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RESEARCH ARTICLE

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The effect of simvastatin induced neurotoxicity on mitochondrial function in human neuronal cells

Lauren Millichap[a], Nadia Turton[b], Razan Alomosh[c], Robert A. Heaton[d], Amy Bateman[c], Nasser Al-Shanti[c], Adam P. Lightfoot[c], Elisabetta Damiani[a], Fabio Marcheggiani[a], Patrick Orlando[a], Sonia Silvestri[a], Luca Tiano[a] and Iain P. Hargreaves[b]

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ABSTRACT

3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR) inhibitors, commonly known as statins, are drugs frequently used in the treatment of hypercholesterolemia and hyperlipidemia. However, the current study has demonstrated that simvastatin induces neurotoxicity and is associated with cellular coenzyme Q_{10} (CoQ_{10}) depletion. CoQ_{10} has a significant role in the mitochondrial electron transport chain (ETC), in addition to being a fundamental lipid-soluble antioxidant. Depletion of CoQ_{10} is frequently associated with impaired mitochondrial function and increased oxidative stress. The aim of this study was to investigate the potential mechanisms of simvastatin-induced neurotoxicity assessing mitochondrial function and evidence of oxidative stress in an *in vitro* SH-SY5Y human neuronal cell line. Fluorescence studies assessed *via* flow cytometry determined significant increases in intracellular and mitochondrial reactive oxygen species production following SH-SY5Y treatment with simvastatin compared to control cells. Additionally, spectrophotometric enzyme studies determined a significant ($p < 0.0001$) inhibition of ETC complex I and II-III activities which accompanied a significant decrease in neuronal CoQ_{10} content ($p < 0.005$) and cell viability ($p < 0.0001$). The results of the present study have indicated evidence of mitochondrial dysfunction and increased oxidative stress, resulting in increased loss of neuronal viability following simvastatin treatment. Thus, these results demonstrate evidence of neurotoxicity associated with statin therapy.

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1. introduction

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR) inhibitors, commonly known as statins, are amphiphilic drugs used in the treatment of hypercholesterolemia and hyperlipidemia (Sirtori 2014; Mollazadeh et al. 2021; Bell et al. 2024). Statins competitively inhibit the enzyme HMGR, the rate-limiting enzyme of the mevalonate (MVA) pathway, therefore reducing intracellular cholesterol synthesis (Bansal and Cassagnol 2024). The MVA pathway is tightly regulated which is essential for the production of MVA-derived intermediates and to protect cells from the accumulation of toxic end-products, including cholesterol (Edwards and Ericsson 1999; Guerra et al. 2021). In addition to cholesterol synthesis, the MVA biosynthetic pathway is essential for the production of cellular metabolism regulators, including lipoproteins, dolichol, ubiquinone (CoQ_{10}), and cholesterol-derived products, such as vitamin D and bile acids. However, as a consequence of a mutual biosynthetic pathway, selective inhibition of the activity of HMGR also results in the depletion of cellular coenzyme Q_{10} (CoQ_{10}) levels, in addition to other MVA-derived metabolites, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Edwards and Ericsson 1999; Littarru and Langsjoen 2007; Guerra et al. 2021).

CoQ_{10} is a lipid-soluble quinone, endogenously synthesized in most cell types and is widely involved in cellular metabolism (Gutierrez-Mariscal et al. 2020; Mantle et al. 2023). It has several vital cellular functions including its essential role in cellular bioenergetics/ATP synthesis *via* mitochondrial oxidative phosphorylation (OXPHOS); its role as a fundamental endogenously synthesized lipid-soluble antioxidant protecting cell membranes from free radical-induced oxidative damage; its role in lysosomes, amino acids, pyrimidine nucleoside, and cholesterol metabolism; and its role in modulating apoptosis and mitochondrial uncoupling protein (Crane 2001; Turunen et al. 2004; Gutierrez-Mariscal et al. 2020; Mantle et al. 2023). Additionally, CoQ_{10} functions as an essential electron carrier in the mitochondrial electron transport chain (ETC), shuttling electrons from complex I (NADH ubiquinone oxidoreductase) and complex II (succinate

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ubiquinone reductase) to complex III (ubiquinol cytochrome *c* reductase), generating a continuous passage of electrons within the chain which is vital for cellular bioenergetics (Crane 2001). The main source of CoQ_{10} is *via* intracellular synthesis and a small amount is acquired *via* the diet (Rauchová 2021).

Statin-induced neurotoxicity may occur as a consequence of reduced cellular CoQ_{10} levels, which can be partially attributed to the decrease in the levels of its lipoprotein transport carriers, which is induced by the therapeutic effect of the statins, as the majority of CoQ_{10} is transported in the circulation by apo B-containing lipoproteins (Littarru and Langsjoen 2007; Mollazadeh et al. 2021). Increasing evidence has shown that the adverse reactions to statins are not exclusively due to their direct cholesterol-lowering effect but as a consequence of statin-induced impaired mitochondrial function (Mollazadeh et al. 2021). Inhibition of CoQ_{10} as a result of the inhibitory effects of statins on the MVA biosynthetic pathway, causes mitochondrial dysfunction, revealed by mitochondrial respiratory chain (MRC) complex inhibition, disruption of the mitochondrial membrane potential, reduced mitochondrial DNA (mtDNA), impaired OXPHOS and cytochrome *c* release and mitochondrial swelling. Furthermore, reactive oxygen species (ROS) production and oxidative stress have been reported to be potential hallmarks of statin-induced neurotoxicity and have a role in regulating mitochondrial biogenesis (Liu et al. 2019). In cells with high-efficient antioxidant systems, statin-induced ROS accumulation is limited and stimulates peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) activity, thus promoting mitochondrial biogenesis (Mollazadeh et al. 2021). However, in cells that have an overwhelmed antioxidant defence system, an increase in ROS production may be responsible for increased oxidative stress and impaired mitochondrial function. Additionally, direct inhibition of MRC complex I has been suggested as the mechanism of simvastatin-induced mitochondrial toxicity (Kwak et al. 2012; Mollazadeh et al. 2021). *In vitro* studies have identified functional impairment of complexes I, III and IV following 24-hour exposure to statins in rat skeletal muscle cells, which were also confirmed in human myocyte studies (Kaufmann et al. 2006). Other studies have demonstrated that simvastatin exposure in CT26 cells resulted in significant apoptotic cell death and perturbations in the antioxidant defence system (Qi et al. 2010). Moreover, chronic statin treatment has been associated with an increased risk of cognitive impairment, which has been demonstrated in several human and animal studies (King et al. 2003; Evans and Golomb 2009; Guo et al. 2021; Mollazadeh et al. 2021) (Figure 1).

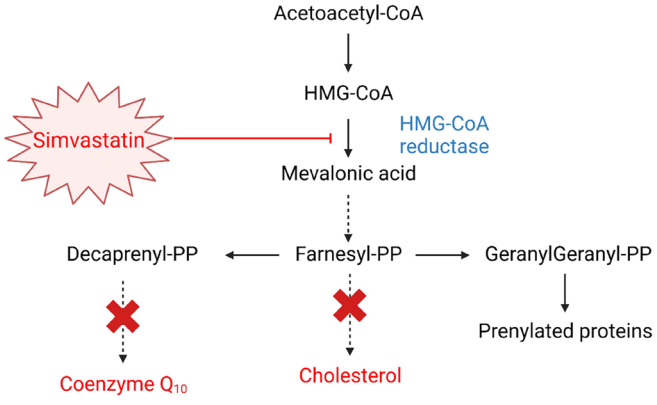

Figure 1. A schematic diagram to show the mechanism of action of simvastatin, resulting in inhibition of Coenzyme Q_{10} (CoQ_{10}) biosynthesis. Statins inhibit HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway (Hargreaves et al. 2020). [created using biorender.com]. CoA: coenzyme A; PP: pyrophosphate.

The present study evaluated the effect of simvastatin-induced neurotoxicity in the SH-SY5Y human neuroblastoma cell line in order to assess the effect of impaired mitochondrial function and increased oxidative stress in an *in vitro* SH-SY5Y human neuronal cell line. Working with neural cell lines offers several advantages over primary neuronal culture, as SH-SY5Y cells are of human origin, meaning that they express the human form of physiological and pathological-related proteins (Strother et al. 2021). There is increasing evidence to support that statin-induced neurotoxicity involves impaired mitochondrial function and increased oxidative stress, in addition to inhibition of CoQ_{10} production. Thus, the effects of impaired mitochondrial function, limited cellular antioxidant status and the effect of elevated oxidative stress and ROS production as a consequence of statin-induced deficits in cellular CoQ_{10} content on the SH-SY5Y neuronal cells were investigated following simvastatin treatment.

The results of this study will provide important insights into statin-induced neurotoxicity on mitochondrial function and human neuronal viability.

2. Materials & methods

2.1. Chemicals

All reagents were of analytical grade.

The following were purchased from Sigma Aldrich (Poole, UK):

Sterile cell culture products: Dulbecco's Modified Eagles Medium (DMEM) high glucose, with L-glutamine and sodium pyruvate; Fetal bovine serum, heat inactivated (EU approved); Trypsin-EDTA (0.25%), phenol red; Sterile 0.4% Trypan blue solution; Penicillin-Streptomycin; PBS (Phosphate-Buffered Saline) tablets; Dimethyl sulphoxide; Simvastatin ≥ 97%; Potassium phosphate, monobasic (KH_2PO_4), 99+%, pure; Potassium phosphate, dibasic (K_2HPO_4), 99+%, pure; Magnesium chloride hexahydrate ($MgCl_2.6H_20$), 99%; Trizma-base (reagent grade); BSA (Bovine Serum Albumin) ≥ 96%; Coenzyme Q_1 ~ 95%; β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate ≥ 97%; Ethylenediaminetetraacetic acid (EDTA), ACS reagent, ≥ 99%; Cytochrome *c* from equine heart ≥ 95%; Potassium cyanide (KCN), ACS reagent, ≥ 96%; Sodium succinate dibasic hexahydrate *ReagentPlus*® ≥ 99%; Antimycin A from *Streptomyces sp.*; Triton X-100; Concentrated hydrochloric acid (HCl) solution; Acetyl coenzyme A sodium salt ≥ 93%; Oxaloacetic acid ≥ 97%; L-Ascorbic acid, cell culture tested; 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) ≥ 98%; Sodium bicarbonate ($NaHCO_3$), ACS reagent, ≥ 99%; L-Buthionine-sulfoximine, ≥ 97%.

The following were purchased from Invitrogen Ltd (Paisley, UK):

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); CM-H_2DCFDA (General Oxidative Stress Indicator); MitoSOX Red™ mitochondrial superoxide indicator; BODIPY™ 665/676 (Lipid Peroxidation Sensor); Monochlorobimane (mBCl).

CellTiter-Glo® 2.0 Cell Viability Assay Kit was purchased from Promega (Promega, UK).

DC total protein assay Reagent A and Reagent B were purchased from Bio-Rad Laboratories Ltd (Hemel Hempstead, UK).

2.2. Cell culture

The SH-SY5Y human neuroblastoma cell line, derived from SK-N-SH, was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum (FBS). Cultures were maintained at 37 °C in an incubator containing 5% CO_2. SH-SY5Y cells were grown to 70–80% confluence before seeding for treatment and analysis. After reaching confluence, cells were harvested and washed twice with warm phosphate buffered saline (PBS), followed by the addition of warm trypsin. Cells were counted with Trypan blue dye solution and dye excluding cells were counted using a hemocytometer. Cells were seeded in 96-well plastic plates at a density of 40,000 cells/well or in T-75 flasks at a density of 400,000 cells/mL prior to treatment with simvastatin.

2.3. Simvastatin treatment

Simvastatin was prepared by dissolving in 100% dimethyl sulfoxide (DMSO), and the final concentration of DMSO was <0.1% (*v*/*v*), showing no signs of toxicity to these cells. Control cells were treated with the vehicle DMSO < 0.1% (*v*/*v*). Cells were treated with simvastatin in DMEM at concentrations of 1 µM and 2 µM, and cells were treated with simvastatin for 48 h. To assess simvastatin toxicity, SH-SY5Y cells were initially exposed to 0, 0.5, 1, 2, and 5 µM simvastatin for 48 h at 37 °C (data not shown). The final concentrations of 1 µM and 2 µM were selected based on a previous study showing bioenergetic defects in CoQ_{10}-deficient fibroblasts (López et al. 2010) and a reduction in neuronal CoQ_{10} content, with cell viability remaining above 50% as indicated by HPLC-ED and MTT assays (Figures 2 and 3).

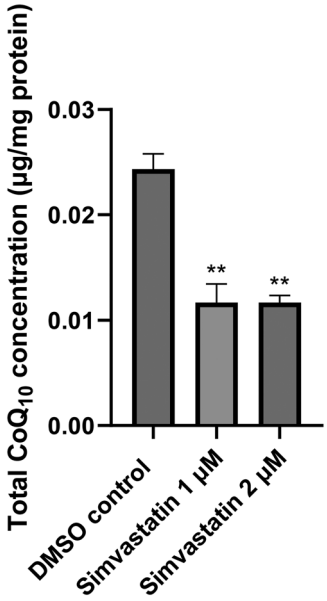

Figure 2. Effect of 48 h simvastatin exposure (1 µM and 2 µM) on total Coenzyme Q_{10} content (µg/mg protein) in SH-SY5Y human neuroblastoma cells. Measurement of neuronal Coenzyme Q_{10} content (µg/mg) in SH-SY5Y human neuroblastoma cells was determined using HPLC-ECD. Error bars represent standard error of the mean (SEM); statistical analysis was carried out using One-Way ANOVA with Tukey's multiple comparison post hoc test; levels of significance $^{**}p < 0.005$ compared to control levels ($n = 3$).

2.4. Cell viability assay

SH-SH5Y cell viability was assessed using the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Invitrogen, UK). As previously described, SH-SH5Y cells were seeded and treated with simvastatin. The MTT assay was carried out according to the manufacturer's instructions (Sylvester 2011). The absorbance was measured at a wavelength of 570 nm using a Tecan Spark Microplate Reader (Tecan; Zurich, Switzerland).

2.5. Measurement of cellular oxidative stress markers

Various oxidative stress markers in SH-SY5Y cells treated with simvastatin were assessed using different fluorescent probes and assays.

The general oxidative stress indicator, CM-H_2DCFDA (Invitrogen; Paisley, UK), was used to measure intracellular ROS levels. A 1 mM stock solution was prepared in DMSO, and a final concentration of 1 µM was used for the experiment. Fluorescence intensity was measured in the FL-1 channel of a BD Accuri™ C6 Flow Cytometer (BD Biosciences; Berkshire, UK).

MitoSOX™ Red was used to assess mitochondrial superoxide production. A 5 mM stock solution was prepared in DMSO,

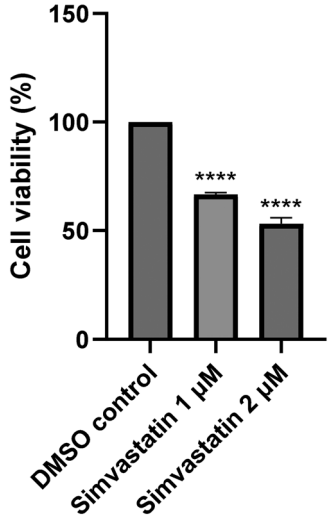

Figure 3. Cell viability (%) of SH-SY5Y human neuroblastoma cells following 48-h incubation with simvastatin (1 µM and 2 µM). Error bars represent standard error of the mean (SEM); statistical analysis was carried out using One-Way ANOVA with Tukey's multiple comparison post hoc test; levels of significance $^{***}p < 0.0001$ compared to control levels ($n = 3$). DMSO: dimethyl sulfoxide.

and a final 5 µM concentration was used. Fluorescence intensity was measured in the FL-3 channel of the same flow cytometer.

Lipid peroxidation was measured using BODIPY™ 665/676 (Invitrogen; Paisley, UK). A 2.2 mM stock solution was prepared in DMSO, and a final concentration of 10 µM was used. Fluorescence intensity was measured in the FL-4 channel. Hydrogen peroxide-induced lipoid peroxidation served as a positive control.

The monochlorobimane (mBCl) fluormetric assay measured GSH levels in SH-SY5Y cells. Fluorescence was measured at Ex = 360 nm and Em = 460 nM using a Tecan Spark Microplate Reader (Tecan; Zurich, Switzerland). L-Buthionine sulphoximine (-L-BSO) was used as a positive control to deplete GSH levels.

All assays followed manufacturer's instructions, with flow cytometry and fluorometric measurements to assess oxidative stress markers.

2.6. Measurement of mitochondrial function

Mitochondrial function and ATP levels in SH-SY5Y cells treated with simvastatin were assessed using various assays.

The activities of the MRC complexes were determined spectrophotometrically, with results expressed as a ratio to CS activity for mitochondrial enrichment. CS activity was normalized to protein concentration. These measurements followed the methods from Duberley et al. (2014) and Lowry et al. (1951), with activities recorded using a Uvikon XS UV-Visible Scanning Spectrophotometer (NorthStar Scientific Ltd.; Bedfordshire, UK).

Cellular ATP levels were measured using the CellTiter-Glo® Cell Viability Assay kit (Promega; Chilworth, UK). Luminescence was recorded using a Tecan Spark Microplate Reader Luminometer, according to the manufacturer's instructions.

Mitochondrial membrane potential was evaluated using Tetramethyl rhodamine, methyl ester (TMRM), normalized to mitotracker green fluorescence as previously described (Thoma et al. 2022, 2024).

2.7. Quantification of cellular CoQ_{10} content

The cellular CoQ_{10} content was assessed in SH-SY5Y human neuroblastoma cells by High-Performance Liquid Chromatography with electrochemical detection (HPLC-ECD). SH-SY5Y cells were seeded in 6-well plates and treated with simvastatin as previously described prior to analysis. The chromatographic system included a Shiseido Co. Ltd. 3005 electrochemical detector (ECD), 2 Shiseido-M 3201 pumps, a Shiseido-M 3023 refrigerated automatic sampler, and a Shiseido-M 3012 switch valve. The total cellular CoQ_{10} content was expressed as µg CoQ_{10}/mg of protein (Lowry et al. 1951).

2.8. Total protein determination

The cellular protein concentration was determined, according to the method of Lowry et al. (Lowry et al. 1951).

2.9. Statistical analysis

Data were presented as mean ± standard error of the mean (SEM), with error bars representing SEM. Comparisons between data sets were made using One-Way Analysis of Variance (ANOVA) with Tukey's multiple comparison post hoc test. A probability value of $p < 0.05$ was considered statistically significant. Statistical analysis and graph creation were performed using GraphPad Prism software (version 10.2.3).

3. Results

3.1. The effect of simvastatin treatment on neuronal CoQ_{10} content

The cellular CoQ_{10} content of the human SH-SY5Y cells was found to decrease significantly after 48-h treatment with simvastatin at concentrations 1 µM and 2 µM (Figure 2). Neuronal CoQ_{10} content was reduced by >50% following simvastatin treatment, compared to control cells treated with the vehicle dimethyl sulfoxide (DMSO).

3.2. The effect on cell viability post-simvastatin treatment

The cellular toxicity induced by treatment with simvastatin was identified to be both time- and dose-dependent. Treatment with simvastatin at a concentration of 0.5 µM had very little effect on cell viability, while 5 µM simvastatin initiated a significant reduction in cell viability (<50%) (data not shown). However, post-simvastatin treatment with 1 µM and 2 µM, cell viability was reduced by 33% ($p < 0.0001$, $n = 3$) and 47% ($p < 0.0001$, $n = 3$), respectively, compared to control cells treated with the vehicle DMSO (Figure 3).

3.3. The effect of cellular ROS production post-simvastatin treatment

A significant increase in intracellular ROS production was observed following neuronal cell treatment with simvastatin at concentrations of 1 µM and 2 µM, increasing by 38% ($p < 0.0001$, $n = 3$) and 42% ($p < 0.0001$, $n = 3$), respectively, compared to control cells treated with the vehicle DMSO (Figure 4(a,c)).

It was also found that mitochondrial superoxide anion production significantly increased post-simvastatin treatment (1 µM and 2 µM), by 37% ($p < 0.05$, $n = 3$) and 44% ($p < 0.005$, $n = 3$), respectively, compared to control values (Figure 4(b)). Furthermore, treatment of neuronal cells with 1 µM and 2 µM simvastatin led to an increase in free radical-induced lipid peroxidation. After treatment with 1 µM simvastatin, a non-significant ($p < 0.1409$, $n = 3$) increase in free radical-induced lipid peroxidation by 23% was observed (Figure 4(d)). However, treatment of neuronal cells with 2 µM simvastatin resulted in a significant increase in free radical-induced lipid peroxidation by 36% ($p < 0.005$, $n = 3$) (Figure 4(d)).

Cellular GSH status was found to decrease significantly by 12% ($p < 0.05$, $n = 3$) and 14% ($p < 0.05$, $n = 3$), respectively, following treatment with 1 µM and 2 µM simvastatin, respectively, compared to control cells treated with the vehicle DMSO (Figure 4(e)).

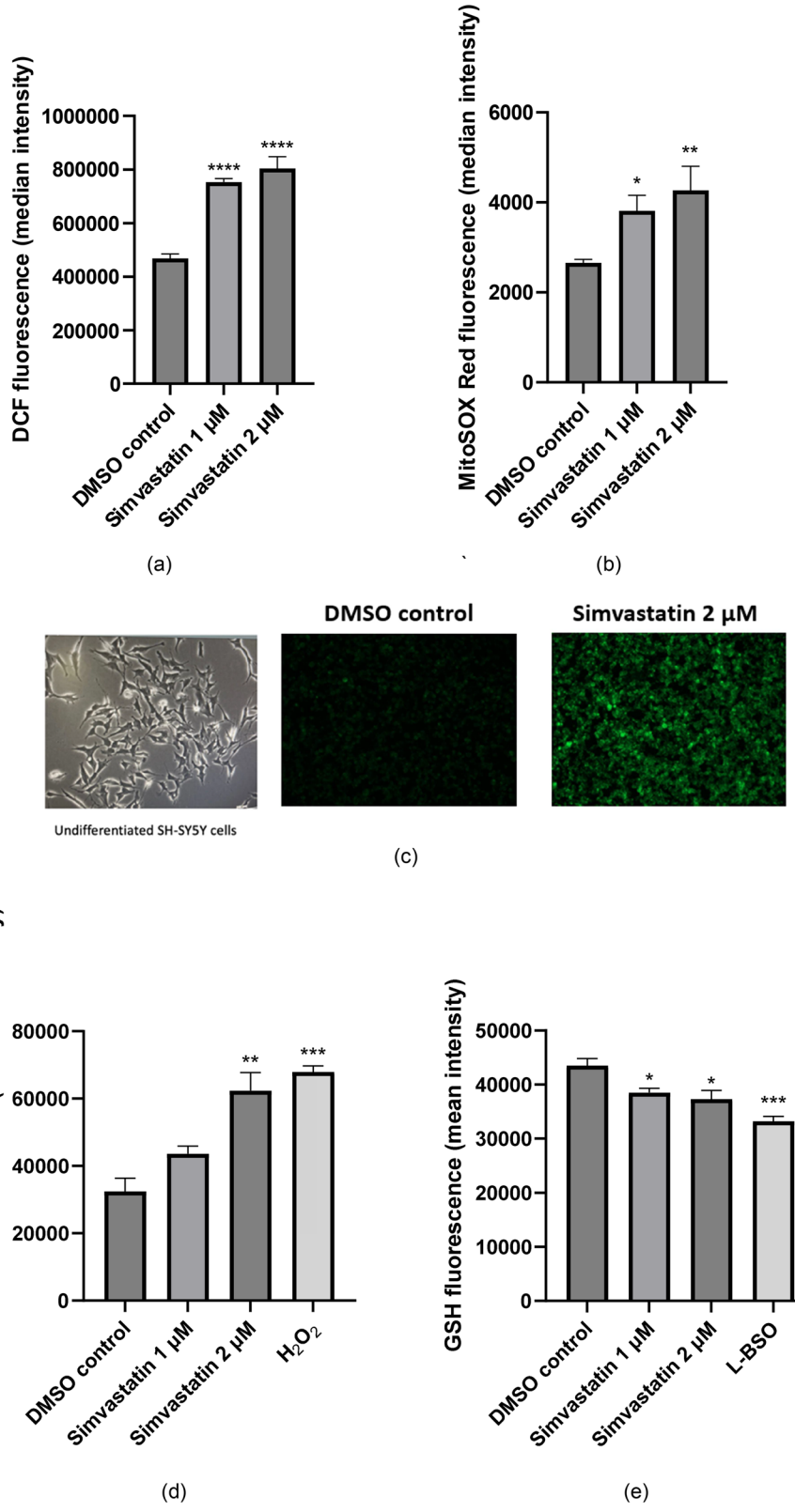

Figure 4. Effect of 48 h simvastatin treatment (1 µM and 2 µM) on cellular reactive oxygen species (ROS) production. (A) Measurement of intracellular ROS production in SH-SY5Y human neuroblastoma cells following staining with CM-H_2DCFDA. (B) Measurement of mitochondrial superoxide production in SH-SY5Y human neuroblastoma cells following SH-SY5Y staining with MitoSOX Red™. (C) Evaluation of intracellular ROS by CM-H_2DCFDA assay in the SH-SY5Y human neuroblastoma cell line following 2 µM simvastatin treatment for 48 h. Representative images taken with a Lionheart Automated Microscope post-CM-H_2DCFDA staining. (D) Assessment of lipid peroxidation in SH-SY5Y human neuroblastoma cells following SH-SY5Y staining with BODIPY™ 665/676. (E) Measurement of intracellular reduced glutathione (GSH) status in SH-SY5Y human neuroblastoma cells following SH-SY5Y staining with monochlorobimane (mBCl). Error bars represent standard error of the mean (SEM); statistical analysis was carried out using One-Way ANOVA with Tukey's multiple comparison post hoc test; levels of significance $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, $****p < 0.0001$ compared to control levels ($n = 3$). DMSO: dimethyl sulfoxide; H_2O_2: hydrogen peroxide; L-BSO: L-Buthionine sulphoximine.

3.4. The effect of mitochondrial function post-simvastatin treatment

SH-SY5Y neuronal cell treatment with 1 µM and 2 µM simvastatin resulted in a significant decrease in MRC complex I activity by 50% ($p<0.0001$, $n=3$) and 61% ($p<0.0001$, $n=3$), respectively, compared to control cells treated with the vehicle DMSO (Figure 5(a)). Additionally, treatment with 1 µM and 2 µM simvastatin resulted in a decrease in MRC complex II-III activity by 56% ($p<0.05$, $n=3$) and 67% (significant; $p<0.005$, $n=3$), respectively, compared to control cells treated with the vehicle DMSO (Figure 5(b)). In addition, a non-significant ($p<0.3295$, $n=3$) increase of 39% in MRC complex IV activity was observed after treatment with 1 µM simvastatin (Figure 5(c)). However, this increase in complex IV activity was no longer present post-treatment with 2 µM simvastatin, and a 5% decrease in complex IV activity (non-significant; $p<0.9898$, $n=3$) was observed, compared to control cells treated with the vehicle DMSO (Figure 5(c)). The activity of citrate synthase (CS), a mitochondrial marker enzyme, was found to increase post-simvastatin treatment (1 µM and 2 µM), increasing by 35% (non-significant; $p<0.3248$, $n=3$) and 61% ($p<0.005$, $n=3$), respectively, compared to control cells treated with the vehicle DMSO (Figure 5(d)).

Following SH-SY5Y neuronal cell treatment with 1 µM and 2 µM simvastatin, inhibition of intracellular ATP synthesis was observed, and the concentration of intracellular ATP was found to be significantly decreased by 19% ($p<0.05$, $n=3$) and 31% ($p<0.005$, $n=3$), respectively, compared to control cells treated with the vehicle DMSO (Figure 5(e)).

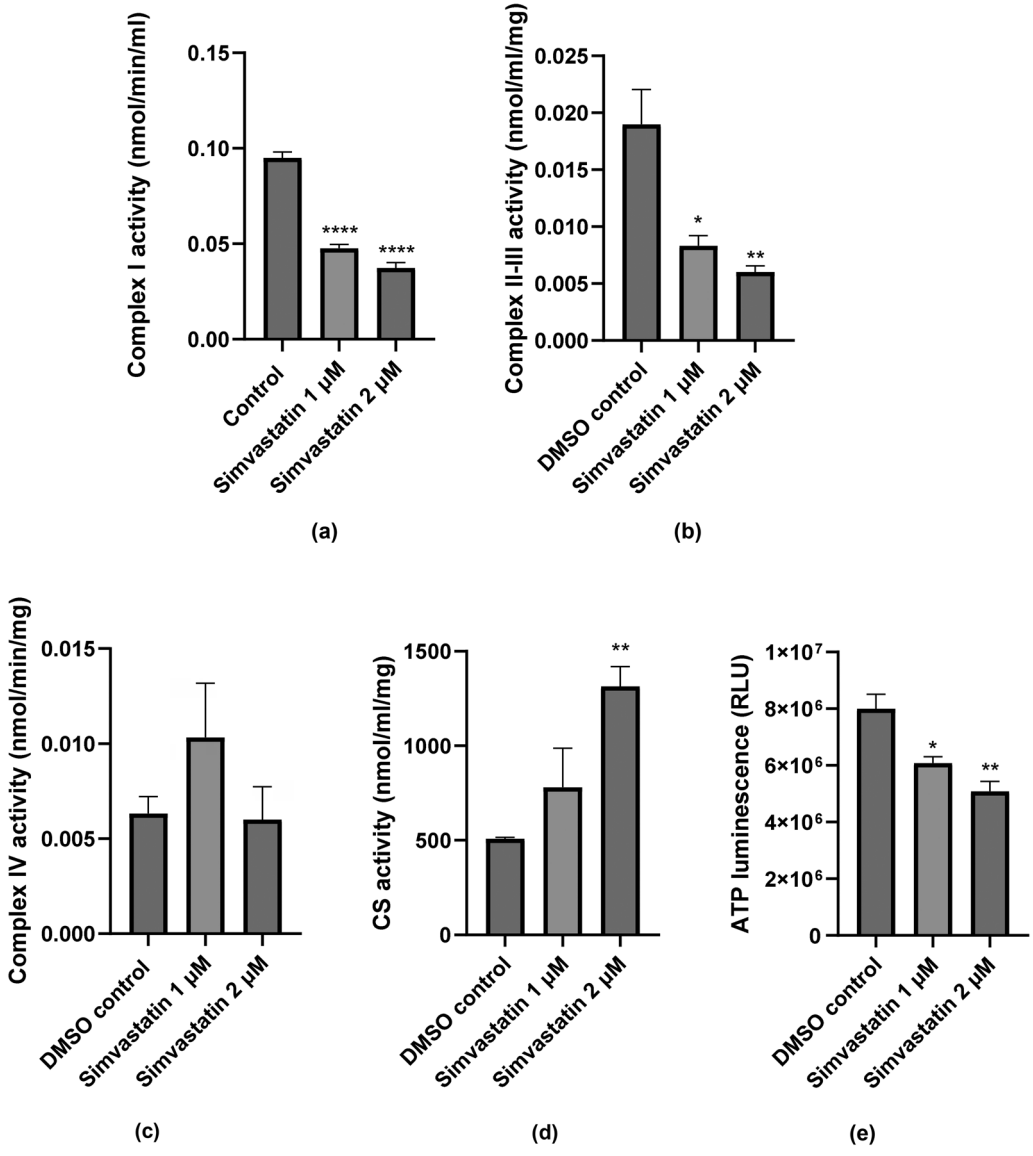

Figure 5. Effect of 48 h simvastatin treatment (1 µM and 2 µM) on mitochondrial function in SH-SY5Y human neuroblastoma cells. (A) Measurement of complex I (NADH-Ubiquinone oxidoreductase) activity measured spectrophotometrically at a wavelength of 340 nm. (B) Measurement of complex II + III (succinate dehydrogenase cytochrome *c* reductase) activity measured spectrophotometrically at a wavelength of 550 nm. (C) Measurement of complex IV (cytochrome *c* oxidase) activity measured spectrophotometrically at a wavelength of 550 nm. (D) Measurement of citrate synthase (CS) activity measured spectrophotometrically at a wavelength of 412 nm. (E) Measurement of intracellular adenosine triphosphate (ATP) status in SH-SY5Y human neuroblastoma cells. Intracellular ATP status was determined by ATP luminescence. Error bars represent standard error of the mean (SEM); statistical analysis was carried out using One-Way ANOVA with Tukey's multiple comparison post hoc test; levels of significance *$p<0.05$, **$p<0.005$, ****$p<0.0001$ compared to control levels. DMSO: dimethyl sulfoxide; ATP: adenosine triphosphate.

3.6. Impact of Simvastatin on mitochondrial membrane potential

Mitochondrial function was evaluated using two distinct mitochondria-based dyes: TMRM, which accumulates solely in active mitochondria with intact membrane potential, and MitoTracker Green FM, which assesses mitochondrial mass. Treatment of SH-SY5Y neuronal cells with simvastatin showed no significant changes in mitochondrial mass, as measured by MitoTracker Green fluorescence intensity. Cells treated with 1 µM and 2 µM simvastatin displayed relative fluorescence intensities of approximately 96.3% and 99%, respectively, compared to DMSO control cells (Figure 6(a)). However, mitochondrial membrane potential, assessed using TMRM fluorescence, showed a dose-dependent decrease following simvastatin treatment. Cells treated with 1 µM simvastatin exhibited a significant decrease in TMRM fluorescence by 4.54% ($p < 0.0216$, $n = 3$), while treatment with 2 µM simvastatin resulted in a more pronounced reduction of 11.41% ($p < 0.0001$, $n = 3$) compared to DMSO control cells (Figure 6(b)). When TMRM fluorescence was normalized to mitochondrial mass (TMRM/MitoTracker Green ratio), the 2 µM simvastatin treatment showed a significant decrease of 10.48% ($p < 0.0001$, $n = 3$) in membrane potential, while the 1 µM treatment showed a slight decrease of 0.7% with no significant change compared to DMSO control cells (Figure 6(c)).

4. Discussion

The results of the present study have provided evidence of simvastatin-induced neurotoxicity which was associated with neuronal CoQ_{10} deficiency resulting in impaired mitochondrial function and increased oxidative stress in SH-SY5Y human neuroblastoma cells.

Chronic statement treatment has been associated with increased risk of cognitive impairment, which may occur as a consequence of statin-induced neuronal CoQ_{10} depletion (Schultz et al. 2018; Mollazadeh et al. 2021). The effect of statins is dose-dependent on CoQ_{10} depletion which has been associated with impaired mitochondrial function (Schultz et al. 2018). CoQ_{10} is present in all areas of the brain and is an important endogenous antioxidant and an essential component of the mitochondrial respiratory chain, therefore it has been widely reported that a deficiency in CoQ_{10} is associated with mitochondrial ETC defects and impaired ATP synthesis, in addition to impaired antioxidant defence systems against free radical-induced oxidative stress (Hidalgo-Gutiérrez et al. 2021).

A CoQ_{10}-deficient neuronal cell line was established in order to assess the effects of statin-induced neurotoxicity on mitochondrial function and neuronal viability. The SH-SY5Y human neuroblastoma cells were incubated with 1 µM and 2 µM simvastatin for 48 h, leading to a 51% ($p < 0.005$) and 52% ($p < 0.005$) decrease in neuronal CoQ_{10} concentration, respectively, in comparison to control cells treated with the vehicle DMSO. The reported deficit in neuronal CoQ_{10} content indicates the ability of simvastatin to induce neuronal CoQ_{10} depletion. Simvastatin, a lipophilic statin (HMGR inhibitor) that is a widely used cholesterol-lowering drug, has reported to have several serious adverse effects associated with the development of oxidative stress and mitochondrial dysfunction (Robinson 2007; Golomb and Evans 2008; Fišar et al. 2016). Statins inhibit HMGR, an enzyme present at an early stage in the mevalonate pathway, resulting in not only

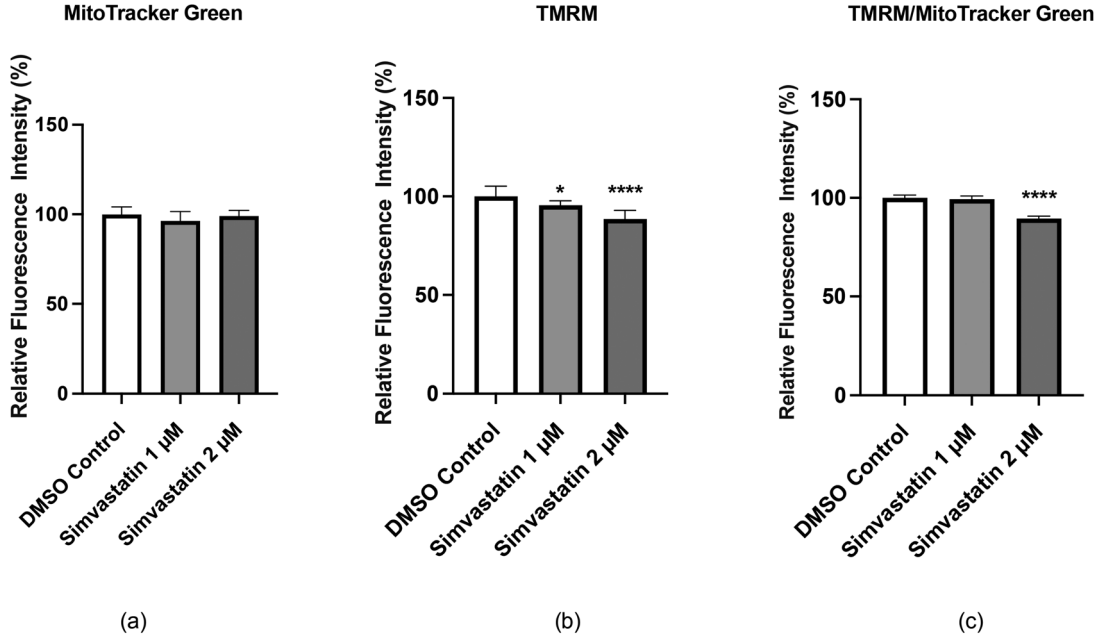

Figure 6. Effect of 48 h simvastatin treatment (1 µM and 2 µM) on mitochondrial mass and membrane potential in SH-SY5Y human neuroblastoma cells. (A) Measurement of mitochondrial mass in SH-SY5Y human neuroblastoma cells following staining with MitoTracker Green FM representing fluorescence intensity changes in mitochondrial mass. (B) Measurement of mitochondrial membrane potential in SH-SY5Y human neuroblastoma cells following staining with TMRM representing fluorescence intensity changes in mitochondrial membrane potential. (C) Fluorescence intensity of TMRM normalised to MitoTracker Green in SH-SY5Y human neuroblastoma cells. Error bars represent standard error of the mean (SEM); statistical analysis was carried out using One-Way ANOVA with Tukey's multiple comparison post hoc test; levels of significance *$p < 0.05$, ****$p < 0.0001$ compared to control levels ($n = 3$). DMSO: dimethyl sulfoxide; TMRM: tetramethylrhodamine methyl ester.

cholesterol synthesis inhibition but also inhibition of the CoQ_{10} biosynthetic pathway (Rahman et al. 2001; Quinzii et al. 2008). The subsequent inhibition of endogenous CoQ_{10} can lead to increased ROS production, impaired mitochondrial function and bioenergetic defects and reduced cell viability (Quinzii et al. 2008). These results enabled further investigation on the effects of simvastatin-induced neurotoxicity as a result of neuronal CoQ_{10} deficiency on neuronal viability and mitochondrial function.

Decreased activity of mitochondrial respiratory chain (MRC) complex II-III is indicative of a CoQ_{10} deficiency, as the activity of these enzymes is dependent upon the availability of endogenous CoQ_{10} (Rahman et al. 2001), therefore the effect of simvastatin treatment on the activity of complex II-III was assessed. Interestingly, the results show that following a significant decrease in neuronal CoQ_{10} content, the activity of complex II-III was found to be significantly reduced, with a reduction of 56% and 67% in enzyme activity post-simvastatin treatment (1 µM and 2 µM), in comparison to control levels. These results support the requirement for CoQ_{10} for optimal complex II-III activity, therefore a deficit in neuronal CoQ_{10} content may indirectly impair MRC activity. However, it has been previously reported that the inhibition threshold for complex III activity is 70-80% before oxidative phosphorylation (OXPHOS) is compromised, therefore the degree of complex II-III activity observed post simvastatin treatment may not be sufficient enough to impair OXPHOS but to cause a decrease in mitochondrial respiration and ETC electron flow, with a subsequent deficit in intracellular ATP synthesis (Davey et al. 1998; Quinzii et al. 2008).

Given that simvastatin-induced CoQ_{10} depletion may directly impair MRC activity, the activities of complex I and complex IV were also assessed following simvastatin treatment in SH-SY5Y cells. The activity of complex I was found to be significantly decreased following simvastatin treatment (1 µM and 2 µM), where the enzyme activity was reduced by 50% and 61%, respectively, compared to control levels. It has been reported by Sirvent et al. (2005a, b) that complex I is the main MRC complex affected by simvastatin as complex I subunits are the site of CoQ_{10} binding, which was supported by findings in human myocytes, where simvastatin acted primarily as a complex I inhibitor (Lenaz et al. 1997; Sirvent et al. 2005a). Although the mechanism of action of complex I inhibition by simvastatin remains to be elucidated, it has been proposed that the interaction between simvastatin and complex I may inhibit electron transfer within the complex or between complex I and II, which may result in the formation of excessive ROS, causing irreversible cell damage and death (Zhao et al. 2019). This may explain to some degree the evidence of loss of cell viability (30–50% loss of viability) indicated by the MTT assay post simvastatin (1 µM and 2 µM) treatment. Furthermore, these results show evidence of altered complex IV activity following simvastatin treatment in SH-SY5Y cells, although these results are non-significant. Following treatment with simvastatin 1 µM, a trend toward a 39% increase in the activity of complex IV was observed, whereas post-treatment with simvastatin 2 µM, there was a trend demonstrating potential weak inhibition (5% decrease) in the activity of complex IV. Several studies have found upregulation of complex IV activity associated with CoQ_{10} diminution. In a study carried out by Durhuus et al. (2020) complex IV activity was increased in platelets in simvastatin-treated patients with myalgia, and evidence of upregulated complex IV activity was identified in postmortem brain tissue from muscular system atrophy (MSA) patients (Foti et al. 2019; Durhuus et al. 2020). It has been reported under certain conditions that the activity of complex IV may be elevated by excess ROS and oxidative stress (Brealey et al. 2002; Foti et al. 2019). Moreover, inhibition of complex IV activity has been associated with age-related neurodegeneration, and it has been reported in astrocytes that reduced CoQ_{10} biosynthesis and a defect in complex IV activity may lead to cytotoxicity (Duncan et al. 2009; Lezi and Swerdlow 2012). However, it cannot be determined whether the decrease in neuronal CoQ_{10} content and complex IV activity is correlated with statin-induced inhibition of the CoQ_{10} biosynthetic pathway (Duncan et al. 2009). It has been suggested that complex IV is not a limiting step of the respiratory chain, as weak inhibition of complex IV activity was found to not affect the global rate of respiration (Lezi and Swerdlow 2012). Nonetheless, statin induced complex IV inhibition may result in elevated oxidative stress as a result of a secondary decrease in cellular CoQ_{10} content (Mazat et al. 1997). These results may explain the reduction in intracellular ATP status observed in the SH-SY5Y cells following simvastatin (1 µM and 2 µM) treatment, as indicated by ATP luminescence assay. Simvastatin-induced CoQ_{10} depletion resulted in a 19-31% inhibition of ATP synthesis. Given that CoQ_{10} is a key component in mitochondrial bioenergy transfer, facilitating electron transfer for ATP generation, a deficit in neuronal CoQ_{10} content may result in a concomitant decrease in ATP synthesis (Deichmann et al. 2010). A decrease in intracellular ATP status further supports the potential of simvastatin-induced CoQ_{10} depletion to perturb MRC function, resulting in increased apoptotic activity and oxidative stress (Duncan et al. 2009; Deichmann et al. 2010; Rzheshevsky 2014).

The significant increase in CS activity post-simvastatin (2 µM) treatment implies that simvastatin-induced CoQ_{10} deprivation triggers upregulation of mitochondrial biogenesis in SH-SY5Y cells, which has been reported as an adaptive mechanism in response to a deficit in OXPHOS (Poole et al. 2015). In order to account for mitochondrial enrichment, the MRC complex activities were expressed as a ratio to CS. Therefore, these results suggest that inhibition of the MRC complexes indicates an impairment of OXPHOS rather than a loss in mitochondrial number (Selak et al. 2000).

Taking into consideration the susceptibility of the MRC complexes to ROS-induced loss of activity, deficits in CoQ_{10} levels may decrease the cellular antioxidant capacity, causing the MRC to become more vulnerable to oxidative stress-induced impairments (Ernster and Forsmark-Andrée 1993; Heales et al. 1996; Duncan et al. 2009). Interestingly, the simvastatin-induced decrease in neuronal CoQ_{10} content and MRC function was found to accompany a significant decrease in the intracellular GSH status of SH-SY5Y cells, suggesting that the antioxidant system may be impaired as a consequence of induced MRC defects caused by a deficit in cellular CoQ_{10} levels. GSH is an important antioxidant within the brain, and it has been

concluded that brain GSH is essential for optimal mitochondrial function, therefore prolonged GSH depletion may lead to loss of neuronal integrity (Heales et al. 1995; Dringen et al. 1997). A reduction in endogenous CoQ_{10} levels with subsequent impairments in OXPHOS and the antioxidant defence system have been implicated in the age-related decline in physiological tissue functions (Mantle and Hargreaves 2019; Hargreaves et al. 2020). GSH biosynthesis is an energy-dependent process, therefore simvastatin-induced MRC defects may cause deficits in the intracellular GSH status, possibly as a consequence of reduced intracellular ATP status and increased oxidative stress (Hargreaves et al. 2005). Furthermore, given the significant role of CoQ_{10} in the cellular antioxidant defence system, diminished neuronal CoQ_{10} content may lead to excessive free radical generation causing increased oxidative damage (Duberley et al. 2013).

Elevated oxidative stress caused by excess free radical generation was detected following simvastatin treatment (1 µM and 2 µM) indicating the ability of simvastatin-induced neurotoxicity to induce elevated levels of intracellular and mitochondrial ROS, indicated by fluorescence using CM-H_2DCFDA and MitoSOX™ Red indicators, which may occur as a result of neuronal CoQ_{10} deficiency.

Further investigation of mitochondrial function in SH-SY5Y cells revealed that simvastatin treatment differentially affects mitochondrial membrane potential and mass. Using MitoTracker Green fluorescence to assess mitochondrial mass, simvastatin treatment (1 µM and 2 µM) did not significantly alter mitochondrial content, suggesting that mitochondrial mass remains intact. However, consistent with previous findings in muscle tissue (Sirvent et al. 2005a), TMRM fluorescence analysis demonstrated a dose-dependent decrease in mitochondrial membrane potential. Specifically, treatment with 1 µM simvastatin resulted in a significant reduction ($p < 0.0216$, $n = 3$), which became more pronounced at 2 µM ($p < 0.0001$, $n = 3$). When TMRM fluorescence was normalized to mitochondrial mass (TMRM/MitoTracker Green ratio), the 2 µM treatment caused a significant decrease of 10.48% ($p < 0.0001$, $n = 3$) in membrane potential, while the 1 µM treatment showed minimal changes. This selective effect on membrane potential without alterations in mitochondrial mass indicates that simvastatin primarily compromises mitochondrial bioenergetics rather than mitochondrial content. The impairment in membrane potential likely stems from the observed CoQ10 depletion, which disrupts electron transfer between complexes I, II, and III of the mitochondrial respiratory chain (Wojcicki et al. 2024). This perturbation of electron transport not only compromises energy production but may also increase electron leakage and promote the formation of the unstable semiquinone isoform of CoQ10 (Turrens et al. 1985; Hargreaves et al. 2020). These findings align with previous studies demonstrating simvastatin-induced mitochondrial dysfunction and membrane depolarization (Sirvent et al. 2005a, 2012) and complement the observed decreases in respiratory chain complex activities.

Despite the compromised bioenergetic function, the preservation of mitochondrial mass suggests an attempted compensatory response by neuronal cells. This adaptation is evidenced by increased citrate synthase activity, potentially reflecting an effort to maintain cellular energy homeostasis through enhanced mitochondrial capacity (Hughes et al. 2014; Zong et al. 2024). However, this compensatory mechanism appears insufficient to overcome the fundamental deficits in electron transport and energy production, ultimately failing to prevent the cascade of mitochondrial dysfunction and oxidative damage (Sas et al. 2007). The observed pattern of maintained mitochondrial mass but impaired membrane potential establishes a critical mechanistic link between simvastatin-induced CoQ10 deficiency and oxidative stress in neuronal cells (Quinzii et al. 2008). The findings suggest that membrane potential disruption initiates a self-perpetuating cycle of mitochondrial dysfunction and ROS generation. The compromised membrane potential, resulting from CoQ10 depletion, appears to promote electron leakage from respiratory chain complexes I and III, leading to increased formation of superoxide radicals as detected by MitoSOX™ Red staining. This elevated ROS production can subsequently damage respiratory chain components and membrane phospholipids, further compromising mitochondrial function and accelerating oxidative stress. This vicious cycle of mitochondrial dysfunction and oxidative damage may explain the progressive nature of simvastatin-induced neurotoxicity and provide insight into potential therapeutic interventions targeting mitochondrial function.

This study also identified an increase in simvastatin-induced oxidative stress indicated by fluorescence using BODIPY™ 665/676 indicator, which was used to identify an increase in the formation of free radicals, particularly peroxyl radicals, an indicator of enhanced lipid peroxidation processes. The increase in mitochondrial oxidative stress following simvastatin-induced neurotoxicity may occur as a consequence of impaired mitochondrial function, caused by inefficient electron transfer between complex I, II and III, leading to increased electron leakage from the MRC and augmented production of the unstable semiquinone isoform of CoQ_{10}, which is a major site of ROS production (Turrens et al. 1985; Hargreaves et al. 2020). These results were supported in a study carried out by Quinzii et al. (Quinzii et al. 2008), where a partial CoQ_{10} deficiency caused oxidative stress associated with increased cell death (Quinzii et al. 2008). It was observed that cultured fibroblasts with severe (< 20% of residual CoQ_{10}) and moderate (> 60% of residual CoQ_{10}) CoQ_{10} deficiencies display low levels of oxidative stress, whereas intermediate (30–40% of residual CoQ_{10}) CoQ_{10} deficiency in cultured fibroblasts display increased ROS production and lipid oxidation, in addition to moderate bioenergetic defects that are associated with increased cell death (Quinzii et al. 2008; Duberley et al. 2013). Moreover, CoQ_{10} can prevent lipid peroxyl radical formation as a result of its antioxidant role, therefore it can be assumed that a deficit in the cellular CoQ_{10} content may stimulate lipid peroxidation, leading to an increase in ROS-induced oxidative damage to biological membranes (Turunen et al. 2004; Zozina et al. 2018). It was suggested by Forsmark-Andrée et al. (Forsmark-Andrée et al. 1997) that activation of the process of lipid peroxidation may cause depletion of the CoQ_{10} pool, which as a result causes a cascade of events leading to MRC impairments and elevated oxidative stress, ultimately leading to apoptosis-mediated neuronal cell death (Forsmark-Andrée et al. 1997).

5. Conclusion

In conclusion, the results of this study have shown evidence to support that mitochondrial dysfunction and increased oxidative stress are consequences of simvastatin-induced neurotoxicity. Interestingly, a 34% decrease in neuronal CoQ_{10} content was shown to induce a loss of neuronal viability, inhibit complex I and complex II-III activity, inhibit antioxidant defence systems as evidenced by decreased intracellular GSH status and inhibition of intracellular ATP synthesis. This study further provided evidence revealing the effect of simvastatin-induced CoQ_{10} depletion on oxidative damage in SH-SY5Y cells caused by elevated oxidative stress, as evidenced by an increase in intracellular and mitochondrial ROS formation. These results highlight the vulnerability of neurons to a deficit in neuronal CoQ_{10} content and may provide important insights into the effects of CoQ_{10} deficiency on neuronal integrity as a result of chronic statin treatment. Consequently, these results may provide important insights into potential therapeutic strategies targeting these parameters. For example, CoQ_{10} supplementation may be beneficial for statin users in order to improve mitochondrial function, which could prevent impaired neurological function. However, this study was conducted with the SH-SY5Y human neuroblastoma cell line, therefore further research in animal models is essential to establish these mechanisms and validate the results of our *in vitro* study.

Authors' contributions

Conceptualization, L.T. and I.P.H.; methodology, L.M., N.T., F.M., P.O., and S.S.; formal analysis, L.M.; investigation, L.M.; resources, L.T., E.D., and I.P.H.; writing- original draft preparation, L.M.; writing- review and editing, L.T., E.D., and I.P.H.; supervision, L.T., E.D., and I.P.H.; funding acquisition, L.T., and I.P.H. All authors have read and agreed to the published version of the manuscript.

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