitine were kindly donated by Poursina Pharmaceutical Co. (Tehran, Iran). Coenzyme Q10 (Roche, Switzerland) was a gift from akbarieh Co. (Tehran, Iran).

Treatment of animals

This study was conducted under the supervision of ethical committee of Isfahan University of Medical Sciences (IR.MUI.REC.1394.3.286) for its accordance with the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences. Male Wistar rats weighing 200-250 g were used in this study. The rats were fed a commercial rodent diet and were housed at 22 ± 2 °C on a 12 h light-dark cycle with free access to food and tap water. The animals were fasted for 12 h before start of the treatment in all groups.

Experimental design

Animals were divided into seven groups of 6 animals in each. Groups received normal saline, atorvastatin (20 mg/kg, p.o.), atorvastatin + Co-Q10 (10 mg/kg, i.p.), atorvastatin + LC (500 mg/kg, i.p.), lovastatin (80 mg/kg, p.o.), lovastatin + Co-Q10 (10 mg/kg, i.p.), and lovastatin + LC (500 mg/kg, i.p.). Drugs were administered once daily for two weeks while both the statin and the supplement (Co-Q10 and LC) were given concurrently. Doses of statins and supplements (Co-Q10 and LC) were chosen based on literature reports (22,23,24). Serum glucose and insulin levels were measured before and after 2 weeks of treatments using a commercial glucometer (Acuu-Check®, Germany) and Insulin ELISA Kit (Mercodia Rat Insulin ELISA, Uppsala, Sweden), respectively. After finishing blood sampling, the animal pancreas was removed and the diabetogenic and apoptotic effects of drugs and supplements on pancreatic mitochondria were assessed.

Isolation of mitochondria from rat pancreas

Mitochondria were prepared from rats' pancreas using differential centrifugation (25). After homogenizing of tissues, the nuclei and broken cell debris were precipitated using a centrifuge at 1500 g for 10 min at 4 ºC and the pellets were discarded. The supernatant was subjected to a further centrifugation at 10,000 g for 10 min. and the supernatant was discarded. After washing and centrifuging (10,000 g for 10 min), the mitochondrial pellets were suspended in Tris buffer containing 0.05 M Tris-HCl, 0.25 M sucrose, 20 Mm KCl, 2.0 mM MgCl$_2$, and 1.0 mM Na$_2$HPO$_4$, pH = 7.4 at 4 ºC, except for the mitochondria used to assess ROS production, mitochondrial membrane potential (MMP) and swelling, which were suspended in respiration buffer (0.32 mM sucrose,10 mM Tris, 20 mM Mops, 50 µM EGTA, 0.5 mM MgCl$_2$, 0.1 mM KH$_2$PO$_4$, and 5 mM sodium succinate), MMP assay buffer (220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH$_2$PO$_4$, 2 mM MgCl$_2$, 50 µM EGTA, 5 mM sodium succinate, 10 mM HEPES, and 2 µM Rotenone), and swelling buffer (70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM tris-phosphate, 5 mM succinate, and 1 µM of rotenone). Mitochondria were prepared freshly for each experiment and kept in a dried condition on ice for a maximum of 4 h to ensure the isolation of high-quality mitochondrial preparation.

Succinate dehydrogenase activity assay

The alteration in succinate dehydrogenase (SDH) or mitochondrial complex II activity was determined by reduction in MTT (26). Briefly, mitochondria (0.5 mg protein/mL) were suspended in Tris buffer. Then 25 µL of MTT was added to 100 µL mitochondrial suspensions and incubated at 37 °C for 30 min. Finally, the produced formazan crystals were dissolved in 75 µL DMSO and the absorbances were measured at 570 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Tecan, Rainbow Thermo, Austria).

Determination of mitochondrial ROS level

The ROS was detected and measured by method previously described (27,28). Briefly, the isolated mitochondria from the pancreas were transferred to respiration buffer. Afterward, dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescent probe used for ROS measurement was added (final concentration, 10 µM) to the mitochondrial suspension and then incubated at 37 ºC.

The fluorescence intensity of dichloro-fluorescein (DCF) was measured using a fluorescence spectro-photometer (Shimadzu RF5000U, Japan) at the excitation and emission wavelengths of 488 nm and 527 nm, respectively.

Determination of the mitochondrial membrane potential

MMP was measured by determination of Rh 123 mitochondrial uptake (cationic fluorescent dye for MMP assay). The fluorescence was monitored using a Shimadzu RF-5000U fluorescence spectrophotometer, Japan at the excitation and emission wavelengths of 490 nm and 535 nm, respectively (29).

Determination of mitochondrial swelling

Analysis of mitochondrial swelling in isolated mitochondria (0.5 mg protein/mL) was estimated by determination of changes in light scattering as monitored spectrophoto-metrically at 549 nm (30 °C) with an ELISA reader (Tecan, Rainbow Thermo, Austria) as described previously (28,29).

Measurement of cytochrome C oxidase activity and assessment of outer mitochondrial membrane damage

Both factors were measured using cytochrome c oxidase assay kit (Sigma, St. Louis, MO) according to the manufacturer protocol. The assay was based on a decrease in the absorbance of ferrocytochrome c at 550 nm caused by its oxidation to ferricytochrome c by cytochrome c oxidase. Briefly, 20 µg freshly isolated mitochondrial fraction was used and diluted in the enzyme dilution buffer (10 mM Tris_HCl, pH = 7.0, containing 250 mM sucrose) with 1 mM n-dodecyl b-D-maltoside and incubated on ice for 30 min. Freshly prepared ferrocytochrome c substrate solution (0.22 mM) was added to the sample. A decrease of the absorbance at 550 nm is related to the oxidizing reaction. Cytochrome c oxidase activities were calculated and normalized for the amount of protein per reaction, and the results were expressed as U/mg mitochondrial protein. Mitochondrial outer membrane integrity was assessed by measuring cytochrome c oxidase activity of mitochondria in the presence or absence of the detergent, n-dodecyl β-D-maltoside, which is one of the few detergents that allow the maintenance of the cytochrome c oxidase dimer in solution at low detergent concentrations. The ratio between activity with and without n-dodecyl β-D-maltoside is a measure of the mitochondrial outer membrane integrity (28,29).

Statistical analyses

The data of this study are generally presented as mean ± SEM (n = 6). The statistical analyses were performed using the Graph Pad Prism software (V. 6). Assays were at least performed three times. Statistical significance ($P < 0.05$) was carried out using one-way ANOVA test.

RESULTS

Effects of statins, coenzyme Q10 and L-carnitine on glucose and insulin serum levels

Atorvastatin and lovastatin administration developed diabetes by increasing fasting glucose levels and decreasing insulin secretion ($P < 0.05$). This diabetogenic activity, on the other hand, was prevented by co-administration of Co-Q₁₀ or LC as showed in Table 1.

Protective effect of coenzyme Q10 and L-carnitine against statin's pancreatic toxicity
Mitochondrial succinate dehydrogenase activity

The viability of pancreatic mitochondria was measured by SDH activity using mitochondria obtained from pancreas following 1 h of incubation. Fig. 1A and 1B shows that administration of atorvastatin (20 mg/kg) and lovastatin (80 mg/kg) significantly decreased the viability of pancreatic mitochondria in comparison with control group (at least $P < 0.01$).

Table 1. Protective effect of Co-Q₁₀ or L-carnitin (LC) on serum glucose and insulin levels of rats treated with atorvastatin (ATV) or lovastatin (LVT).

Parameters mean ± SD	Control	ATV	ATV + Co-Q10	ATV + LC	LVT	LVT + Co-Q10	LVT + LC
Glucose (mmol/L)	8.00 ± 1.6	16.4 ± 2.0*	13.6 ± 2.4#	11.8 ± 1.3#	13.7 ± 2.0*	10.3 ± 2.4#	9.50 ± 3.3#
Insulin (µU/mL)	24.4 ± 7.0	14.1 ± 1.1*	21.2 ± 5.3#	22.1 ± 6.0#	16.3 ± 2.1	22.6 ± 9.0#	23.3 ± 8.2#

Data are presented as mean ± SEM, n = 6. * Significantly different from control. #Significantly different from same statin-treated group.

The results also showed that the administration of CO-Q10 significantly increased SDH activity in comparison with statins treatment alone (at least $P < 0.05$). In LC groups, the effect of lovastatin on SDH activity was increased significantly ($P < 0.05$) with LC pretreatment however; this supplement had no effect on atorvastatin treated group ($P > 0.05$).

Mitochondrial ROS Level

As shown in Fig. 2A and 2B, administration of atorvastatin and lovastatin induced significant ($P < 0.001$) H_2O_2 formation demonstrated as fluorescence intensity emitted from highly fluorescent DCF in the mitochondria obtained from the pancreas. The results also showed that the administration of CO-Q10 or LC decreased the toxic effect of statins on ROS formation.

The results also showed that atorvastatin was probably more effective than lovastatin towards ROS formation.
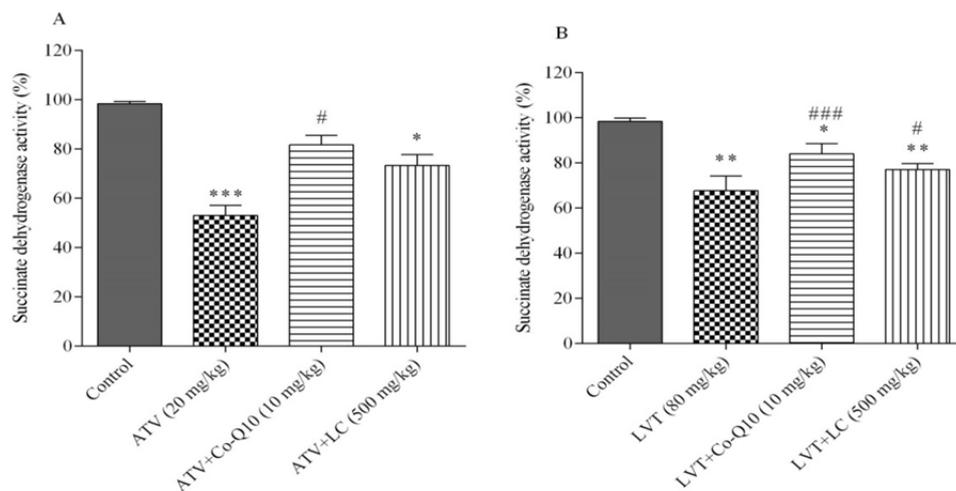

Fig. 1. The effects of atorvastatin (ATV) and lovastatin (LVT) alone or in combination with Co-Q10 or L-carnitine (LC) on succinate dehydrogenase (SDH) activity. SDH activity was measured using MTT assay. Data are presented as mean ± SEM (n = 6). One-way ANOVA was performed. *, **, and *** significantly different from the control ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively). # and ### significantly different from atorvastatin and lovastatin alone groups ($P < 0.05$ and $P < 0.001$, respectively).
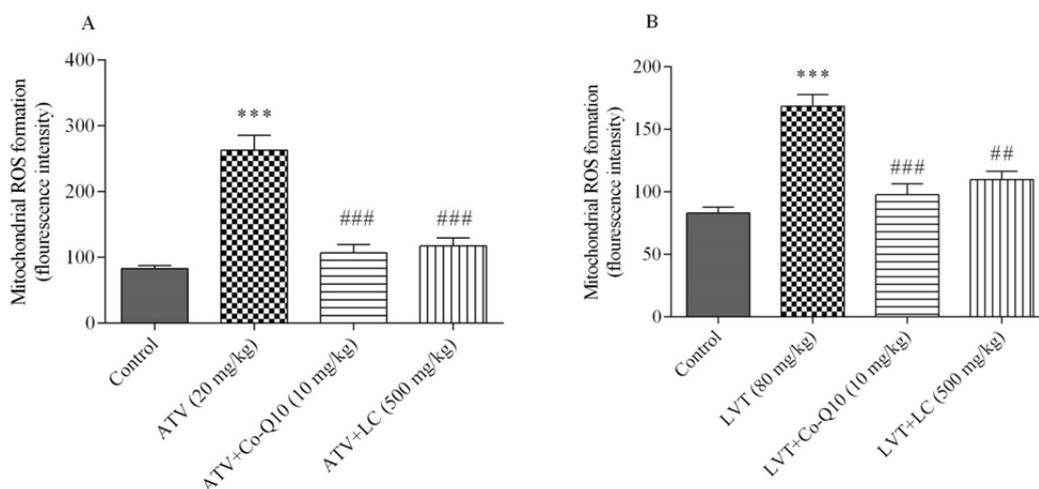

Fig. 2. The effects of atorvastatin (ATV) and lovastatin (LVT) alone or in combination with Co-Q10 or L-carnitine (LC) on mitochondrial ROS formation in rat pancreas. ROS formation was measured fluorometrically using DCF-DA. Data are presented as mean ± SEM (n = 6). One-way ANOVA was used to analyse the data. . *** significantly different from the control ($P < 0.001$). ## and ### significantly different from atorvastatin and lovastatin alone groups ($P < 0.01$ and $P < 0.001$, respectively).

Mitochondrial membrane potential

MMP, as an important factor in assessment of mitochondrial functionality, showed that atorvastatin and lovastatin caused mitochondrial dysfunction and intensity of fluoresce (Fig. 3A and 3B). Co-administration of CO-Q10 and LC effectively protected cellular mitochondria against statins induced mitochondrial injury as revealed by an improvement in mitochondrial membrane potential ($P < 0.01$).

Mitochondrial swelling

The mitochondrial swelling as a subsequent event after mitochondrial permeability transition pore opening was also assayed during this study. Our findings indicate that statins increased mitochondrial swelling (decrease of absorbance). In contrast, CO-Q10 and LC effectively reversed the mitochondrial swelling in isolated mitochondria ($P < 0.001$) when compared with statins treatment alone (Fig. 4A and 4B).
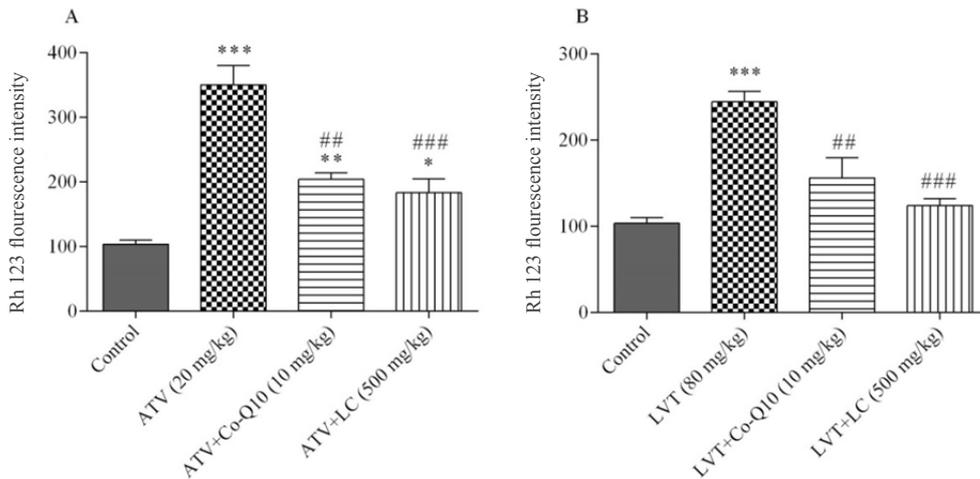

Fig. 3. The effects of atorvastatin (ATV) and lovastatin (LVT) alone or in combination with Co-Q10 or L-carnitine (LC) on the mitochondrial membrane potential (MMP) in pancreas. MMP was measured by cationic probe rhodamine 123. Data are presented as mean ± SEM (n = 6). One-way ANOVA was used to analyse the data. . *, **, and *** significantly different from the control ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively). ## and ### significantly different from atorvastatin and lovastatin alone groups ($P < 0.01$ and $P < 0.001$, respectively).
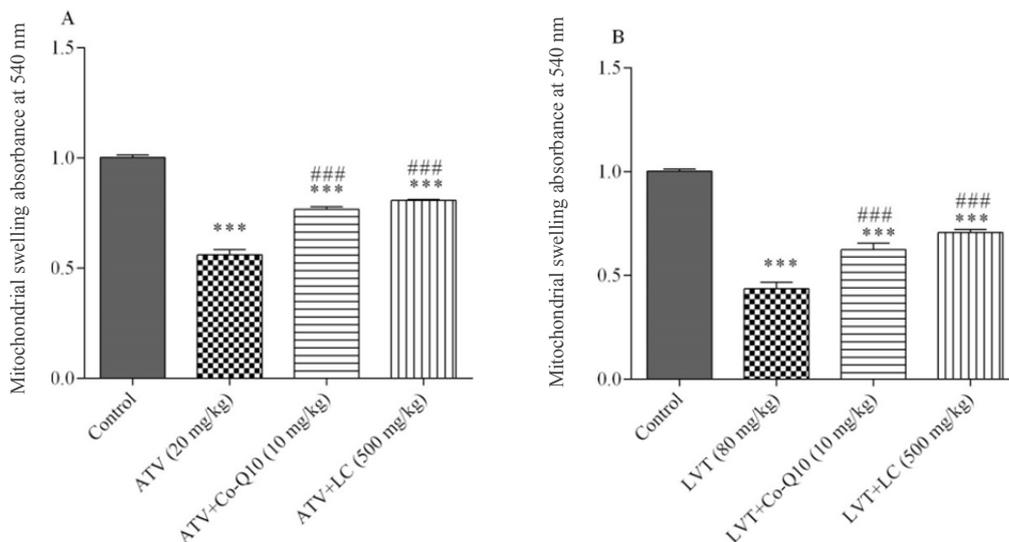

Fig. 4. The effects of atorvastatin (ATV) and lovastatin (LVT) alone or in combination with Co-Q10 or L-carnitine (LC) on the mitochondrial swelling in pancreas mitochondria. Mitochondrial swelling was measured through the determination of absorbance at 549 nm. Data represented as mean ± SEM (n = 6). One-way ANOVA was used to analyse the data. *** Significantly different from the control ($P < 0.001$). ### Significantly different from atorvastatin or lovastatin alone groups ($P < 0.001$).

Measurement of cytochrome c oxidase activity and assessment of outer mitochondrial membrane damage

As shown in Fig. 5A and 5B and Fig. 6A and 6B, results indicate that atorvastatin and lovastatinat at doses of 20 mg/kg and 80 mg/kg induced significant ($P < 0.001$) reductions in the activity of enzyme cytochrome c oxidase and disruption of mitochondrial outer membrane integrity in the pancreas mitochondria. Pre-treatment of rats with Co-Q10 or LC prevented this decrease in the activity of enzyme cytochrome c oxidase and disruption of mitochondrial outer membrane in comparison with groups treated with atorvastatin and lovastatin alone ($P < 0.01$).
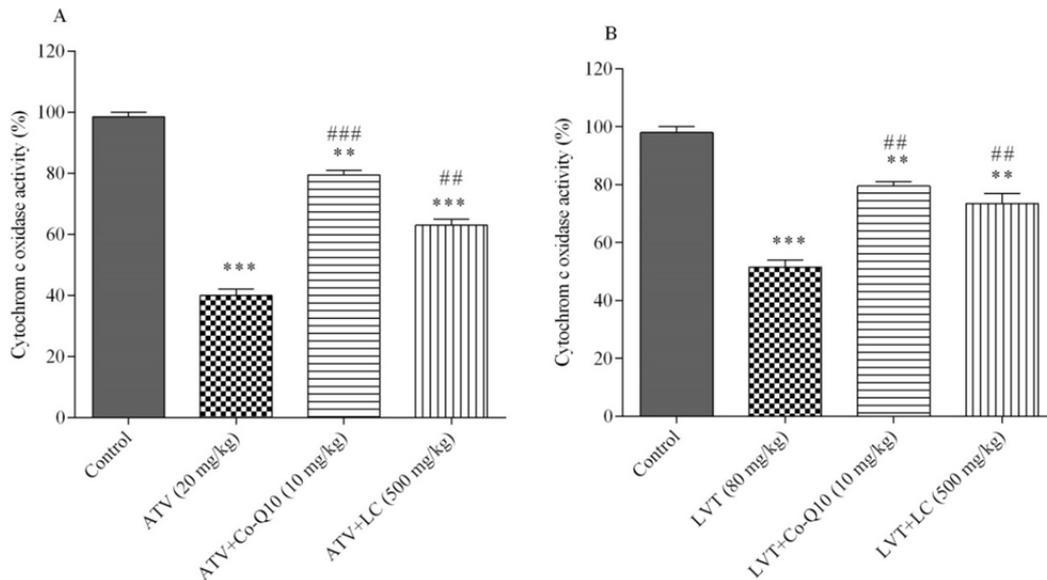

Fig. 5. The effects of atorvastatin (ATV) and lovastatin (LVT) alone or in combination with Co-Q10 or L-carnitine (LC) on the cytochrome c oxidase (complex IV) activity. Data are presented as mean ± SEM (n = 6). One-way ANOVA was used to analyse the data. ** and *** significantly different from the control ($P < 0.01$ and $P < 0.001$, respectively). ## and ### significantly different from atorvastatin and lovastatin alone groups ($P < 0.01$ and $P < 0.001$, respectively).
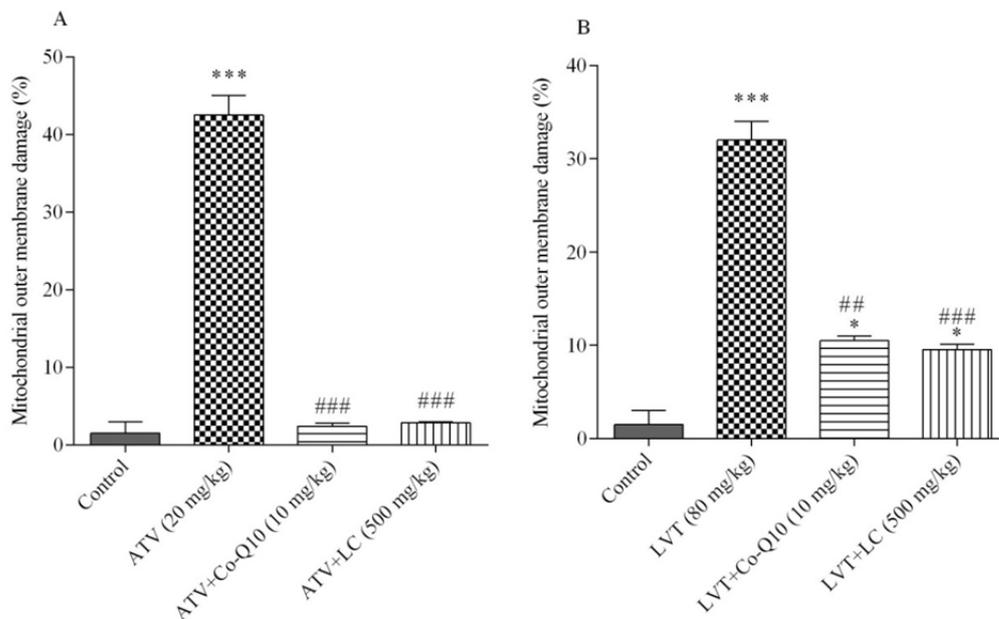

Fig. 6. The effects of atorvastatin (ATV) and lovastatin (LVT) on mitochondrial outer membrane integrity. Data are presented as mean ± SEM (n = 6). One-way ANOVA was used to analyse the data. * and *** significantly different from the control ($P < 0.5$ and $P < 0.001$, respectively). ## and ### significantly different from atorvastatin or lovastatin alone groups ($P < 0.5$ and $P < 0.001$, respectively).

DISCUSSION

In this study, administration of atorvastatin and lovastatin developed diabetes by increasing blood glucose level and decreasing insulin secretion in rats. Additionaly the statins increased ROS formation, mitochondrial swelling, decresed mitochondrial membrane potential, and declined cytochrome c oxidase activity. Co-administration of tested statins with LC and Co-Q10, on the other hand, significantly protected pancrese against statins toxicity.

The statins are one of the most widely recommended medications in the world for their benefits in prevention of cardiovascular diseases though, their adverse effects such as myopathy (9), diabetes risk (8,30), renal injury and rhabdomyolysis (31) have been also reported.

Emerging evidences show that therapy with statins (e.g. rosuvastatin, atorvastatin, and simvastatin) raises the risk of type 2 diabetes mellitus (32,33). Although, the mechanisms of induction of diabetes with the consumption of statins have not been fully understood, published studies have indicated that one of the mechanisms of statin-induced diabetes is an increase in insulin resistance, which is reflected through hyperglycemia (34,35). Hyperglycemia induces free radical production such as H_2O_2 that impairs the endogenous antioxidant defense system in patients with diabetes and leads to domination of the condition called oxidative stress (36).

Here it was shown that atorvastatin and lovastatin could potentially induce hyperglycemia and decrease insulin secretion in the pancreas. It seems that mitochondrial damage due to statins is principally responsible for pancreatic toxicity, however, in recent study increased ROS formation and mitochondrial damage were concurrently occurred with hyperglycemia and also a decline in serum insulin concentration. In acute phase it is unlikely that hyperglycemia could result in pancreatic damage, however, in long term period and after diabetes aggravation it might be possible.

Statins can impair mitochondrial biogenesis by production of a large amount of ROS and trigger deleterious effects on mitochondrial function (37). It seems that same mechanisms for disruption of mitochondrial function in skeletal muscles and in pancreatic tissue are probably taking place by statins (32). Our data showed that statins induce significant changes in mitochondrial membrane potential. Abdoli, *et al.* (38) showed that treating hepatocytes with statins produces a significant amount of ROS, induces lipid peroxidation, reduces MMP, and promotes cytotoxicity.

The results indicate that atorvastatin and lovastatin induced significant decline in cytochrome c oxidase activity (complex IV) and disrupted mitochondrial outer membrane integrity in pancreas mitochondria. Therefore, we hypothesized that statins might induce release of cytochrome c from mitochondria into cytosolic medium and initiate apoptosis signaling. Kato, *et al.* (39) showed that treatment with statins caused an increase in cytochrome c activity of mitochondria, indicating activation of an intrinsic pathway.

Co-Q10 and LC are recently considered as two important protectors of statins side effects on isolated mitochondria (12,13). Our results are in accordance with other studies demonstrating that usage of supplements such as Co-Q10 and LC reduced oxidative stress and toxic effects of statin-induced injury (12,40). Co-Q10 and LC act as potent antioxidants by scavenging ROS resulting in protection of the cells against oxidative stress in many disease conditions (41,42).

In the present study, the results indicated that Co-Q10 and LC can reduce the toxic effects of statins and improve mitochondrial dysfunction during 2-week treatment. Previous studies reported that statin treatment (atorvastatin and lovastatin) reduced the levels of Co-Q10, which is part of electron transport chain involved in the process of ATP production (32,43).

On the other hand, Co-Q10 pretreatment inhibited mitochondrial damage, expression of cytochrome c and cell apoptosis (44). The same inhibitory effect was observed with LC administration (45). This study, therefore, addresses the possible anti-apoptotic activity of Co-Q10 and LC in rats treated with atorvastatin and lovastatin.

In addition Co-Q$_{10}$ and LC reversed the statins-induced MMP impairement and mitochondrial swelling and significantly prevented statins-induced decline in cytochrome c oxidase activity. These observationsare in accordance with the previous study which showed that Co-Q$_{10}$ remarkably inhibited the mitochondrial swelling in rat liver treated with statins (46). Therefore, current results provide good evidences that coadministration Co-Q$_{10}$ or LC with statins reduce statins toxicity especially those related to pancreatic mitochondrial biogenesis and activity.

CONCLUSIONS

Taken together, it is suggested that Co-Q$_{10}$ and LC supplementation could be considered as a combination therapy strategy for patients treated with statins and are prone to higher levels of oxidative stress and inflammation. Furthermore, this supplementation therapy can reduce the incidence of side effects of statins and patients could better tolerate their statin therapy.

ACKNOWLEDGEMENTS

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