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Toxicity of Atorvastatin on Pancreas Mitochondria: A Justification for Increased Risk of Diabetes Mellitus

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Abstract: Statins (including atorvastatin) are a widely used class of drugs, and like all medications, they have a potential for adverse effects. Recently, it has been shown that statins also exert side effects on the pancreas. *In vitro* studies have suggested that this class of drugs induced a reduction in insulin secretion. Also, the use of statins is associated with a raised risk of diabetes mellitus (DM), but the mechanisms underlying statin-induced diabetes are poorly known. Literature data indicate that several statins are able to induce apoptosis signalling. This study was designed to examine the mechanism of atorvastatin on mitochondria obtained from rat pancreas. In our study, mitochondria were obtained from the pancreas and then exposed to atorvastatin and vehicle to investigate probable toxic effects. The results showed that atorvastatin (25, 50, 75, 100 and 125 μM) increased reactive oxygen species (ROS) production, mitochondrial swelling, collapse of mitochondrial membrane potential and cytochrome *c* release, the orchestrating factor for mitochondria-mediated apoptosis signalling. Atorvastatin also reduced the ATP levels. These results propose that the toxicity of atorvastatin on pancreas mitochondria is a key point for drug-induced apoptotic cell loss in the pancreas and therefore a justification for increased risk of DM.

Statins (HMG-CoA reductase inhibitors) as anti-hyperlipidaemic drugs are widely used for prevention of stroke. Epidemiological studies have reported conflicting results associated with statin use and pancreatic cancer. Thus, several studies have shown beneficial effects of statins on pancreatic cancer risk through the induction of apoptosis signalling, but some meta-analyses did not confirm this effect [1,2]. Decreased mitochondrial function was presumed as an important reason of statin-induced myopathy. Long-term atorvastatin therapy induced impaired mitochondrial function in human striated muscles [3,4]. Simvastatin-treated H9c2 cells showed a decline in the mitochondrial membrane potential (MMP) ($\Delta\psi m$) and a decreased activity of various enzyme complexes in the electron transport chain (ETC) in the mitochondria [5].

Previous research showed that some statins (especially lovastatin) induced apoptosis via the mitochondrial pathway in neuronal cell lines [6]. Other studies reported that treatment with statins such as lovastatin, atorvastatin and simvastatin could induce acute pancreatitis [7–9]. It was reported that the use of statins affects the mitochondria. Besides, mitochondrial mechanisms have been implicated in muscle adverse effect many times [10].

It has been shown that mitochondria are used as a biosensor for the detection of drug-induced toxicity. This organelle plays a main role in energy production, biosynthesis and generation of reactive oxygen species (ROS) and apoptosis. Mitochondrial dysfunction is connected with several disorders such as diabetes [11].

Today, concerns have increased regarding enhanced incidence of new-onset diabetes mellitus (DM) observed in clinical trials of statin treatment [12]. Diabetes is a class of metabolic disorders characterized by abnormally high level of blood sugar resulting from defects in insulin production (insulin deficiency), insulin action (insulin resistance) or both [13]. Previous studies reported that therapy with statin (including atorvastatin and simvastatin) is accompanied by increased risk of type 2 diabetes (T2DM) through decreases in insulin secretion and insulin sensitivity [14,15]. It has also been documented that incident DM associated with statin use is possibly more common in older people, in women and in Asians [16]. However, the key mechanisms of statin-induced DM are poorly known. Mitochondrion is a key player in bridging glucose metabolism to insulin secretion and is therefore the main controlling site for glucose-stimulated insulin secretion in pancreatic ß cells. The mitochondrial defects associated with ROS production might result in impaired metabolic coupling and induction of apoptosis in the ß cell [17,18].

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In recent decades, due to a further conception of mitochondrial involvement, newer assays such as mitochondrial ROS production, decline in the MMP and induction of mitochondrial permeability transition (MPT) (such as mitochondrial swelling or cytochrome *c* expulsion) have been designed [19].

Due to lack of detailed mechanistic information about toxicity of atorvastatin on the pancreas and obvious role of ATP depletion in pancreatic cells in the pathogenesis of T2DM, we decided to determine the toxic mechanisms of atorvastatin on pancreas mitochondria obtained from the rat.

Materials and Methods

Animals. Male Sprague Dawley rats (200–300 g) fed a standard chow diet and given water *ad libitum* were used in all experiments. The animals were purchased from Institute Pasteur (Tehran, Iran). All animals were maintained in a controlled room temperature of 20–25°C and a humidity of 50–60% and were exposed to a 12-hr light/dark cycle. The protocols approved for the study were conducted according to the ethical standards and the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran. All efforts were made to minimize the number of animals used and their suffering.

Isolation of mitochondria from rat pancreas. Mitochondria were prepared from the Sprague Dawley rat pancreas using differential centrifugation. Pancreas tissues were excised and minced in a cold mannitol solution (0.225 M D-mannitol, 75 mM sucrose and 0.2 mM EDTA). In the next step, minced tissue was homogenized and then centrifuged to remove nuclei, unbroken cells and other non-subcellular debris. Subsequently, supernatant was subjected to a further centrifugation and the superior layer was carefully discarded from the pellet containing mitochondria [20,21]. For the evaluation of each experiment, mitochondria should be prepared freshly and kept in a dried condition on ice for a maximum of 4 hr. In the toxicity assessment using isolated mitochondria technique, we suspended the mitochondria in different water-based working buffers at laboratory temperature (25°C) before the addition of drug or poison. In this regard, we performed toxicity studies within 60 min., because all the destructive proteases and phospholipases will be active in the isolated mitochondria at a temperature above 4°C. This is why we determined all mitochondrial toxicity end-points within 60 min. after drug exposure to the mitochondria.

Complex II or succinate dehydrogenase activity assay. The alteration in succinate dehydrogenase (SDH) or mitochondrial complex II activity by atorvastatin, simvastatin, lovastatin and pravastatin was measured by reduction in MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide). Mitochondria (500 μg protein/ml) obtained were suspended in Tris buffer. In the next step, 100 ml mitochondrial suspensions was incubated with all applied concentrations of atorvastatin, simvastatin, lovastatin and pravastatin (0, 25, 50, 75, 100 and 125 μM) at 37°C for 30 min. Finally, the product formazan crystals was dissolved in 75 ml DMSO and absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow Thermo, Austria) [22]. As the IC_{50} we obtained for atorvastatin on pancreatic mitochondria with MTT assay was about 125 μM, we decided to select a concentration range lower than IC_{50} for atorvastatin inclusion (25, 50, 75, 100 and 125 μM) to avoid very toxic conditions. The IC_{50} concentration for a chemical treated on isolated mitochondria is defined as concentrations that inhibit the activity of mitochondria SDH (respiratory complex II) by 50%.

Determination of mitochondrial ROS level. In this experiment, isolated mitochondria from the pancreas were placed in respiration buffer. Afterwards, DCFH-DA (dichloro-dihydro-fluorescein diacetate, fluorescent probe used for ROS measurement) was added (final concentration, 10 μM) to the mitochondria suspension and then incubated for 15 min. at 37°C. In the next step, the fluorescence intensity of DCF (dichlorofluorescein) as a ROS level determination after the addition of various concentrations of atorvastatin (0, 25, 50, 75, 100 and 125 μM) at different time intervals within 60 min. (5, 15, 30, 45 and 60 min.) of incubation in the mitochondria obtained from pancreases rats was measured using a fluorescence spectrophotometer at the EXλ = 488 nm and EMλ = 527 nm [22].

Determination of the mitochondria membrane potential. Briefly, 10 μM of Rhodamine 123 (Rh 123) (cationic fluorescent dye for MMP assay) was added to the mitochondrial suspensions in the corresponding buffer. The MMP was measured after the addition of various concentrations of atorvastatin (0, 25, 50, 75, 100 and 125 μM) at different time intervals within 60 min. (5, 15, 30, 45 and 60 min.) of incubation in the mitochondria obtained from the rat pancreas. The cytosolic Rh 123 fluorescence intensity which indicates the redistribution of the Rh 123 from mitochondria into the cytosol was determined at the EXλ = 490 nm and EMλ = 535 nm [23].

Determination of mitochondrial swelling. In this experiment, isolated mitochondria from pancreas were suspended in swelling buffer and incubated at 30°C with 25, 50, 75, 100 and 125 μM of atorvastatin. Afterwards, absorbance was assayed at 549 nm at within 60 min. (5, 15, 30, 45 and 60 min.) of incubation in the mitochondria obtained from the rat pancreas with all applied concentration of atorvastatin with an ELISA reader (Tecan). The decrease in absorbance represents an increased swelling of the mitochondria [23].

Cytochrome c *assay.* The release of cytochrome *c* from mitochondria by atorvastatin was assayed by the Quantikine Rat/Mouse cytochrome *c* Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, MN, USA).

ATP assay. In this study, the ATP level was assayed through the luciferase enzyme [24].

Statistical analysis. All results in this study are presented as mean ± S.D. (n = 3). The statistical analyses were performed with the GraphPad Prism software (version 5) (GraphPad Software, Inc.7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA). Assays were performed three times. Statistical significance (set at $p < 0.05$) was carried out using one-way and two-way ANOVA tests.

Results

Mitochondria (500 μg protein/ml) obtained from the pancreases were suspended in the corresponding buffers. Three animals were used for mitochondrial isolation in each test. The mitochondria parameters were assayed after the addition of different concentrations of atorvastatin (0, 25, 50, 75, 100 and 125 μM) at several time intervals within 60 min. (5, 15, 30, 45 and 60 min.) of incubation. Also, the one-way ANOVA test was used as a specific statistical analysis for the determinations of SDH activity, cytochrome *c* release and ATP level. The two-way ANOVA test was used for the determinations of mitochondrial ROS level, MMP and mitochondrial swelling.

Atorvastatin reduced activity of SDH.

It was reported that the mitochondrial dysfunction in ß cells is associated with the pathogenesis of diabetes. The effects of atorvastatin, simvastatin, lovastatin and pravastatin (0, 25, 50, 75, 100 and 125 μM) on SDH activity (determined as % of enzyme activity) were measured by the MTT assay, using mitochondria obtained from pancreas after 1 hr of incubation. Our results showed that only atorvastatin in high concentrations (75, 100 and 125 μM) significantly ($p < 0.05$) reduced the activity of complex II in the isolated pancreas mitochondria (fig. 1). Simvastatin, lovastatin and pravastatin in all applied concentrations (0, 25, 50, 75, 100 and 125 μM) did not significantly affect the SDH activity. Our results showed that atorvastatin was much more toxic than other statins towards pancreatic mitochondria.

Atorvastatin induced ROS formation.

Reactive oxygen species affect the insulin signalling cascade. On the other hand, pancreatic ß-cells are especially vulnerable to ROS because their natural enzymatic antioxidant defence is lower than other tissues. It has been shown that hyperglycaemia can stimulate free radical production. As shown in fig. 2, various concentrations of atorvastatin (50, 75, 100 and 125 μM) induced significant ($p < 0.05$) H_2O_2 formation demonstrated as fluorescence intensity emitted from highly fluorescent DCF in the mitochondria obtained from the pancreas. Results showed that the lowest concentration of atorvastatin (25 μM) did not significantly increase H_2O_2 within 60 min. of incubation.

Atorvastatin declined MMP.

It is well understood that mitochondria play a vital role in apoptosis via the redistribution of intermembranous mitochondrial pro-apoptotic proteins (including cytochrome *c*) and the MMP causes opening of the MPT pore. The findings shown in fig. 3 indicate that atorvastatin in concentrations of 50, 75, 100 and 125 μM significantly ($p < 0.05$) decreased the MMP (demonstrated as fluorescence intensity emitted from Rh123

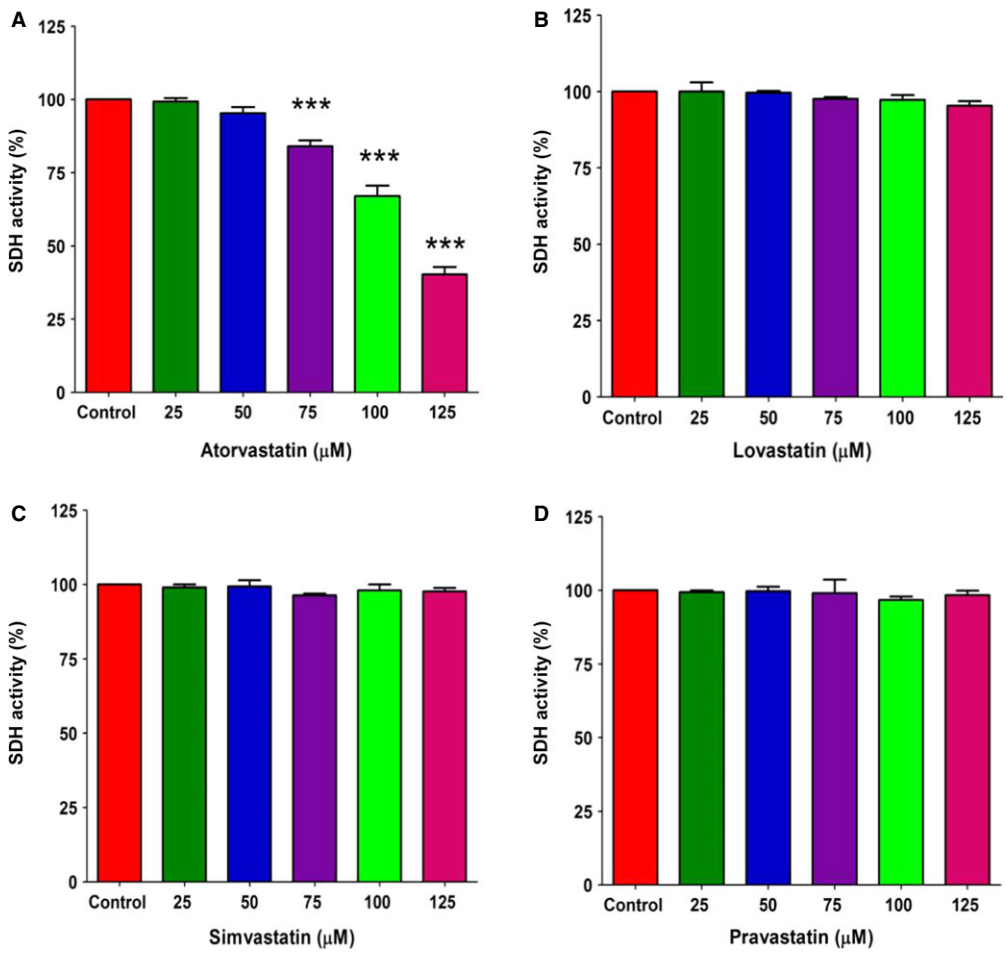

Fig. 1. The effect of atorvastatin on succinate dehydrogenase (SDH) activity. SDH activity was measured using 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT). The pancreas mitochondria (0.5 mg/ml) were incubated for 1 hr with various concentrations of (A) atorvastatin (0, 25, 50, 75, 100 and 125 μM) (B); lovastatin (0, 25, 50, 75, 100 and 125 μM); (C) simvastatin (0, 25, 50, 75, 100 and 125 μM) (D) and pravastatin (0, 25, 50, 75, 100 and 125 μM). Data represented as mean ± S.D. of data determined from three separate experiments. Pancreas mitochondria obtained from three separate rats (n = 3) in each experiment in comparison with the control group. The one-way ANOVA test was performed. ***Significantly different from the corresponding control (atorvastatin concentration = '0 μM') ($p < 0.001$).

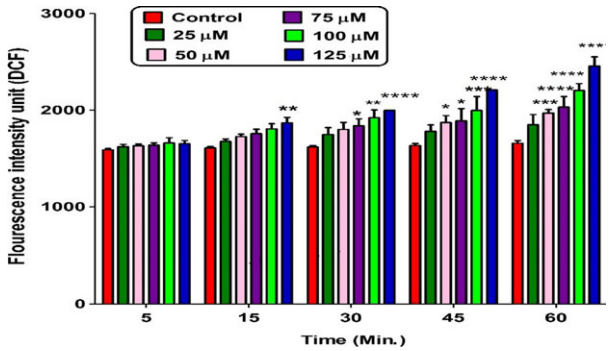

Fig. 2. Measurement of mitochondrial reactive oxygen species (ROS) formation. The ROS level was assayed after the addition of various concentrations of atorvastatin (0, 25, 50, 75, 100 and 125 μM) at different time intervals within 60 min. (5, 15, 30, 45 and 60 min.) of incubation in the mitochondria obtained from rat pancreas. Data represented as mean ± S.D. of data determined from three separate experiments. Pancreas mitochondria obtained from three rats in each experiment in comparison with the control group. The two-way ANOVA test was performed. *, **, *** and ****significantly different from the corresponding control (atorvastatin concentration = '0 μM') ($p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively).

redistributed from damaged mitochondria into the cytosol) in a time- and concentration-dependent manner in the rat pancreas mitochondria compared with control mitochondria. Also, our finding indicates that atorvastatin at 25 μM did not significantly decrease MMP within 60 min.

Atorvastatin induced mitochondrial swelling.

The mitochondrial swelling and cytochrome *c* release as subsequent events after MPT pore opening were also assayed during this study. In the next step, we assayed the decrease in absorbance of mitochondria suspensions at 540 nm to determine swelling in the mitochondrion, which is an indicator of MPT. Our findings indicate that most of the concentrations of atorvastatin led to significant ($p < 0.05$) mitochondrial swelling. Only lower concentrations (25 and 50 μM) did not significantly increase mitochondrial swelling within 5–60 min. of incubation (fig. 4).

Atorvastatin increased cytochrome c *release.*

Cytochrome *c* (Cyt *c*) as a pro-apoptotic protein plays a main role in the induction of apoptosis signalling. As shown in fig. 5, results indicate that atorvastatin at a concentration of 75 μM induced significant ($p < 0.05$) expulsion of cytochrome *c* (ng/mg mitochondrial protein) from the mitochondria into the buffer media. Pre-treatment of atorvastatin (75 μM)-treated mitochondria with the MPT inhibitors like cyclosporine A (CsA) and antioxidants such as butylated hydroxyl toluene (BHT) decreased atorvastatin-induced cytochrome *c* release ($p < 0.05$).

Atorvastatin decreased ATP level.

Studies have shown an association between mitochondrial oxidative energy and alterations in mitochondrial function, including declined production of ATP. As shown in fig. 6, mitochondrial ATP level significantly declined by different concentrations of atorvastatin (75, 100 and 125 μM) in a concentration-dependent manner in comparison with the control

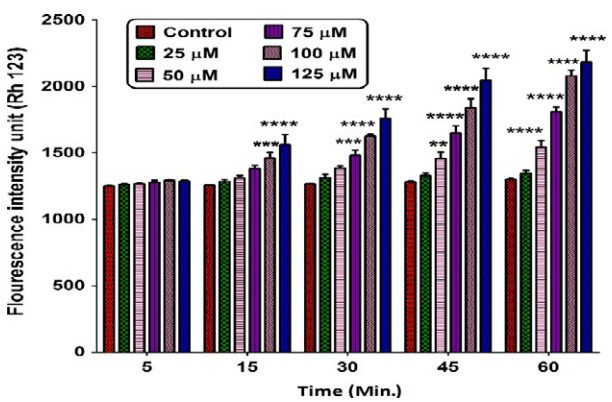

Fig. 3. Evaluation of the decline in the mitochondrial membrane potential (MMP). The decline of MMP was assayed after the addition of various concentrations of atorvastatin (0, 25, 50, 75, 100 and 125 μM) at different time intervals within 60 min. (5, 15, 30, 45 and 60 min.) of incubation in the mitochondria obtained from rat pancreas. Data represented as mean ± S.D. of data determined from three separate experiments. Pancreas mitochondria obtained from three rats in each experiment in comparison with the control group. The two-way ANOVA test was performed. *, ** and ****significantly different from the corresponding control (atorvastatin concentration = '0 μM') $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively.

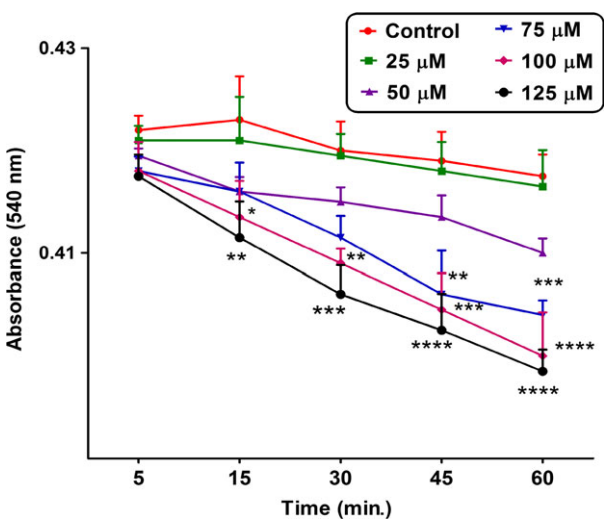

Fig. 4. Mitochondrial swelling assay. The mitochondria swelling was assayed after the addition of various concentrations of atorvastatin (0, 25, 50, 75, 100 and 125 μM) at different time intervals within 60 min. (5, 15, 30, 45 and 60 min.) of incubation in the mitochondria obtained from rat pancreas. Data represented as mean ± S.D. of data determined from three separate experiments. Pancreas mitochondria obtained from three rats in each experiment in comparison with the control group. The two-way ANOVA test was performed. *, **, *** and ****significantly different from the corresponding control (atorvastatin concentration = '0') ($p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively).

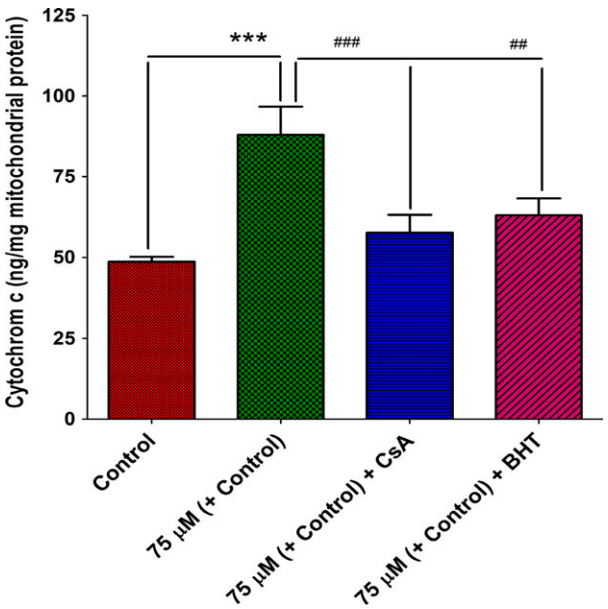

Fig. 5. cytochrome *c* expulsion assay. Cytochrome *c* expulsion was determined by corresponding rat/mouse cytochrome *c* ELISA kit. The cytochrome *c* expulsion is increased after the addition of atorvastatin (75 μM) on the mitochondria obtained from the pancreas. Data represented as mean ± S.D. of data determined from three separate experiments. Pancreas mitochondria obtained from three rats in each experiment in comparison with the control group. The one-way ANOVA test was performed. *** Significantly different from the corresponding control (atorvastatin concentration = '0') ($p < 0.001$). ## and ### Significant difference in comparison with atorvastatin (75 μM)-treated mitochondria $p < 0.01$ and $p < 0.001$, respectively.

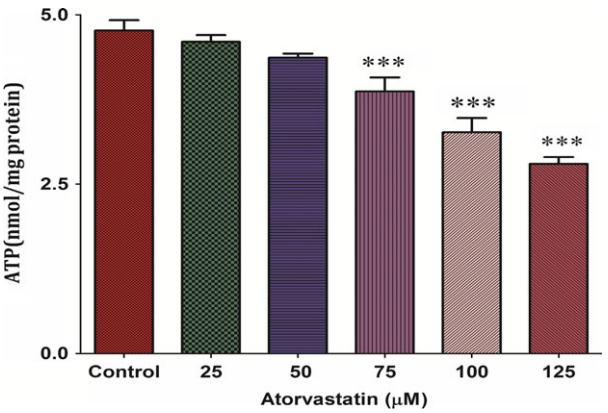

Fig. 6. ATP level assay. Effect of atorvastatin (0, 25, 50, 75, 100 and 125 μM) on mitochondrial ATP level. Pancreas mitochondria (500 μg protein/ml) was incubated with various concentrations of atorvastatin (0, 25, 50, 75, 100 and 125 μM) assayed using Luciferin/Luciferase Enzyme System. Data represented as mean ± S.D. of data determined from three separate experiments. Pancreas mitochondria obtained from three rats in each experiment in comparison with the control group. The one-way ANOVA test was performed. ***Significantly different from the corresponding control (atorvastatin concentration = '0') ($p < 0.001$).

group. Atorvastatin, however, at lower concentrations of 25 and 50 μM did not significantly induce change in the ATP content.

Discussion

These anti-hyperlipidaemic agents are one of the most widely recommended groups of drugs in the globe and beneficial in prevention of cardiovascular disease. It was reported that the use of statins is associated with any adverse effects, such as peripheral neuropathy, myopathy, rhabdomyolysis and raised liver enzymes. In many studies, the risk of DM in patients receiving statins (e.g. rosuvastatin, atorvastatin and simvastatin) has been investigated [25,26]. DM is a metabolic disorder that is characterized by an abnormally high level of blood sugar (hyperglycaemia). Type 1 diabetes (T1DM) is due to an autoimmune defect of the insulin producing from pancreas β-cells, which usually leads to complete insulin deficiency. Studies have shown that the origins of T2DM are multi-factorial. Factors such as obesity or overweight, ethnic origin, age and family history of diabetes play an important role in the development of T2DM [27]. Statistics show that diabetes among adults will be considerably increased by 2030 [27].

Emerging evidence shows that therapy with statins (such as simvastatin, atorvastatin and rosuvastatin) raises the risk of T2DM. However, the mechanisms connected with the consumption of statins and induction of diabetes are not fully understood. So far, two major mechanisms, including defects in insulin secretion and insulin resistance, have been suggested [14,28,29]. Previously published studies have indicated – for the first time – that one of the mechanisms leading to DM in patients consuming statins is an increase in insulin resistance, which is reflected through hyperglycaemia [14,28,29]. In our study, it was shown that atorvastatin could potentially induce an increase in blood glucose and decrease in insulin level in the pancreas.

Evidence has suggested that hyperglycaemia can stimulate free radical production such as H_2O_2. The weak and inefficient defence system of the body becomes unable to counteract the elevated ROS production and as a result the status of misbalance among ROS and their protection occurs leading to domination of the condition called oxidative stress [30]. ROS affect the insulin signalling cascade. Pancreatic ß-cells are especially vulnerable to ROS; the reason is that their natural enzymatic antioxidant defences are lower than other tissues [27]. In addition, pancreatic ß-cells lack the ability to adapt their low enzyme activity levels in response to stresses such as high glucose or high oxygen [27]. Our study showed that atorvastatin was able to increase the generation of ROS (especially of H_2O_2) in pancreas mitochondria. Possibly the mechanism of increased ROS by statin is hyperglycaemia caused by insulin resistance. The mitochondrial dysfunction in ß cells, skeletal muscles and adipocytes has been associated with the pathogenesis of diabetes. As statins are known to cause mitochondrial dysfunction in skeletal muscles, it is plausible that a similar mechanism is also responsible for their diabetogenic effect in the pancreas [25].

It has also been reported that therapy with this class of drugs (such as atorvastatin and lovastatin) reduces the levels

of CoQ10, which is part of ETC involved in the process of ATP production [25,31,32]. Our other findings showed that atorvastatin induced significant changes in MMP and mitochondrial swelling. These changes are the result of increased generation of ROS by hyperglycaemia. ROS (such as H_2O_2) trigger apoptosis via oxidation of mitochondrial inner membrane (MIM) phospholipids in β–cells [27,33]. It is well documented that the mitochondria are the main source of ROS generation. Also, mitochondria are the powerhouse of cells that provides over 90% of ATP consumed by the body. On the other hand, ATP production via oxidative phosphorylation process is one of the most important applications of mitochondria. Furthermore, the ATP level determines the mode of cell death in target cells (apoptosis or necrosis). The reduction in mitochondrial ATP content indicates the mitochondrial dysfunction leading to decrease in the ability of oxidative phosphorylation for ATP production. Also, the decrease in the ATP content may exacerbate the ROS production [34,35]. The onset of T2DM is accompanied by an advanced decrease in β-cell mass that results from a significant rise in β-cell apoptosis, and mitochondria are known to play an important role in regulating apoptosis signalling [33].

Considering the target organ toxicity of statins *in vitro*, the question arises to what extent our results are relevant to the *in vivo* situation. In atorvastatin consumption, plasma concentration levels after oral doses of 20–40 mg are in the range of 0.1 μM [36–38], which is significantly lower than the concentrations assayed in our examination. So, data on statin concentrations in affected tissues (i.e. skeletal muscle, liver and pancreas, adipose) of human beings are lacking so far; possible tissue concentrations obtained have to be evaluated. The volume of distribution (VD) of lipophilic statins such as atorvastatin is high. At least for atorvastatin, accumulation in peripheral tissues (adipose and skeletal muscle) can be supposed. Patients affected by pancreas problems and also muscular problems may possibly have an underlying mitochondrial disease, rendering them more sensitive to lower statin concentrations [37,39]. Taken together, our results may be relevant to the *in vivo* situation in sensitive human beings; however, the statin concentrations associated with mitochondrial toxicity found in our research appear to be high. In conclusion, the lipophilic statins (especially atorvastatin) impair various functions of skeletal muscle (perhaps other sensitive tissues) mitochondria. On the other hand, the hydrophilic pravastatin does not reveal relevant mitochondrial toxicity *in vitro*. However, results showed that the toxic concentrations of the statins on isolated mitochondria are significantly higher than their plasma concentration levels in human beings. Mitochondrial toxicity may trigger rhabdomyolysis and other statin-related organ toxicity including diabetes in sensitive patients.

Eventually, our results showed that atorvastatin induced expulsion of cytochrome *c* from the mitochondria into the buffer media. Furthermore, inhibition of cytochrome *c* release from pancreas mitochondria indicates that the induction of apoptosis signalling by atorvastatin in pancreatic cells resulted from an oxidative stress and opening of the mitochondrial MPT pores. Reports indicate that cytochrome *c* expulsion from mitochondria is an important step in the starting of apoptosis signalling and finally provides a satisfactory justification for the massive cell loss in pancreas and increased risk of DM caused by atorvastatin.

Conclusion

Our study provides evidence that mitochondrial targeting is the mechanism by which atorvastatin could potentially induce increased blood glucose and decreased insulin level in the pancreas.

Conflict of Interest

There are no conflicts to disclose.

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