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Original Article

Toxicity of Atorvastatin on Isolated Brain Mitochondria

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Abstract

Background: Although the bio kinetics, metabolism and chemical toxicity of Atorvastatin are well known, until recently little attention was paid to the potential neurotoxic effect of Atorvastatin (Atv). Regarding the concrete evidences indicating Atv may reduce Coenzyme Q10 (CoQ10) levels through blockage of metalonate cycle, the present work aims to determine if Atorvastatin may provide toxic effects on brain mitochondria and whether its supplementation with two lipidicantioxidants, CoQ10 and Vit E may improve such outcomes.

Methods: to evaluate mitochondrial toxicity, male NMRI mice were first treated with Atorvastatin(bo; 20 and 60 mg/kg) every other day with or without supplementation with CoQ10 (200 mg/kg) or Vit E (40 u/kg). After a period of 4 weeks, the animals were euthanized and brain cortices were harvested ad subjected to mitochondria isolation procedure. ROS release, mitochondrial membrane potential (MMP) and cytochrome c release were performed to precisely address the probable mitochondrial injury.

Findings: Our data showed increased ROS formation, MMP collapse, mitochondrial swelling and cytochrome c release in Atorvastatin treated mice, suggesting that mitochondrial ROS formation and apoptosis signaling are likely involved in Atorvastatin neurotoxicity. Importantly according to the data from animals receiving either CoQ10 or Vit E, supplementation with these lipophilic antioxidants, remarkable reverted the corresponding Atorvastatin mitochondrial toxicity markers.

Concusion: Conclusively the present work addresses chronic Atorvastatin toxic effects on brain mitochondria and supports advantages of Vit E or CoQ10 supplementation. Whether such neurotoxic effect is correlated with CoQ10 depletion or if the improving effects of the due supplementation contribute toAtorvastatin receiving patients, needs more investigations.

Key word: Atorvastatin; Mitochondria; Apoptosis; brain; E vitamin; CoQ10

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1. Introduction

Atorvastatin is a member of the drug class known as statins, which catalyzes the conversion of HMG-CoA to mevalonate and is used for decreasing blood cholesterol(1).Statins (HMG-CoA reductase inhibitors) as antihyperlipidemic drugs are widespread used for prevention of stroke, heart attack [1,2]. There are conflicting evidence showed that atorvastatin can produce potential teratogenicity during the first trimester of pregnancy in experimental animals. As intensive statin therapy is likely to be a main step of cardiovascular disease prevention strategy, gestational exposure to statins is an issue of significant clinical importance[(2),(3)].In toxicity studies, it was shown that HMG-CoA reductase inhibitors could inducecellular toxicity in different tissues (brain and pancreatic cells) inrats. CoQ10 is an essential proton–electron carrier in the lipid phase of the inner mitochondrial membrane. It has been reported to have high antioxidant efficacy and considerable protective effects against mitochondrial toxicity[(4)]. It was shown that oral administration of CoQ10 increased its cerebral cortex mitochondrial concentrations and provided further evidence that CoQ10 could exert neuroprotective effects that might be useful in the treatment of neurodegenerative diseases[(5)]. Vitamin E is a fat-soluble vitamin with numerous biological functions[(6),(7)]. It is the most effective chain breaking lipid soluble antioxidant in biological membranes, and protects cellular structures against damage from oxygen free radicals and reactive products of lipid peroxidation[(8),(9)]. The antioxidant activity of vitamin E has persuaded many groups to study its ability to prevent chronic diseases, especially those believed to have an oxidative stress component, such as cardiovascular diseases, atherosclerosis, and cancer[(10)]. Although cellular, molecular, and toxicity of Atrovastatin on brain have not yet been clarified, devastated mitochondrial function and as a consequence impaired cellular energy state is an attractive hypothesis for describing the toxicity of Atrovastatin on brain. The brain is very sensitive to oxidative stress which is due to high oxygen consumption, high concentrations of polyunsaturated fatty acids, and low levels of some antioxidant enzymes[(11)]. Mitochondrion is a sub-cellular structure in the cell that has respiratory chain that helps to produce energy. In some investigations, reduced levels of ATP were found in the frontal lobe, temporal lobe, and basal ganglia of brain tissue[(12)].It is well known that mitochondria are the main source of reactive oxygen species (ROS) production and also are the major cellular site of energy production in brain[(13)]. Moreover, the brain is very dependent on mitochondrial energy fuels

for normal functioning, therefore normal function of mitochondria is essential to maintain brain homeostasis[(14)]. Since the brain are enriched in mitochondria[(15)]and it is thought that mitochondria are the most vulnerable organelle in brain tissue, therefore, any factors that impaired the mitochondrial function can affect the normal function of nervous system[(16)].Therefore, it is important to know about the potential hazards and their cellular mechanisms in ratbrain, because in common point of view, atorvastatin has the toxicity position among all statins for human lipid lowering drug therapy. Therefore, toxicological and pathological research to determine the effects of atorvastatin in brain and its possible mitochondrial damage in brain cells will be more than necessary.Outcomes of this research will provide protective strategies which can counter the toxic effects of atorvastatin in high risk brain.

2. Materials and Methods

2.1. Animals

Balb C male mice obtained from Pasture institute were habituated to the animal room in controlled temperature (37°C) and light/dark cycle with food and water *ad libitum*. Tryhydrate (Atv) pure powder was purchased from Alborz Daroo Pharmaceuticals every other day for a one-month period. Due to its very low solubility Atv suspension was prepared readily before gavage in dosages of 20, 60, and 120 mg/kg. Coenzyme Q10 (CoQ 10; Bulk supplement, Usa) and vitamin E (Vit E; Osveh Pharmaceutical) were also administered immediately after Atv while being dissolved in Almond oil (Pharmaceutical grade) to reach to the oral dosages of 20 or 60 mg/kg each. All the drug administrations were carried out with a skilled person to minimize the animals' suffering by the frequent gavages and also all the animalsmanipulations performed were approved by the board of research ethics at the Neuroscience Research Center of ShahidBeheshti Medical University in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised1996). Following the one-month period of treatment with either Atv, CoQ10 and Vit alone or in combination, the animals were euthanized with CO2 inhalation and thenwere decapitated and their brains were harvested for the following molecular analysis on either hippocampi or cortices.

2.2. Isolation of Mitochondria from Rat Brain

Mitochondrion was prepared from Sprague-Dawleyrat brain using differential centrifugation. Brain tissue was 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, the 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[(17-19)].For the evaluation of each experiment, mitochondria should be prepared fresh and can be kept in dried condition maximum for 4 h on ice temperature. But in toxicity assessment using isolated mitochondria technique, we have to suspend our mitochondria in different water based working buffers at laboratory temperature (25 ºC) before addition of certain drug or poison. In this regard, we have only 60 minute times to performe toxicity studies, because all the distractive proteases and phospholipases will be active in the isolated mitochondria at temperature above than for 4 ºC. That is why we determined all the mitochondrial toxicity endpoints within 60 minutes of drug exposure to the mitochondria.

2.3. Complex II or Succinate Dehydrogenase (SDH) Activity Assay

The activity of mitochondrial complex II was measured by determination of reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Briefly, 100μL mitochondrial suspensions (normalized to 0.5 mg/ml of mitochondrial protein) were incubated with different concentration of atorvastatin (10,20 and 60μM) at37 °C, then 0.4% of MTT was added to the solution and incubated at 37°C for 30 min. The product formazan crystals were then dissolved in 100 μL DMSO and the absorbance at 570 nm was measured with an ELIZA reader (Tecan, Rainbow Thermo, Austria) [(20)].

2.4. Determination of Mitochondrial ROS Level

In this experiment, isolated mitochondria from brain 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 mitochondria suspension and next incubated for 15min at 37◦C.In the next step, the fluorescence intensity of DCF (dichlorofluorescein) as a ROS level determination after addition of various concentrations of Atorvastatin (10,20 and 60μM) at different time intervals within 60 min (5,15,30,45 and 60 min) of incubation in the mitochondria obtained from brain rats was measured using fluorescence spectrophotometer at the EX= 488 nm and EM=527 nm[(21)].

2.5. Determination of the Mitochondria Membrane Potential (MMP)

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 addition of various[(17)].

2.6. Determination of the Mitochondria Cytochrome C Release

The amount of cytochrome c released to the medium from isolated mitochondria was determined at 450 nm according to the instructions provided by the manufacturer of the Quantikines Rat/Mouse Cytochrome c Immunoassay Kit (Minneapolis, MN). All

analysis stages were carried out using an ELISA reader (InfiniteM 200, TECAN) at desired concentrations in all groups.

2.7. Statistical Analysis

The all results in this study are presented as mean ±SD (n=3). The statistical analyses were performed using the GraphPad Prism software (version 5). The assays were performed 3 times. Statistical significance (set at $P< 0.05$) was carried out by using the one-way and two-way ANOVA test.

3. Results and Discussion

3.1. Effects of Atorvastatin on Mitochondria Complexii(SDH) Activity

SDHactivity which is the measure for mitochondrial functionality was also assessed using the MTTtest after 1h of incubation of isolated brain mitochondria with different concentrations of atorvastatin (10, 20, and 60μM).Figure 1, shows a significant decrease in theSDH activity determined by mitochondrial metabolic conversion of MTT to formazan following addition ofall concentrations of atorvastatin (10, 20 and 60μM) isolated brain mitochondria.

3.2. Effects of Atorvastatin, Vitamin E And Q10 on Mitochondrial ROS Level

As shown in Figure 2, the rate of ROS formation in isolated brain mitochondria significantly increased compared to control group within 5, 15, 30, and 60 min of exposure

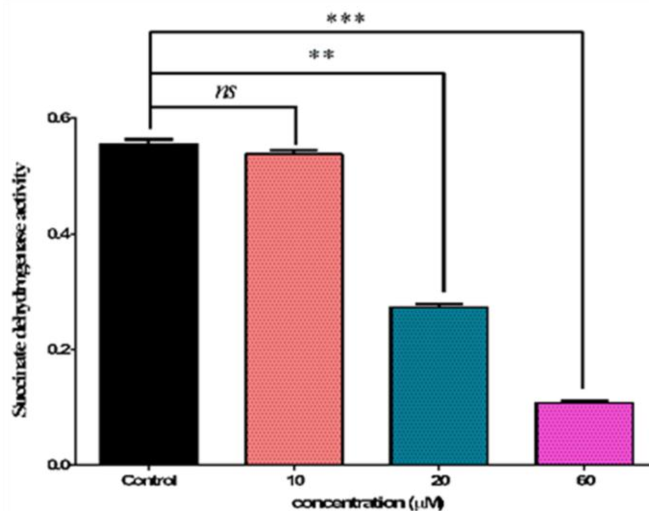

Figure 1. The effect of atorvastatin on the SDH activity. SDH activity was measured using MTT. The brain mitochondria (0.5 mg/ml) were incubated for 1 h with various concentrations of (A) atorvastatin (20 and 60μM) (B); vitamin E (20 and 60μM); (C) CoQ10 (20 and 60μM). Data represented as mean ± SD from three separate experiments. Brain Mitochondria obtained from 3 separate rats (n=3).In each experimentin each experiment all findings compared with the control group. The one-way ANOVA test was performed. *, **, *** and ****significantly different from the corresponding control (p<0.05, p<0.01, p<0.001 and p<0.0001, respectively).

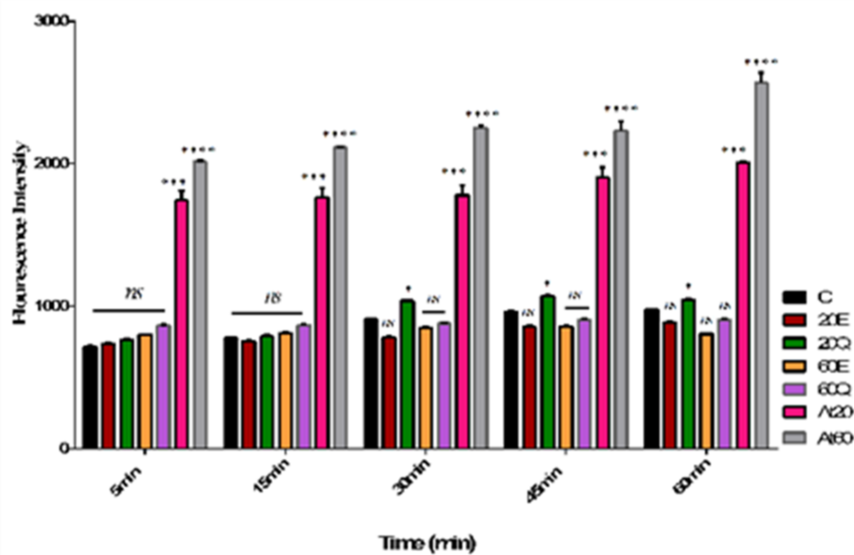

Figure 2. Measurement of mitochondrial ROS formation. The ROS level was assayed after administration of various concentrations of (A) atorvastatin (20 and 60µM) in the absence and presence of lipid antioxidants (B) vitamin E 40 IU /kg and (C) CoQ10 200mg/kg at different time intervals within 60 min (5, 15, 30, 45 and 60 min) following isolation from brain of rats. Data represented as mean ± SD from three separate experiments. Brain Mitochondria obtained from 3 separate rats (n=3).In each experiment all findings compared with the control group.The two-way ANOVA test was performed. *, **, *** and ****significantly different from the corresponding control ($p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively).

with all applied concentrations of atorvastatin (20 and 60µM).But, co-administration of vitamin E 40 IU /kg and CoQ10 200mg/kg with two concentrations of atorvastatin (20 and 60µM) didnot significantly increase mitochondrial ROS formation compared with control group.

3.3. Effects of Atorvastatin, Vitamin E And Q10 on Mitochondrial MMP Level

As shown in Figure 3, the rate of mitochondrial membrane potential (MMP) level in isolated brain mitochondria significantly decreased compared to control group within 5,15 ,30, and 60 min of exposure with all concentrations of atorvastatin (20 and 60µM).MMP decrease was determined by increased fluorescence of expelled rhodamine 123 from mitochondria into incubation buffer. But co-administration of vitamin E 40 IU /kg and CoQ10 200mg/kg with two concentrations of atorvastatin (20 and 60µM) did not significantly alter mitochondrial membrane potential compared with control group.

3.4. Effects Of Atorvastatin, Vitamin E,And Q10 on Mitochondrial Swelling Leve:

Any decrease of absorbance of mitochondria suspension at 540 nm wasconsidered as mitochondrial swelling, which is another

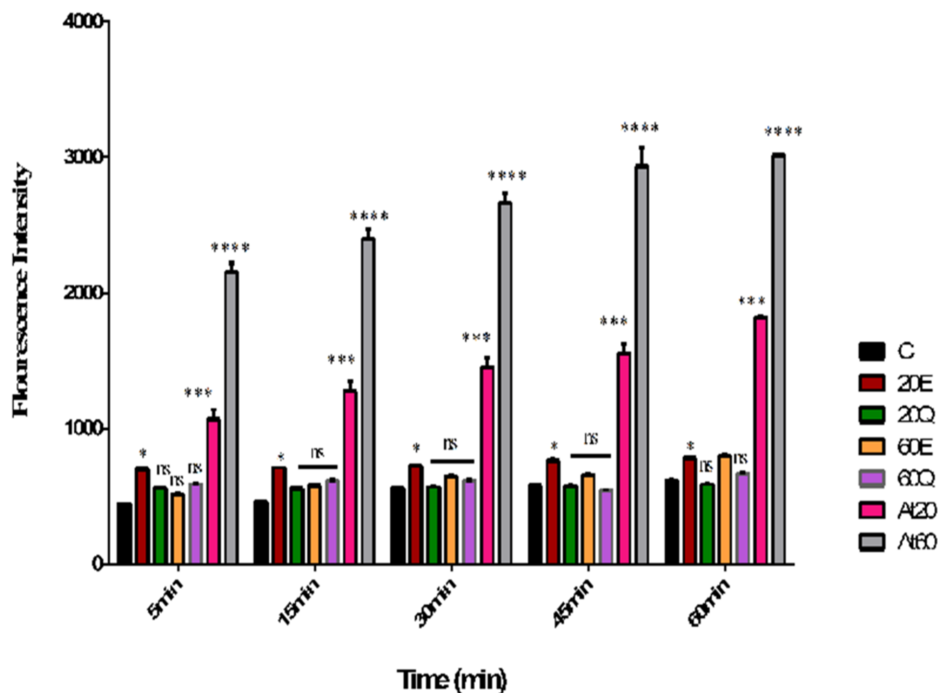

Figure 3. The decline of MMP was assayed after addition of various concentrations of (A) atorvastatin (20 and 60μM) in the absence and presence of lipid antioxidants (B) vitamin E 40 IU /kg and (C) CoQ10 200mg/kg at different time intervals within 60 min (5, 15, 30, 45 and 60 min) following isolation from brain of rats. Data represented as mean ± SD from three separate experiments. Brain Mitochondria obtained from 3 separate rats (n=3).In each experimentin each experiment all findings compared with the control group. The two-way ANOVA test was performed. *, ** and ****significantly different from the corresponding control p<0.05, p<0.01 and p<0.0001, respectively.

indicator of mitochondrial membranepermeability transition (MPT). Our results showed that addition of atorvastatin (20 and 60μM) on isolated rat brain mitochondria leads to mitochondrial swelling within 5-60 min of incubation. But co-administration of vitamin E 40 IU /kg and CoQ10 200mg/kg with two concentrations of atorvastatin (20 and 60μM) did not significantly alter mitochondrial swelling compared with control group (Figure 4).

3.5. Effects of Atorvastatin on Mitochondrial Cytochrome C Release

As shown in Figure 5, results indicate that addition of atorvastatin (20 and 60μM) on isolated rat brain mitochondria leads to significant (*P*< 0.05) expulsion of cytochrome c (ng/mg mitochondrial protein) from the mitochondria into the buffer media.But co-administration of vitamin E 40 IU /kg and CoQ10 200mg/kg with two concentrations of atorvastatin (20 and 60μM) did not significantly

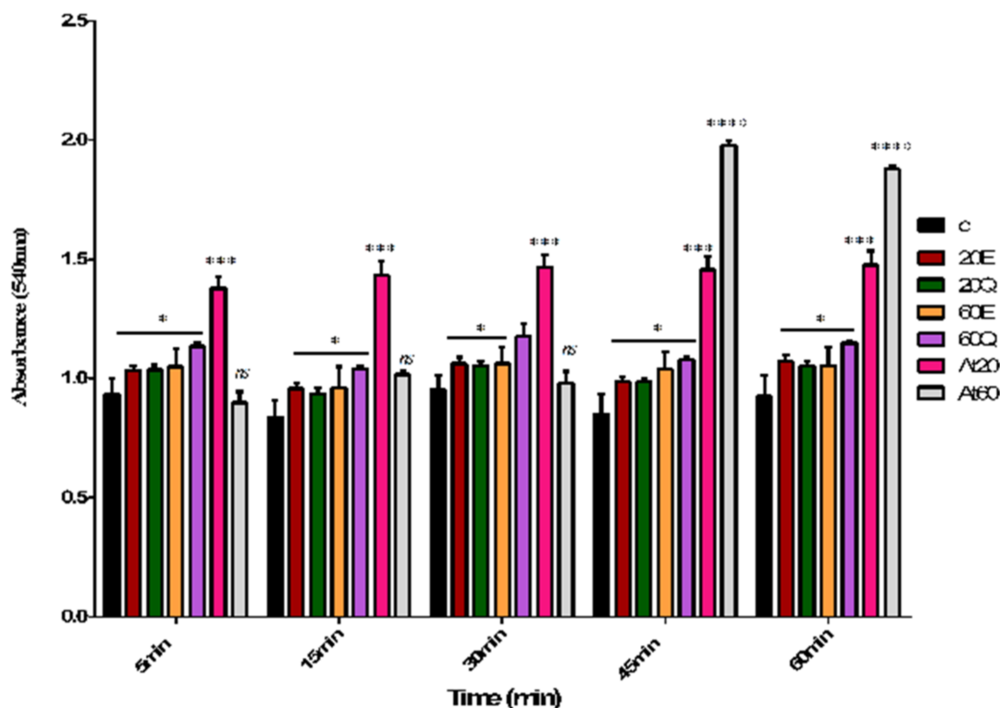

Figure 4. Mitochondrial swelling assay. The mitochondria swelling was assayed after addition of various concentrations of (A) atorvastatin (20 and 60µM) in the absence and presence of lipid antioxidants (B) vitamin E 40 IU /kg and (C) CoQ10 200mg/kg at different time intervals within 60 min (5, 15, 30, 45 and 60 min) following isolation from brain of rats. Data represented as mean ± SD from three separate experiments. Brain Mitochondria obtained from 3 separate rats (n=3). In each experimentin each experiment all findings compared with the control group. The two-way ANOVA test was performed. *, **, *** and ****significantly different from the corresponding control (p<0.05, p<0.01, p<0.001 and p<0.0001, respectively).

alter mitochondrial cytochrome c releasewith control group (Figure 5).

3.6. Discussion

These anti hyper lipidemic 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[(1)]. In our study, it was shown that atorvastatin could potentially induce toxicity in brain which can be prevented byCoQ10 and vitamin E.Apoptotic cell death can be mediated via several pathways. One pathway involves the mitochondria. Mitochondria are semi-autonomous organelles that play essential roles in cellular metabolism and programmed cell death. Many diseases can induce cellular stress, which may also lead to mitochondrial perturbation and, finally, cell death. Mitochondrial dysfunction is usually resulted from mitochondrial membrane permeability

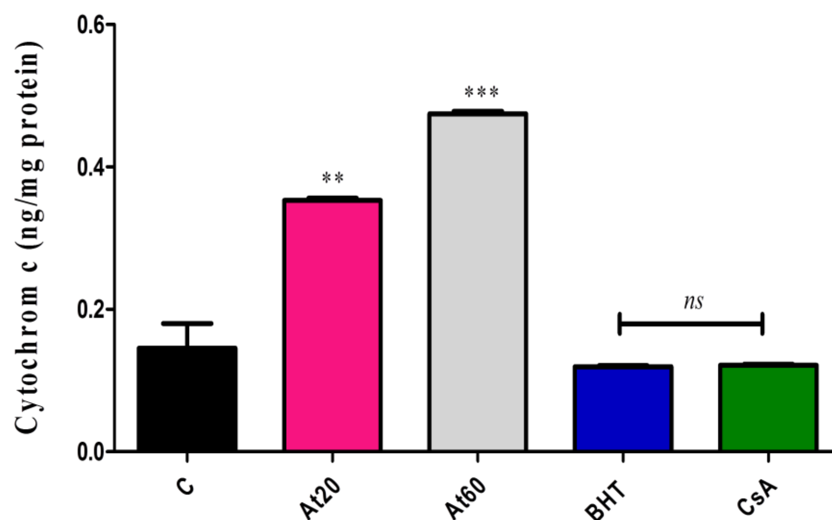

Figure 5. Mitochondrial cytochrome c release. The mitochondria cytochrome c release was assayed after addition of various concentrations of (A) atorvastatin (20 and 60μM) in the absence and presence of lipid antioxidants (B) vitamin E 40 IU /kg and (C) CoQ10 200mg/kg at different time intervals within 60 min (5, 15, 30, 45 and 60 min) following isolation from brain of rats.Data represented as mean ± SD of data determined from three separate experiments. Brain Mitochondria obtained from 3 separate rats (n=3).In each experimentin each experiment all findings compared with the control group. The two-way ANOVA test was performed. *, **, *** and ****significantly different from the .(corresponding control (p<0.05, p<0.01, p<0.001 and p<0.0001, respectively.

transition, dissipation of the inner membrane potential, osmotic swelling of the matrix, rupture of the outer mitochondrial membrane, release of cytochrome c and other apoptogenic proteins from the mitochondria, and formation of the caspase-3 activation complex, the apoptosome[(22)]. Permeability transition involves the opening of a channel named permeability transition pore complex (MPT pore) [[17]]. Our data suggested that mitochondrial ROS formation in brain mitochondria causes MPT, leading to the collapse of mitochondrial membrane potential, mitochondrial swelling, and cytochrome c release. But pretreatment with CoQ10 and vitamin E prevented ROS production in brain. ROS production is the starter of apoptosis signaling. The release of cytochrome c from isolated mitochondria would slow down the electron transfer from complex III to complex IV and therefore enhances the ROS generation at the Q-cycle[17]. In the other words, ROS generationcan occur even earlier than cytochrome c release from mitochondria. This may explain the puzzle between the ROS generation and cytochrome c release, which is usually considered a delayed event [17]. The increased mitochondrial ROS formation can cause oxidation of a lipid membrane resulting in disruption of the mitochondrial membrane and consequently the collapse of mitochondrial

membrane potential (MMP) and cytochrome c release[17].Previous studies showed that MMP represents the integrity of the mitochondrial membrane and its metabolic activity as a key indicator of mitochondrial functionality[(22)]. Furthermore, MMP is a crucial factor for the regulation of mitochondrial activity and the MMP collapse is the important stimuli for apoptosis [(22)]. Altered regulation of the apoptotic cascade can potentially reduce neuronal and glial viability at various stages of neural development and contribute to the volumetric and functional brain deficits observed in brain damage of atorvastatin [(23)]. Previous studies showed that CoQ10 and vitamin E prevented cell death signaling in brain through mitochondria. In particular, apoptosis which was identified as a potential underlying mechanism for evidence of progressive gray matter volume loss reported in atrovastatin induced psychosis[(22)]. Our results also provided evidence that mitochondrial ROS formation and apoptosis signaling are involved in cellular pathology induced by atorvastatin in brain.

4. Conclusion

This study addresses the mechanism of brain toxicity of atrovastatin on isolated mitochondria. These results suggested the perspective on the role of oxidative stress and ATP depletion in the cellular toxicity of atrovastatin on brain cells. This investigation bridged the induction of oxidative stress by atrovastatin to the initiation of apoptosis signaling through mitochondrial pathway by the determination of upstream events including decline of MMP, increased mitochondrial swelling and finally the release of cytochrome c in the isolated brain mitochondria, which can then be considered as a satisfactory justification for the reported brain strock following drug misuse**.**

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