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Mitochondrial dynamics imbalance and mitochondrial dysfunction contribute to the molecular cardiotoxic effects of lenvatinib

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ABSTRACT

Lenvatinib is a tyrosine kinase inhibitor (TKI) approved for the treatment of resistant differentiated thyroid cancer, advanced renal cell carcinoma, unresectable hepatocellular carcinoma, and endometrial carcinoma. Although it is successful in cancer treatment, it can cause life-threatening side effects such as cardiotoxicity. The molecular mechanism of cardiotoxicity caused by lenvatinib is not fully known.

In this study, the molecular mechanism of lenvatinib's cardiotoxicity was investigated focusing on mitochondrial toxicity in the H9c2 cardiomyoblastic cell line. Lenvatinib inhibited cell viability at 48 and 72 h exposure with three selected concentrations (1.25 μM, 5 μM and 10 μM); and inhibited intracellular ATP after 72 h exposure compared to the control group. Mitochondrial membrane potential was decreased after 48 h and did not show significant changes after 72 h exposure. Evaluated with real-time PCR, mitochondrial dynamics (*Mfn1, Mfn2, OPA1, DRP1, Fis1*) expression levels after lenvatinib treatment significantly changed. Lenvatinib triggered the tendency from fusion to fission in mitochondria after 48 h exposure, and increased both fusion and fission after 72 h. The mtDNA ratio increased after 48 h and decreased after 72 h. ASK1, JNK and *AMPKα2* increased. *UCP2* showed downregulation, *SOD2* level showed upregulation and *Cat* levels decreased after drug treatment. *Nrf1* and *Nrf2* also changed concentration-dependently. Protein carbonyl levels increased significantly after lenvatinib treatments indicating oxidative stress. The protein levels of the electron transport chain complexes, LONP1, UCP2, and P21 showed significant differences after lenvatinib treatment.

The outcome of our study is expected to be a contribution to the understanding of the molecular mechanisms of TKI-induced cardiotoxicity.

1. Introduction

Tyrosine kinases are enzymes that act by reversible phosphorylation affecting signal pathways that control many vital activities such as, cell cycle, cell proliferation, cell death, differentiation and repair in the regulation of cell biology (Force and Kolaja, 2011; Roskoski, 2015). After understanding that mutations and disorders in kinases play a role in the emergence of cancer, diabetes, autoimmune diseases, cardiovascular, inflammatory and nervous system diseases, the kinase enzyme family has become one of the most important drug targets. In order to interfere with the activity of kinases in tumor cells, the small molecule agents tyrosine kinase inhibitors (TKIs) have been developed as effective drugs.

TKI drugs have been successful in keeping cancer under control by inhibiting activated signal pathways in cancer cells. Although these drugs are generally considered safer than chemotherapeutic cytotoxic agents, they have been shown to cause adverse effects such as proteinuria, stomatitis, hypertension, skin toxicity, anemia, neutropenia, thrombocytopenia, diarrhea, nausea, vomiting, edema and hypothyroidism. One of the serious side effects observed is cardiotoxicity which can force the physician and patient to reduce the dose and eventually discontinue and choose an alternative treatment (Chen et al., 2008; Narayan and Ky, 2018).

Lenvatinib is a tyrosine kinase inhibitor anticancer drug that is taken orally and is effective by inhibiting multiple tyrosine kinase receptors such as vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), Platelet-derived growth factor receptor-alpha (PDGFR-alpha) which are important for angiogenesis along with RET and KIT proto-oncogenes. It was approved by the Food and Drug Administration (FDA) in 2015 for the treatment of differentiated thyroid

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cancer resistant to radioactive iodine therapy (Bruheim et al., 2011; Hussein et al., 2017; Krajewska et al., 2015; Schlumberger et al., 2016). Other indications of lenvatinib approved by FDA are advanced renal cell carcinoma in 2016, unresectable hepatocellular carcinoma in 2018 and endometrial carcinoma in 2019. Research continues on the use of lenvatinib in the form of monotherapy or combination therapy against solid tumors including melanoma, non-small molecule lung carcinoma, glioblastoma multiforme, ovarian and endometrial carcinoma (U.S. FDA, 2019). In the "Warnings" section of the product information of lenvatinib; hypertension, cardiac dysfunction, arterial thromboembolic events, QT interval prolongation are stated (Schlumberger et al., 2015; Scott, 2015; U.S. FDA and CDER, 2015).

Drug-induced cardiotoxicity following cancer therapy is defined as a group of adverse events negatively affecting myocardial functions, which is reported as the most common cause of death in long-term survivors of cancer (Giudice et al., 2020). There is indeed a variability in the underlying mechanism of TKIs induced cardiotoxicity. Some side effects can be anticipated based on the receptor tyrosine kinase profile. Hypothesized mechanisms of TKIs induced cardiotoxicity are mainly involved with the following aspects: Effects on vasculature, mitochondrial dysfunction, modulation of mitogen-activated protein kinase activity, PDGFR inhibition, and ion channel inhibition (Lamore et al., 2019; Mellor et al., 2011). Although several hypotheses related to TKI-induced cardiotoxicity have been proposed, the molecular mechanisms of cardiotoxicity are not fully understood (Chen et al., 2008; Shim et al., 2017).

The heart muscle is very dependent on aerobic metabolism and is very sensitive to drugs that disrupt mitochondrial metabolism or homeostasis due to the high energy use for cardiac tissue contractility. Mitochondria account for around 30% of the total volume of cardiac myocytes by giving energy to strengthen the continuous cardiac contractility needed to pump blood and nutrients throughout the body in mammals (Barth et al., 1992). Mitochondrial damage is one of the factors in the onset of cardiovascular diseases and it is stated that one of the mechanisms of cardiotoxicity caused by TKIs may be mitochondrial toxicity (French et al., 2010; Gorini et al., 2018; Greineder et al., 2011; Tridente, 2017).

Our aim in this study was to examine the underlying causes of cardiotoxicity induced by the TKI drug lenvatinib at the molecular level in a cardiomyocyte model (H9c2). We have focused on investigating the essentials of mitochondrial function including intacellular ATP level and mitochondrial membrane potential (MMP), mitochondrial dynamics and expression of proteins related to mitochondria.

2. Materials and methods

2.1. Cell culture

Rat cardiomyoblastic H9c2 cell line from the American Type Culture Collection (ATCC) was used. The cells were cultured in DMEM-F12 medium with 10% FBS (fetal bovine serum) and 1% penicillin-streptomycin-amphotericin (all from Gibco) at 37 °C with 5% CO_2 and 92% humidity. Subculturing was done every 3 days (60–70% confluency) with trypsinization and cells with passage number under 25 were used in the experiments to ensure myoblastic cell population. Lenvatinib Treatment.

Selected exposure concentrations of lenvatinib (Santa Cruz Biotechnology) were 1.25 μM, 5 μM and 10 μM and two endpoints were chosen as 48 h and 72 h and not 24 h since the effect of extended lenvatinib exposure time was more reasonable to investigate considering that the drug is used by the patients for a long period of time. The selected concentrations and endpoints in this study were determined considering the available data in the literature (Rodríguez-Hernández et al., 2020; Zhang, 2017) and the results of our conducted cell viability tests and intracellular ATP content measurement. The cells of the "control group" in the experiments were treated with medium containing the same ratio of DMSO with the treatment groups.

2.2. MTT cytotoxicity assay

Before exposure, 10^4 cells/well were seeded in a 96 well plate. After a 24 h incubation, the cells were exposed to lenvatinib. After the drug exposure times (48 h and 72 h) 20 μL MTT solution (Biomatik, 5 mg/mL in PBS) was added to each well and kept in the dark for 3 h at 37 °C with 5% CO_2 and 92% humidity. Following the 3 h incubation time, the medium was removed and the formazan crystals were dissolved in 100 μL DMSO. The absorbance was measured with a microplate reader at 590 nm (BioTek, Epoc). Cell viability percentage of the treatment group cells was calculated by dividing the recorded absorbance to the absorbance of the solvent control cells.

2.3. Determination of ATP content

ATP content of the cells was determined by using the ATP Bioluminescence kit (Invitrogen). According to the instructions, 5×10^4 cells were seeded into each well of a 24-well plate and drug treatment was conducted after a 24 h incubation. Following lenvatinib treatment, cells were shaken for 5 min with a lysis buffer (200 mM Tris (PH:7.5), 2 M NaCl, 20 mM EDTA, 0.2% Triton X-100, 1% DTT). 8 μL of the lysed cell suspension was added to 92 μL of standard reaction solution in a luminometer suitable 96-well plate. The luminescence intensity was measured with Multimode Plate Reader (EnSpire-PerkinElmer). Protein concentrations were measured using the Bicinchoninic acid (BCA) Protein Assay kit (Intron Biotechnology) from the lysates and the results are given as a percentage relative to the control group.

2.4. Mitochondrial membrane potential (MMP)

Measurement was done using tetraethylbenzimidazolcarbocyanide iodide (JC-1). 2.5×10^5 cells were seeded in T25 cm^2 flasks and after 24 h incubation the cells were treated with lenvatinib. After the drug exposure time, cells were collected in clear fluorescence-activated cell sorting (FACS) tubes with trypsinization and washed with PBS. JC-1 dye was added and the cells were incubated for 15 min at 37 °C. Afterwards, the cells were suspended in PBS. MMP was measured with a flow cytometry (FACSCalibur, Becton-Dickinson). The results were analyzed in FL-1 and FL-2 channel using a BD software program (BD Biosciences).

2.5. Evaluation of gene expression levels

Gene expressions of Mitofusin 1 (*Mfn1*), Mitofusin 2 (*Mfn2*), Optic atrophy 1 (*OPA1*), Dynamine related protein (*DRP1*), Fission 1 (*Fis1*), Apoptosis signal-regulating kinase 1 (*ASK1*), C-Jun N-Terminal Kinase (*JNK*), 5′ Adenosine monophosphate-activated protein kinase-α2 (*AMPKα2*), Uncoupling protein 2 (*UCP2*), Superoxide dismutase 2 (*SOD2*), Catalase *(Cat)*, Nuclear factor erythroid 2 related factor 1 and 2 (*Nrf1* and *Nrf2*) were evaluated by reverse transcription polymerase chain reaction (RT-PCR) and Beta actin was used as house-keeping gene. Total RNA extraction was done according to the manufacturer's instructions of High Pure RNA Isolation Kit (Roche Diagnostics). cDNA synthesis was done using Roche Transcriptor cDNA synthesis kit (Roche Diagnostics). RT-PCR was performed in StepOnePlus (Thermo Fisher Scientific Inc.) by PowerUp SYBR green master mix (Thermo Fisher Scientific Inc.). Gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The studied primer sequences can be found in Table 1.

2.6. Evaluation of mtDNA content

For the determination of mtDNA content of the cells, total DNA isolation was done using G-spin Total DNA Extraction Kit (Intron Biotechnology). The mtDNA content was determined by real-time PCR

Table 1
Primer sequences for determination of gene expression levels.

Gene	Forward primer (FP)	Reverse primer (RP)	Melting (°C)
Mfn1	AGAATTTCCTTGGGCTCCAT	AGAAAGGAGGTGGACGGTTT	60
Mfn2	GTGACTGGACTCCCCAGAAA	ATCACAATGCCAGACACCAA	60
OPA 1	GCATCTGGTTTCCCGAAGTA	GAGCCTTGCAGCTAACCTTG	60
DRP1	GTAGGTGATCAGCCCAAGGA	CATCTGGATCTACCTCTCTGGAA	60
Fis1	AAATGATGCTACGCAGGCTT	CCTGGACCATGACCAAGTTT	60
ASK1	GAATCGCCTTCGAATCAAAC	TCCATCTTCATCCTCCCAAG	60
JNK	TCGGAGCATGAGCGTGTTCT	AGACGGCTTCTCAGCGATCA	63
AMPKα2	CTGCCGTGGACTACTGTCAC	TCTTAGCATTCATCTGGGCG	61
UCP2	CCACAGCCACCGTGAAGTT	CGGACTTTGGCGGTGTCTA	61
SOD 2	CCTGACTATCTGAAAGCCATTTGG	CACAGTGCTGCAATGCTCTACAC	63.2
Cat	GGGTGTGACTCATTCTTCCG	AACAGCCCATCACCTACCAA	61
Nrf1	GCAACAGGGAAGAAACGG	GTGTCTGCTGTCTCTTTCGGATA	61
Nrf2	TCACCTACATTTCAGTCGCTTG	AAACTTGCTCCATGTCCTGC	61
BETA ACTIN	ATGGTGGGTATGGGTCAGAA	CTTTTCACGGTTGGCCTTAG	60

in StepOnePlus using SYBR green master mix. The studied primer sequence list for mtDNA content evaluation can be found in Table 2. mtDNA copy number was calculated by a described method using the following formula (Quiros et al., 2018).

$$\Delta CT = Ct_{nuclear\ DNA\ gene} - Ct_{mtDNA\ gene}$$

$$MtDNA\ content = 2 \times 2^{\Delta CT}$$

2.7. Evaluation of protein expression levels

The total protein of the cardiomyocyte cells was isolated using the RIPA lysis buffer system (Santa Cruz Biotechnology). Protein concentrations were measured using the BCA Protein Assay kit (Intron Biotechnology). 40 μg of protein samples were loaded with the final volume of 25 μL into SDS-PAGE after mixing with NuPAGE sample loading buffer and NuPAGE reducing agent and water. After the electrophoresis process, protein blotting on nitrocellulose or PVDF membrane and blocking with 5% non-fat-dry milk were done. Incubation with primer antibodies overnight (+4 °C) was done and the membrane was washed with tris buffered saline with Tween 20 (TBST). The membrane was then incubated for 2 h with HRP-conjugated seconder antibody at room temperature (antimouse (sc-2357, Santa Cruz Biotechnology) and antirabbit (sc-516,102, Santa Cruz Biotechnology) (1:10000 dilution). Following another washing step, the proteins were visualized by Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientifc Inc.) using the ChemiDoc MP Imaging System (BioRad). The density of the bands was analyzed using Image J software HRP-conjugated β-actin (sc-47,778, Santa Cruz Biotechnology) was used as the loading control.

The primer antibodies were as follows; total oxidative phosphorylation (OXPHOS) antibody cocktail (ab110413, Abcam), Lon protease 1 (LONP1) (28020S, Cell Signaling), P21 (sc-469, Santa Cruz Biotechnology), uncoupling protein 2 (UCP2) (89326S, Cell Signaling).

2.8. Evaluation of protein carbonyl level

The level of protein carbonylation was measured as an oxidative stress biomarker. Protein carbonyl enzyme-linked immunosorbent (ELISA) assay kit (Bioassay Technology Laboratory, China) based on the biotin double antibody sandwich technology has been used for detecting the level of protein carbonylation in the cell lysates in accordance with the manufacturer's protocol. The protein carbonyl level of cells was measured at 450 nm using a microplate reader.Protein concentrations were measured using the BCA Protein Assay kit (Intron Biotechnology) and the results are given as ng/mg protein.

Table 2
Primer sequence list for determination of mtDNA content.

Gene	5′- 3′primer sequences	Melting (°C)
NADH dehydrogenase (Complex I)	F: ACCTCACCCCCTTATCAACC	60.1
	R: GGAGCTCGATTTGTTTCTGC	60.0
BETA ACTIN	F: ATGGTGGGTATGGGTCAGAA	60.0
	R:CTTTTCACGGTTGGCCTTAG	59.7

2.9. Imaging mitochondria with transmission electron microscope (TEM)

The cells were trypsinized and washed with PBS after treatment with lenvatinib. The cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4 °C and washed three times with PBS. Afterwards, another fixation with 1% OsO_4 in 0.1 M phosphate buffer for one hour was done and the cells were washed three times. After the second fixation, the cells were embedded in small blocks of 4% agar and were dehydrated in ethanol (50%, 70%, 96% and 100% ethanol) and again embedded in araldite resin. Ultra-thin sections (60 nm) were prepared using an ultramicrotome (Leica Ultracut), stained with uranyl acetate-lead citrate, and examined with JEOL JEM 1220 TEM. The images were evaluated by two independent blind reviewers.

2.10. Statistical analysis

All experiments were performed in biological and technical triplicate. The results were expressed as the mean ± SD and comparison of groups was done by the one-way ANOVA test followed by the Tukey's test using Graphpad Prism Software. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of lenvatinib on cell viability

According to the results of the MTT assay, the cell viability of 48 h lenvatinib treated cells at 5 μM and 10 μM concentration was decreased compared to the control group. After 72 h treatment a significant decreasement ($p < 0.001$) was recorded in all lenvatinib-treated groups, in a dose dependent manner (Fig. 1).

3.2. Effect of lenvatinib on intracellular ATP content

As shown in Fig. 2, after 48 h lenvatinib treatment the ATP content of the cells was reduced in the 10 μM treatment group and a significant decrement was seen in all three concentration groups after 72 h treatment.

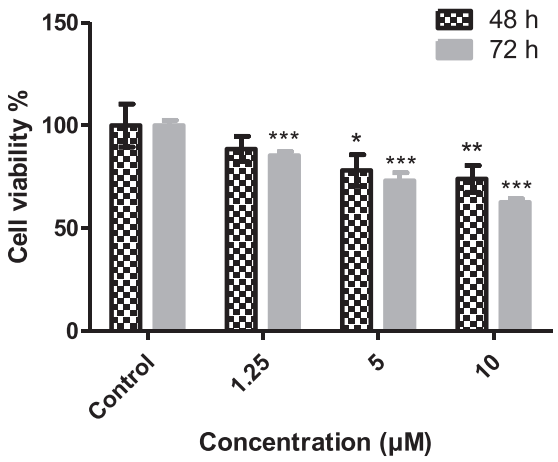

Fig. 1. MTT assay results, effect of lenvatinib on H9c2 cell viability following 48 h and 72 h treatment.
Cells were treated with 1.25 μM, 5 μM and 10 μM lenvatinib for 48 h and 72 h. Data are expressed as mean ± SD, $*p < 0.05$ $**p < 0.01$ and $***p < 0.001$ versus control group. MTT: 3-(4,5 dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide.

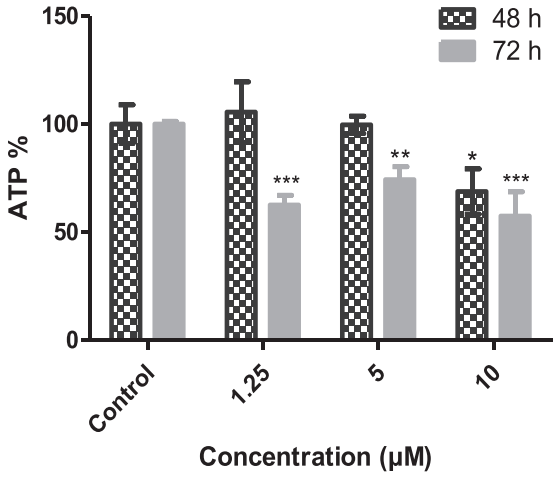

Fig. 2. Effect of lenvatinib on the ATP content of H9c2 cells after 48 h and 72 h treatment.
Cells were treated with 1.25 μM, 5 μM and 10 μM lenvatinib for 48 h and 72 h. Data are expressed as mean ± SD, $*p < 0.05$ $**p < 0.01$ and $***p < 0.001$ versus control group.

3.3. Effect of lenvatinib on MMP

As shown in Fig. 3, loss of MMP was determined in the 5 μM and 10 μM concentration groups after 48 lenvatinib treatment ($p < 0.05$). MMP did not show significant changes after 72 h exposure.

3.4. Effect of lenvatinib on gene expression levels

3.4.1. Mitochondrial dynamics

The expression of genes related to mitochondrial dynamics (fusion and fission genes) significantly changed after 48 h and 72 h lenvatinib treatment (Fig. 4). After 48 h of lenvatinib treatment there was a significant increase of the fusion gene *Mfn1* in the 1.25 μM concentration group. There was no significant difference in the 5 μM group while in the 10 μM treatment group inhibition was observed. After 72 h of lenvatinib exposure, an increase of the *Mfn1* gene level in the 5 μM and 10 μM treatment groups was observed. The upregulation after 1.25 μM lenvatinib treatment was not significant (Fig. 4a). A dose-dependent inhibition was observed after 48 h of exposure in the fusion gene *Mfn2*. *Mfn2* levels were upregulated after 72 h of exposure in all of the concentration groups (Fig. 4b). After 48 h and 72 h of lenvatinib treatment, the fusion gene *OPA1* level increased in all of the treatment groups although insignificantly in the 1.25 μM concentration group (Fig. 4c).

The fission gene level *DRP1* increased significantly after 48 h 10 μM lenvatinib treatment. Likewise, a significant increase has been recorded after 72 h 5 μM and 10 μM lenvatinib treatment (Fig. 4d). The other fission gene *Fis1* level showed significant upregulation after 48 h and 72 h in the 10 μM treatment groups (Fig. 4e).

3.4.2. mtDNA content after lenvatinib treatment

According to the results, upregulation in the mtDNA content has been observed in all three of the 48 h treatment groups (Fig. 4f). Conversely, in the 72 h treatment groups, the mtDNA content shows downregulation in all of the treatment groups.

3.4.3. ASK1, JNK, AMPKα2, UCP2, SOD2, Cat, Nrf1, and Nrf2

The apoptosis signal regulating kinase *ASK1* gene level shows significant upregulation in the 5 μM and 10 μM treatment groups after 48 h exposure. All of the 72 h lenvatinib treatment groups showed significant upregulation in the *ASK1* gene level (Fig. 5a). The kinase gene *JNK* level showed significant upregulation in the 48 h 1.25 μM and 5 μM treatment groups and in the 72 h 10 μM treatment group (Fig. 5b). *AMPKα2* level shows insignificant upregulation in the 48 h treatment groups and in the 10 μM treatment group of 72 h (Fig. 5c).

The uncoupling protein *UCP2* level shows significant downregulation in all of the treatment groups significantly except the 72 h 1.25 μM treatment group (Fig. 5d). The mitochondrial antioxidant gene *SOD2* level shows upregulation in the 48 h 5 μM treatment group and in the 72 h 10 μM treatment group (Fig. 5e). The antioxidant enzyme *Cat* level shows significant downregulation in all of the treatment groups (Fig. 5f). The antioxidant pathway regulators *Nrf1* and *Nrf2* show downregulation in the 48 h treatment groups while upregulation is observed in the 72 h treatment groups (Fig. 5 g, h).

3.5. Effect of lenvatinib on protein expression level

3.5.1. Mitochondrial electron transport chain (ETC) complexes protein levels

As can be seen in Fig. 6, after 48 h 1.25 μM lenvatinib treatment Complex I and Complex III protein level showed significant increase. The 48 h 5 μM treatment group showed inhibition in Complex II, Complex III and Complex IV. In the 48 h 10 μM treatment group, there has been a significant inhibition in Complex I, Complex II, Complex III and Complex IV whereas a significant increase has been observed in Complex V.

After 72 h 1.25 μM lenvatinib treatment there has been an increase in Complex I and Complex IV. The 72 h 5 μM treatment group showed inhibition in Complex V, whereas an increase has been observed in the Complex IV protein level. In the 72 h 10 μM treatment group the protein levels of all of the Complexes have declined.

3.5.2. LONP1 protein level

As can be seen in Fig. 7a, significant inhibition in the protein level of LONP1 has been observed in the 1.25 μM and 5 μM treatment groups of 48 h exposure. The change seen in the 48 h 10 μM treatment group was not found statistically significant. After 72 h lenvatinib exposure, all of the concentration groups showed significant downregulation.

3.5.3. P21 protein level

After 48 h lenvatinib exposure a significant downregulation has been observed in the 10 μM concentration group (Fig. 7b) and after 72 h lenvatinib exposure the protein level showed a significant increase only in the 1.25 μM treatment group.

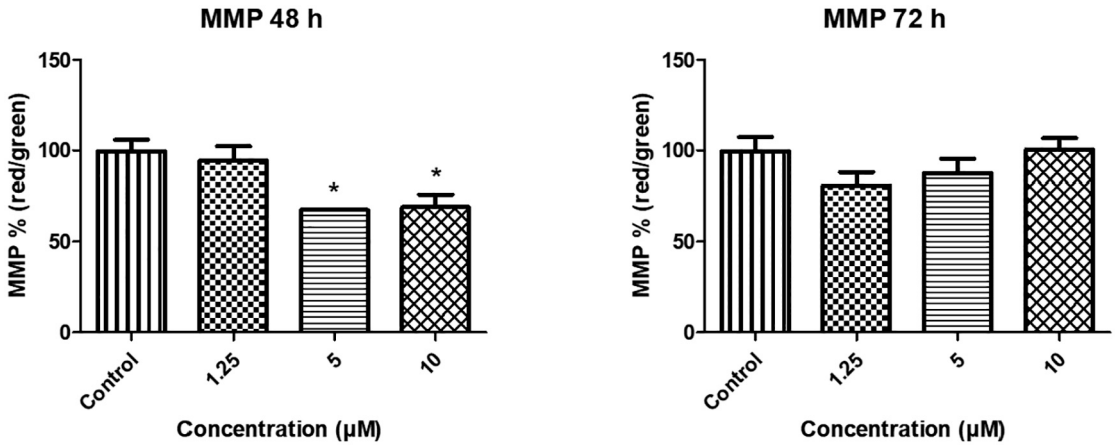

Fig. 3. Effect of lenvatinib on MMP of H9c2 cells following a) 48 h and b) 72 h treatment.
Cells were treated with 1.25 μM, 5 μM and 10 μM lenvatinib for 48 h and 72 h. Data are expressed as mean ± SD, $^{\#}p < 0.05$ versus control group. MMP: Mitochondrial membrane potential.

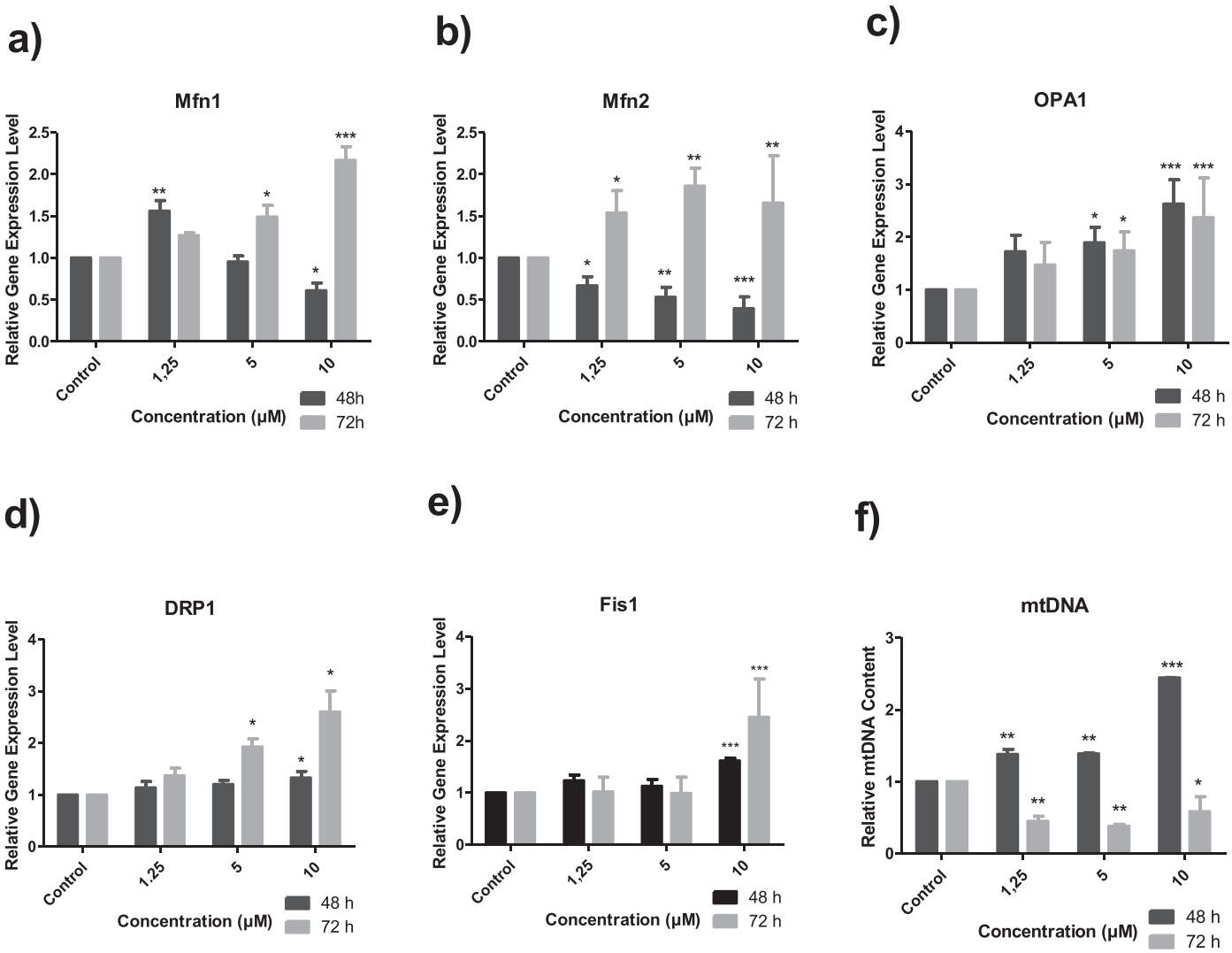

Fig. 4. Effect of lenvatinib on the expression of the mitochondrial fusion, fission, and mitochondrial DNA genes in H9c2 cells following 48 h and 72 h treatment. Cells were treated with 1.25 μM, 5 μM and 10 μM lenvatinib for 48 h and 72 h. Data are expressed as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control group. Mfn1: Mitofusin 1, Mfn2: Mitofusin 2, OPA1: Optic atrophy 1, Fis1: Mitochondrial fission 1, DRP1: Dynamin-related protein, mtDNA: Mitochondrial DNA.

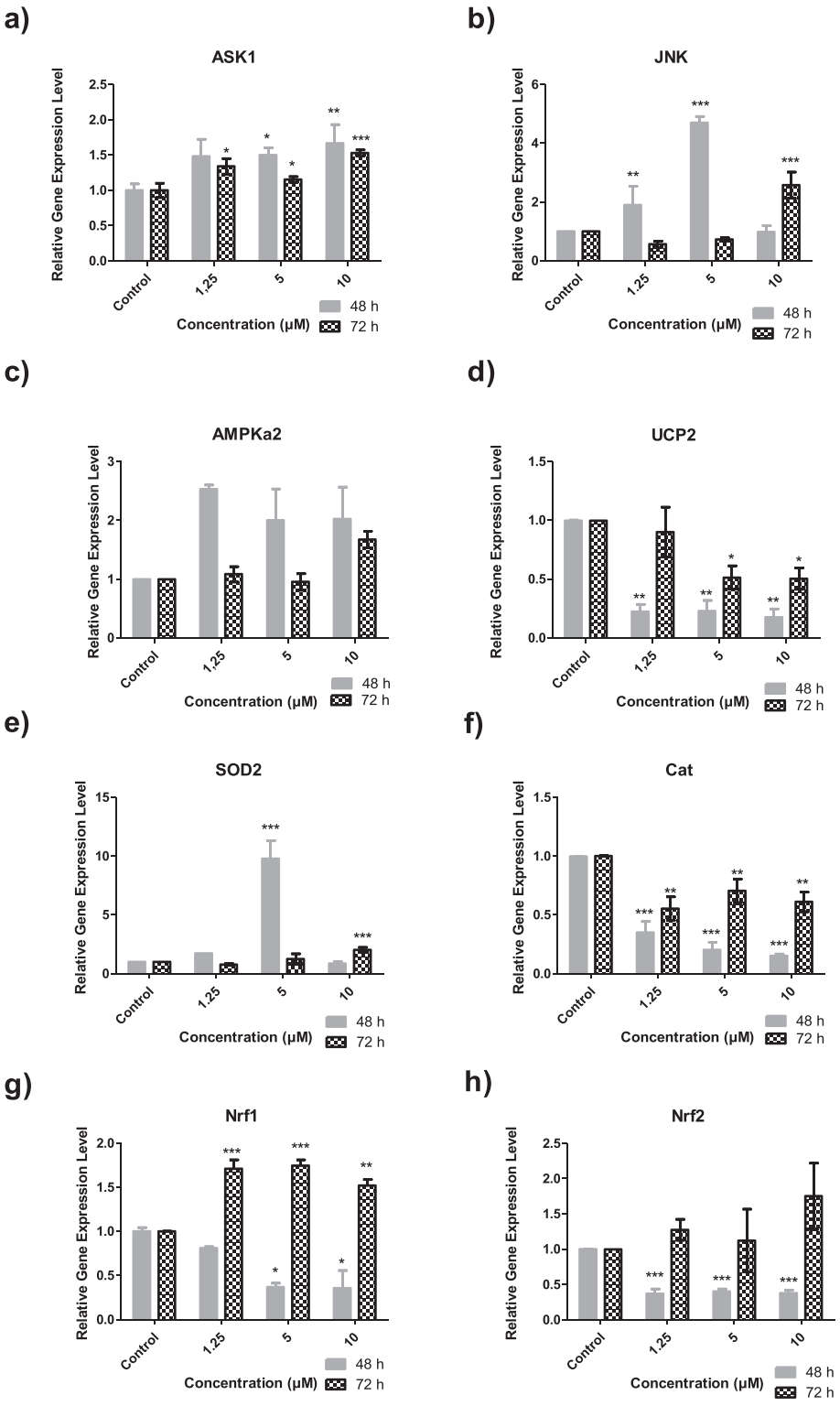

Fig. 5. Effect of lenvatinib on the expression of the redox signaling related genes in H9c2 cells following 48 h and 72 h treatment.
Cells were treated with 1.25 μM, 5 μM and 10 μM lenvatinib for 48 h and 72 h. Data are expressed as mean ± SD, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ versus control group. ASK1: Apoptosis signal regulating kinase, JNK: C-Jun N-Terminal Kinase, AMPKα2: Adenosine monophosphate-activated protein kinase-α2, UCP2: Uncoupling protein 2, SOD2: Superoxide dismutase 2, Cat: Catalase, Nrf1: Nuclear factor erythroid 2 related factor 1, Nrf2: Nuclear factor erythroid 2 related factor 2.

3.5.4. *UCP2 protein level*

Inhibition of the protein level of UCP2 has been observed after 48 h and 72 h lenvatinib exposure significantly in the treatment groups of 5 μM and 10 μM (Fig. 7c).

3.6. *Protein carbonyl level*

The protein carbonyl levels after lenvatinib treatments showed increase in all of the groups (Fig. 8), of which, the change in the 5 μM and 10 μM treatment groups after 48 h exposure and 10 μM after 72 h exposure was found significant.

3.7. *TEM image of the mitochondria*

Mitochondrial morphology was observed to be affected after 48 h and 72 h, 5 μM and 10 μM lenvatinib exposure. Mitochondrial elongation, mitochondrial content increment and damaged cristae structures were observed (Fig. 9).

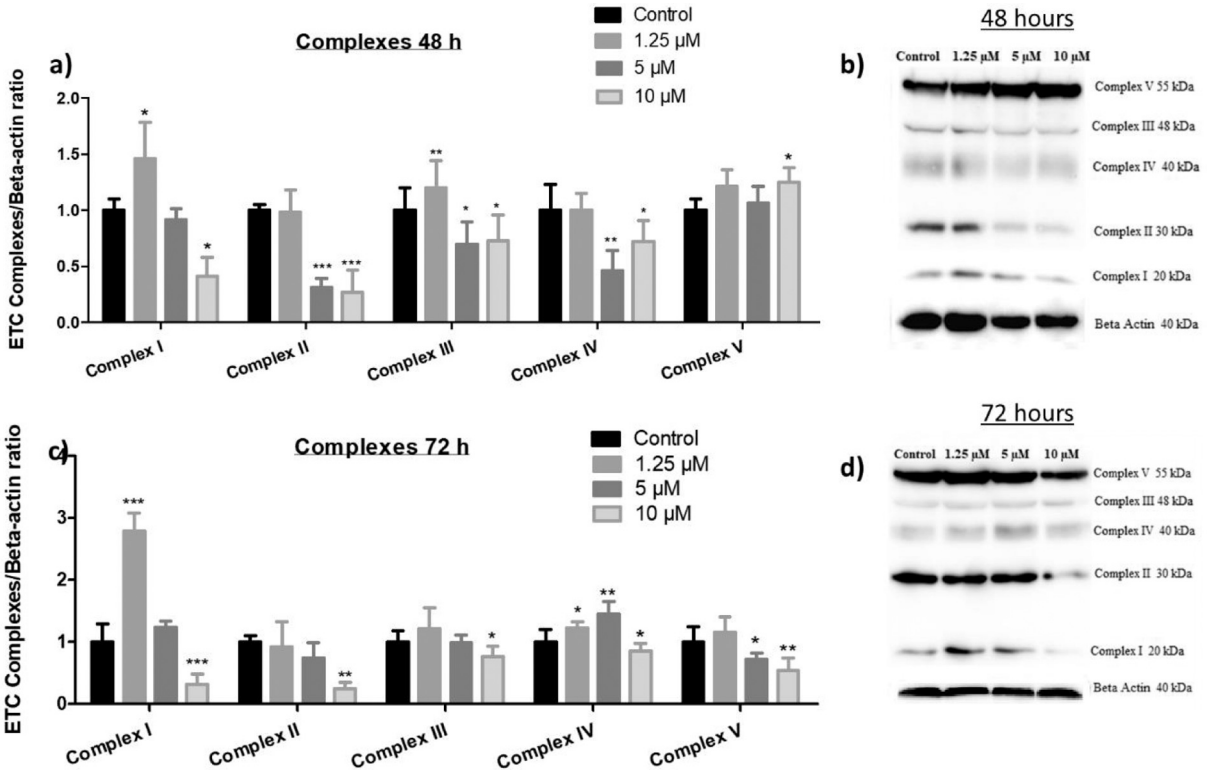

Fig. 6. Effect of lenvatinib on the protein expression levels of the ETC complexes in H9c2 cells following a), b) 48 h and c), d) 72 h treatment. The subunits of the Complex Proteins are as follows: NDUFB8 for Complex I, SDHB for Complex II, UQCRC2 for Complex III, MTCO1 for Complex IV, ATP5A for Complex V.
Cells were treated with 1.25 μM, 5 μM and 10 μM lenvatinib for 48 h and 72 h. Data are expressed as mean ± SD, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ versus control group.

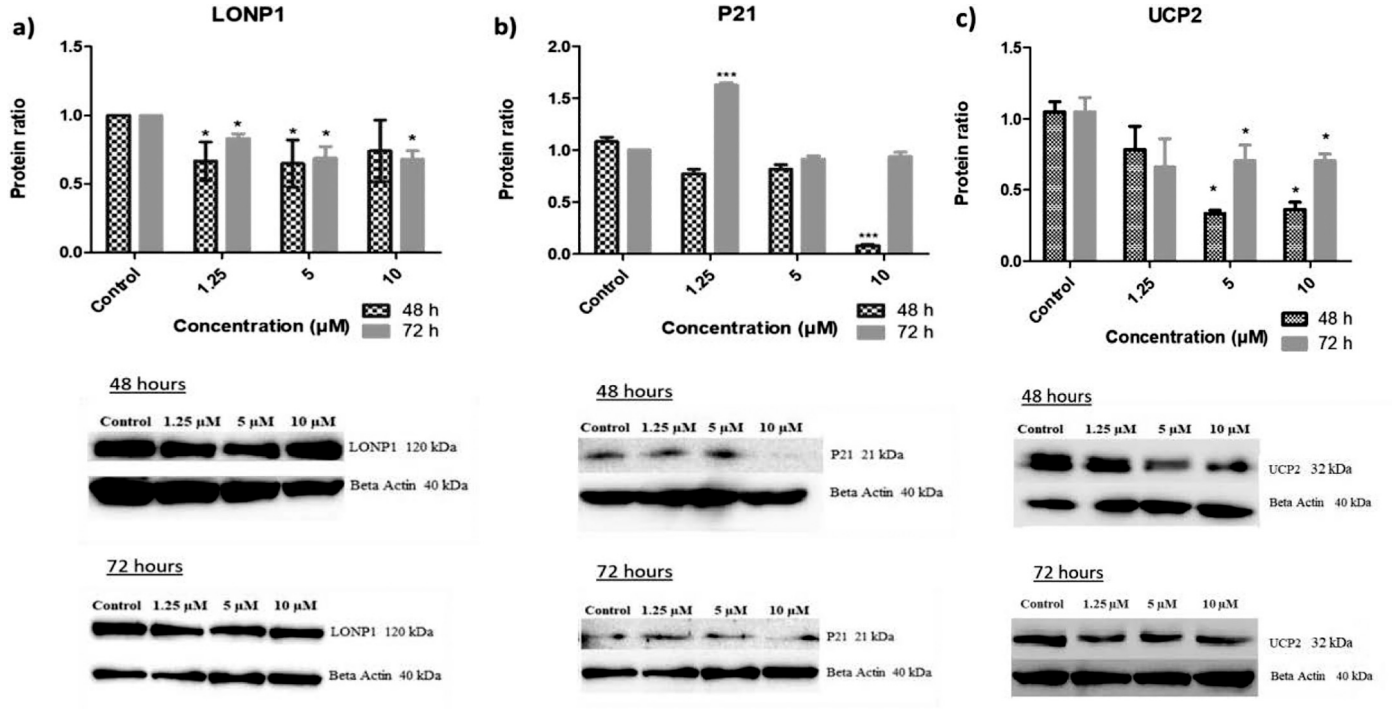

Fig. 7. Effect of lenvatinib on the protein expression levels of a) LONP1, b) P21, and c) UCP2 in H9c2 cells following 48 h and 72 h treatment.
Cells were treated with 1.25 μM, 5 μM and 10 μM lenvatinib for 48 h and 72 h. Data are expressed as mean ± SD, $*p < 0.05$, $***p < 0.001$ versus control group.
LONP1: Lon protease 1, UCP2: Uncoupling protein 2.

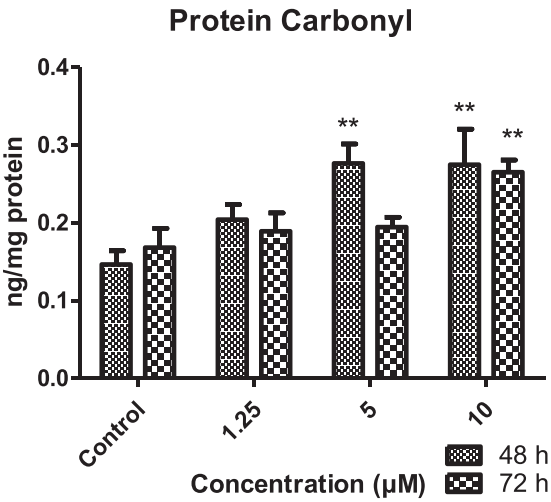

Fig. 8. Effect of lenvatinib on protein carbonyl levels of the H9c2 cells following 48 h and 72 h treatment.
Cells were treated with 1.25 μM, 5 μM and 10 μM lenvatinib for 48 h and 72 h. Data are expressed as mean ± SD, $^{**}p < 0.01$ versus control group.

4. Discussion

The molecular cardiotoxic mechanism of the TKI antineoplastic drug lenvatinib was evaluated in this study by focusing on different parameters on mitochondrial function using the H9c2 cardiomyoblastic cell line. It has been reported that 7% of differentiated thyroid cancer patients using lenvatinib experienced cardiac dysfunction, while another study reported that 10% of renal cell carcinoma patients using the drug encountered cardiac failure (Schlumberger et al., 2015; Scott, 2015; U.S. FDA and CDER, 2015). Despite the increasing body of evidence for TKI-induced cardiotoxicity the molecular mechanisms of lenvatinib-induced cardiotoxicity are not fully known. The cardiac cells, as they are highly bound to aerobic metabolism, are sensitive to drugs that disrupt mitochondrial metabolism or homeostasis. There are studies showing that TKIs such as imatinib, sunitinib and sorafenib modulate heart mitochondrial function directly or indirectly due to their toxicological and pharmacological properties (Cohen et al., 2011; Force et al., 2007; French et al., 2010; Gorini et al., 2018; Greineder et al., 2011; Tridente, 2017; Varga et al., 2015).

The H9c2 cardiyomyblastic cell-line is a simple reliable *in vitro* model to predict cardiotoxic effects of chemotherapeutic drugs (Boran et al., 2021; De Lorenzo et al., 2018; Govender et al., 2018; Pecoraro et al., 2020; Zhao et al., 2010). Our research team previously showed molecular cardiotoxic effects of regorafenib and mitochondrial involvment in these effects (Boran et al., 2021). In this study we aimed to investigate underlying mechanisms of cardiotoxic effects of lenvatinib *in vitro*. Here, for the first time we showed that lenvatinib-induced cardiotoxicity is related with disturbed mitochondrial dynamics and mitochondrial functions.

The Cmax value of 24 mg/day lenvatinib is shown to be 0.68 μM as reported in the product labeling at the FDA website (U.S. FDA, 2020) and the selected concentrations of lenvatinib (1.25 μM, 5 μM, and 10 μM) was found to inhibit cell viability. Indeed, evaluating mitotoxicity using higher levels than Cmax can be used to help identifying mitotoxic TKIs as an early safety screen (Zhang, 2017). There are *in vitro* studies evaluating the cytotoxic activity of lenvatinib using different concentrations on different cell lines and the results show that lenvatinib sensitivity may change from cell line to cell line (Ferrari et al., 2018; Ogasawara et al., 2019; Wei et al., 2018).

Cardiomyocytes need ATP for contraction functions to continuously

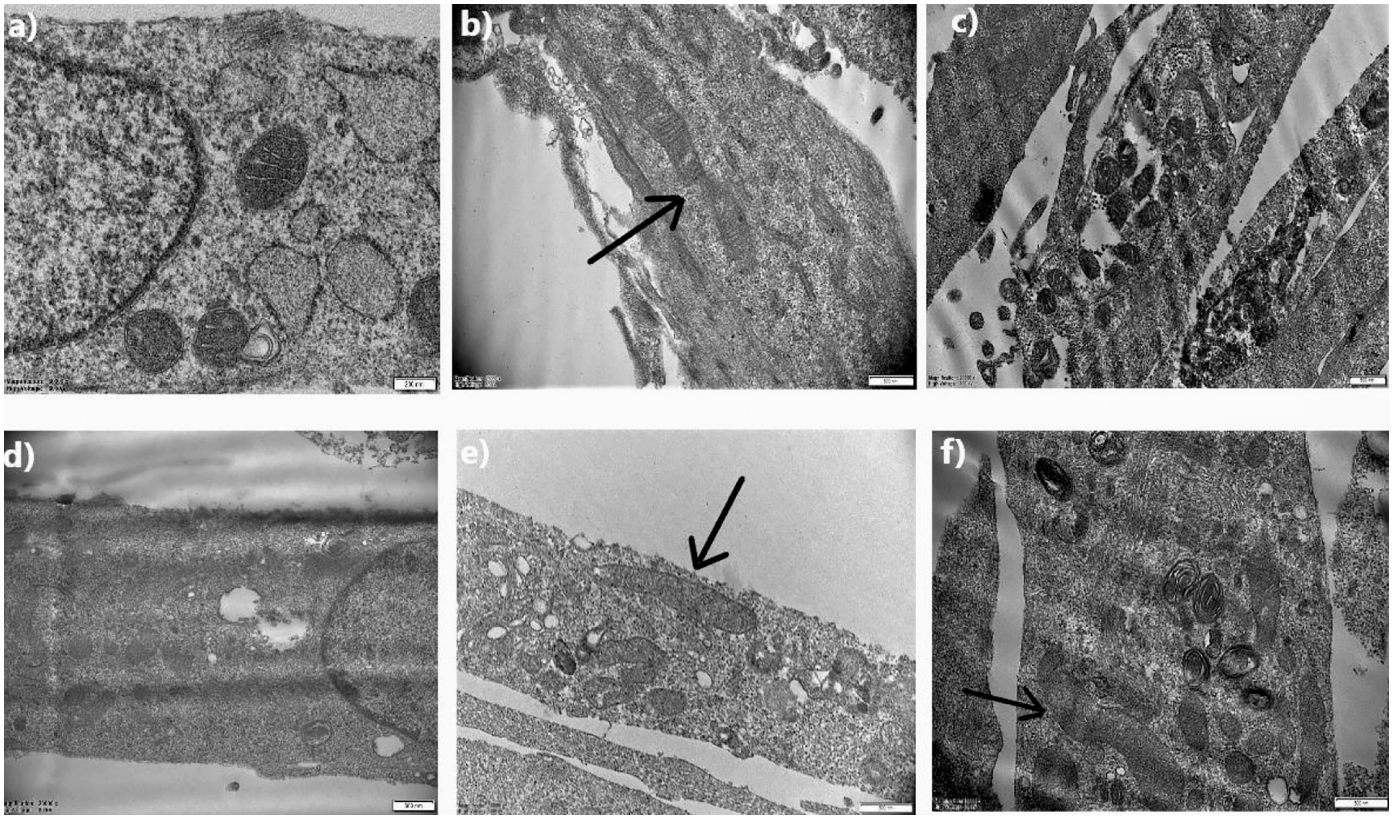

Fig. 9. TEM images of mitochondrial morphology of H9c2 cells after lenvatinib exposure a) 48 h control group b) 48 h 5 μM treatment group c) 48 h 10 μM treatment group d) 72 h control group e) 72 h 5 μM treatment group f) 72 h 10 μM treatment group. The arrows show elongated and structurally changed mitochondria. Cells were treated with 5 μM and 10 μM lenvatinib for 48 h and 72 h. TEM: Transmission electron microscopy. Scale bars: 500 nm and 200 nm.

pump blood. In order for mitochondria to perform ATP synthesis by oxidative phosphorylation, it is essential that they maintain the mitochondrial membrane potential (MMP) by creating a proton gradient between the membranes. MMP produced by electron transport chain (ETC) proteins is an important component in the ATP productionprocess during oxidative phosphorylation (Gergely et al., 2002; Perl et al., 2004; Zorova et al., 2018). Loss of MMP is reported to be an important mechanism of cerebral infarction after myocardial infarction and ischemia/reperfusion (Hausenloy et al., 2002; Sims and Muyderman, 2010; Sunaga et al., 2014). Adenosine monophosphate-activated protein kinase (AMPK) is sensitive to AMP:ATP ratio, activated in response to energy depletion, and it is a stimulator of mitochondrial biogenesis (Osataphan et al., 2020; Siasos et al., 2018). In our study lenvatinib decreased the amount of intracellular ATP level in the highest concentration after 48 h exposure, and significantly reduced the ATP level after 72 h exposure with all concentrations studied. The slight increase we found in AMPKα2 gene expression levels can be read as a reaction of the inhibition of ATP levels. It has been reported that nilotinib, another TKI, causes a significant decrease in MMP as a result of 24 h exposure to H9c2 cells in the concentration range of 0.1–15 μM (Lekes et al., 2016). Similarly, MMP decreased at medium and high concentrations after 48 h of exposure in our study.

Mitochondrial dynamics are essential for controlling mtDNA levels (Liu et al., 2020) and mtDNA copy number may be indicative of mitochondrial dysfunction (Malik and Czajka, 2013). We found that lenvatinib has a noteworthy effect on mitochondrial dynamics of the H9c2 cells. 48 h high concentration lenvatinib treatment causes upregulation in mitochondrial fission genes along with mitochondrial DNA (mtDNA) copy number. However, extended lenvatinib exposure causes upregulation in both fission and fusion genes while mtDNA copy number shows decrease. This is thought to be related to the cells trying to reduce the impact of the damage to the mitochondria with mitochondrial dynamic alterations which may contribute to compensate mitochondrial stress as shown before, H9c2 cells with reduced mtDNA ratio were more resistant to stress (Lee et al., 2015).

To maintain “healthy” mitochondria population and continue to the generation of ATP, damaged mitochondria are constantly replaced by mitochondrial biogenesis. Also, fission and fusion dynamically form mitochondrial network and regulate mitochondiral morphology. Consequently, cellular stress can result in mitochondiral fission and fusion (Meyer et al., 2017; Varga et al., 2015). Paech et al. found that in HepG2 cells, regorafenib and ponatinib triggered the tendency from fusion to fission as a protective mechanism (Paech et al., 2018). Likewise, after 48 h lenvatinib exposure, the mitofusins’, *Mfn1* and *Mfn2*, levels showed downregulation along with fission increment in our study which may have facilitated mitochondrial fragmentation. This may also be related to mitochondrial depolarization and reduced ATP production which seems similar to the work of Jheng et al. in which they found increased mitochondrial fission associated with mitochondrial dysfunction in skeletal muscle cells (Jheng et al., 2012). The decrease in Mfn2 can lead to heart hypertrophy (Chen et al., 2011; Chen and Dorn II, 2013; Fang et al., 2007) and Mfn2 is necessary for the quality control of cardiac mitochondria, thus, the recorded downregulation of Mfn2 may cause disturbed mitochondrial quality in cardiomyocytes (Chen and Dorn II, 2013). However, the significant upregulation of the fusion genes seen in the 72 h treatment group in our study along with MMP amelioration and reduced mtDNA content may be considered a protective response mechanism given by the cells against pro-longed stress. Supportively, it is found that the increase of mitochondrial fusion contributes to the protection of the oxidative phosphorylation capacity that meets the demand of cardiomyocytes and is one of the protective response mechanisms of the cell against stress (Youle and Van Der Bliek, 2012). In addition, low doses and chronic exposures of substances increase fusion and slightly increases fission, thereby causing strengthening of the mitochondrial network to remove damaged components (Westermann, 2010). Studies have found that myocardial contractile dysfunction is associated with an increase in mitochondrial fission in diabetic patients, and acute inhibition of excessive mitochondrial fission at the onset of reperfusion can protect the heart against ischemia/reperfusion injury (Ding et al., 2018a, 2018b; Disatnik et al., 2013; Ong et al., 2010).

The incompatibility between mitochondrial dynamics can cause the healthy mitochondrial balance to deteriorate. Our study results show that lenvatinib causes stress in the cells and creates damaged mitochondria by potentially disrupting the fusion/fission balance. This data is supported by the TEM images of mitochondria. After 48 h of exposure small fragmented mitochondria were detected to be higher, which is a sign of increased fission. This is in parallel with the observed increase in the fission genes and decrease of the fusion genes. Furthermore, in the 72 h treatment group elongated mitochondria along with mitochondrial swelling and damage are detected.

There is a tight relationship between changes in the levels of ETC proteins and disruption of MMP and therefore ATP production (Leipnitz et al., 2018). In our study, after 48 h of exposure to high concentration of lenvatinib, MMP and ATP decreased in parallel with the decrease in protein levels of ETC complexes (I-IV). On the other hand, after 72 h of high concentration lenvatinib exposure, a decrease in the protein levels of all complexes (I-V) was determined, similarly the ATP amounts decreased, but the decrease in the MMP level was not significant. This condition at the MMP level can be considered as an amelioration in MMP as a prerequisite for survival in mitochondria. Also in a study using H9c2 cells, amelioration of MMP disruption was reported to protect cardiomyocytes from cell death (Sunaga et al., 2014).

The declined protein level of ETC complexes at the highest concentration of lenvatinib after 48 and 72 h exposure can be considered as a result of prolonged high concentration lenvatinib exposure leading to chronic inhibition of protein expression in ETC complexes. Similarly, there are studies showing that sorafenib inhibits Complex I, II, III, and V activity (Will et al., 2008; Zhang, 2017). Forkink et al. stated that chronic Complex I inhibition is maintained with the inhibition of the other mitochondrial complexes (Forkink et al., 2014).

A group working on mitochondrial toxicity of sorafenib stated that they found JNK activation in HepG2 cells concluding that JNK activation triggers mitochondrial dysfunction (Heslop et al., 2020). The expression of the two interrelated apoptotic pathway genes was examined in our study and the results suggest that apoptosis may be activated via the ASK1/JNK pathway in the treatment groups where these two genes were upregulated together.

It has been shown that sorafenib attenuates P21 in renal cell carcinoma and hepatocellular carcinoma cells (Inoue et al., 2011). Similarly in our study the inhibition in the P21 protein may have attempted to facilitate cells to go to cell death by reducing the function of stopping cell growth. On the other hand, cell cycle regulation is accomplished by the upregulation of the P21 protein (Leonard et al., 2014). Therefore, kinase inhibition with lenvatinib may lead to cell cycle arrest and thus, the cells may have decreased their P21 protein expression to bring stability. Also, it has been reported that the cardiotoxic drug doxorubicin also inhibits P21 protein, and that P21 can protect against cardiotoxicity (Cheng et al., 2015).

Early *SOD2* expression upregulation may be due to immediate response to oxidative stress which is accompanied by the downregulation of the ETC complexes’ protein levels since toxic substances that inhibit the function of the ETC complexes can increase the production of mitochondrial ROS (Dröse and Brandt, 2012). Oxidative stress is a leading cause of cardiotoxicity due to mitochondrial damage (Malik and Czajka, 2013). Mitochondrial uncoupling protein 2 (UCP2) can protect against ROS by reducing mitochondrial respiration (Cadenas, 2018; Rousset et al., 2004). The downregulation of UCP2 mRNA and protein levels suggests that lenvatinib can be an inhibitor of UCP2 resulting in ROS accumulation and oxidative stress. *SOD2* upregulation along with *Cat* downregulation also seems to contribute to oxidative stress which is also supported by the high protein carbonyl levels after

lenvatinib treatments. Low levels of ROS induce the antioxidant enzymes SOD2 and Cat while sublethal levels may inhibit Cat (Dhoke et al., 2018).

Key modulators of the antioxidant pathway, *Nrf1* and *Nrf2*, were observed to show downregulation after 48 h in contrast to upregulation after 72 h levatinib exposure. This can be interpreted as high oxidative stress in 48 h and an effort of amelioration after 72 h exposure to prolonged stress. Doxorubicin-induced cardiotoxicity has also been linked with the inhibition of Nrf1 and Nrf2 (Osataphan et al., 2020). In a study with H9c2 cells it has been found that sunitinib induced mitochondrial oxidative stress can lead to cardiotoxicity (Bouitbir et al., 2019) and another recent study shows that imatinib and dasatinib cause mitochondrial dysfunction leading to oxidative stress in C2C12 myotubes and human rhabdomyosarcoma cells (Bouitbir et al., 2020). It has also been disclosed that increased oxidative stress could lead to enhanced mitochondrial biogenesis and increased mtDNA ratio (Malik and Czajka, 2013) which is in accordance with our findings.

LONP1 (mitochondrial Lon) is one of the most important proteases of mitochondria and a decrease in its level leads to impairment in mitochondrial proteostasis (Venkatesh and Suzuki, 2020). In our study, a significant decrease was observed in the protein level of LONP1. LONP1 inhibition has been shown to cause oxidative damage to ETC proteins, which reduces mitochondrial respiratory activity and causes contractile dysfunction in the heart (Hoshino et al., 2014). LONP1 regulates redox homeostasis in the mitochondrial matrix and protects cardiomyocytes from ischemia/reperfusion injury (Venkatesh et al., 2019). Venkatesh et al. states that LONP1 reduces myocyte cell death by reprogramming mitochondrial bioenergetics on account of reducing oxidative damage to biologic macromolecules (Venkatesh and Suzuki, 2020). Rendón and Shoubridge stated that the loss of LONP1 reveals the result of an integrated stress response, and that mtDNA number decreased after LONP1 inhibition (Rendón and Shoubridge, 2018). Similarly in our study, LONP1 inhibition may have triggered mtDNA content inhibition after 72 h exposure as a result of pro-longed stress.

Two studies investigating mitochondrial toxicity of lenvatinib have been conducted so far and both of the studies have used liver cells. The first one is to investigate the mitochondrial damage of 31 FDA-approved drugs from Zhang et al. on the mitochondria isolated from rats, and in this study, lenvatinib disrupted mitochondrial oxygen consumption and interestingly it was concluded that the drug exposure had no effect on ETC complexes and MMP (Zhang, 2017). In the second study, Roberts et al. found that lenvatinib inhibits the expression of mitochondrial protective proteins in liver cancer cells when used with histone deacetylase inhibitor entinostat (Roberts et al., 2020).

5. Conclusions

The results we obtained from the study show that lenvatinib causes cellular stress in H9c2 cells depending on the exposure time and consequently affects mitochondrial membrane potential, ATP levels, mitochondrial proteins related to oxidative phosphorylation and cellular proteostasis by inducing oxidative stress, and also potentially induces mitochondrial damage disrupting mitochondrial dynamics and biogenesis.

Our findings are expected to contribute to the understanding of the molecular mechanism of cardiotoxicity, an important side effect frequently encountered during treatment with lenvatinib and other TKI medications. However, the findings presented here were obtained with an in vitro cardiomyocyte model and this can be considered as a limitation of the study. To translate our results to the clinic further *in vitro* and *in vivo* studies should support these findings. Possible implications of the study results can be used toward developing cardioprotective strategies for the patients under TKI treatment and understanding the molecular mechanisms may enable rational design of drugs with higher cardiovascular safety.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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