

Elamipretide protects H9c2 Rat Cardiomyoblasts against Doxorubicin-Induced Disruption of Mitochondrial Quality Control by Restoration of Fusion and Fission Balance

Marwa A. Mahmoud^{*1}, Inam S. Arif¹ and Muthanna I. Al-Ezzi¹

¹Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq

*Corresponding author

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Abstract

Doxorubicin (DOX) has been used to treat malignant diseases for over 40 years. The main constraint to its clinical applications is dose-dependent cardiotoxicity. Mitochondrial dysfunction is considered the main contributor and it involves disruption of mitochondrial quality control, mainly impaired fusion, and enhanced fission processes. Compounds that specifically target the mitochondria and restore fusion and fission balance are considered promising tool to protect or treat cardiomyopathy and thus could be investigated as a novel strategy to alleviate DOX-induced cardiac toxicity, one of which is elamipretide (ELAM). Methods: firstly, the tetrazolium bromide (MTT) assay was used to evaluate the cytotoxic effects of DOX and ELAM on the viability of the H9c2 cell line and to compare the effect of ELAM pre- and co-treatments on DOX-induced H9c2 cell damage. Secondly, western blot was utilized to investigate the effect of 72-hour DOX, 72-hour ELAM treatment, and 48-hour ELAM pre-treatment on the expression of the mitochondrial GTPases: mitofusin2 (MFN2) and dynamin-related protein1 (DRP1) that orchestrate mitochondrial fusion and fission respectively. Results: MTT assay revealed that DOX induces a significant reduction in cell viability which is both time and dose-dependent whereas ELAM has no significant effect on the viability of the relevant cells at most of the concentrations used. Additionally, western blot analysis showed a significant reduction in the expression of MFN2 in the DOX-treated group compared to the control ($p^{***} < 0.001$) whereas the fission protein DRP1 was significantly upregulated in DOX-treated cells compared to the control ($p^{**} < 0.01$) and normalization of both proteins was achieved when 10 μ M ELAM introduced 48-hour prior to DOX therapy. Conclusion: ELAM could exert an interesting cardioprotective role against DOX-induced cardiotoxicity by restoration of mitochondrial fusion and fission balance.

Keywords: Doxorubicin, Elamipretide, MFN2, DRP1, H9c2 cell line

الإيلامبريتايد يحمي خلايا عضلة قلب الجرذان H9c2 من اختلال التحكم في جودة الميتوكوندريا المستحثة بواسطة دوكسوروبيسين عن طريق استعادة التوازن بين الاندماج والانقسام
مروة عبد الكريم محمود¹، انعام سامح عارف¹، مثنى ابراهيم العزي¹

افرع الادوية والسموم، كلية الصيدلة، الجامعة المستنصرية، بغداد، العراق

الخلاصة

يستخدم دوكسوروبيسين (DOX) لعلاج الأمراض الخبيثة لأكثر من 40 عامًا. عائق الرئيسي لتطبيقه السريري هو السمية القلبية المعتمدة على الجرعة. يعتبر الخلل الوظيفي في الميتوكوندريا المساهم الرئيسي في السمية القلبية التي يسببها DOX وينطوي على اضطراب مراقبة جودة الميتوكوندريا والتي تتضمن بشكل أساسي ضعف عملية الاندماج وتعزيز عملية الانقسام. تعتبر المركبات التي تستهدف الميتوكوندريا على وجه التحديد وتعمل على استعادة التوازن بين عمليات الاندماج والانقسام أداة واعدة لحماية أو علاج اعتلال عضلة القلب، وبالتالي يمكن فحصها كاستراتيجية جديدة للتخفيف من السمية القلبية التي يسببها DOX، أحدها هو الإيلامبريتايد (ELAM). في هذه الدراسة، كشف اختبار MTT أن DOX يؤدي إلى انخفاض كبير في قابلية نمو خلايا H9c2 والتي تعتمد على الوقت والجرعة في حين أن ELAM ليس له تأثير كبير على قابلية نمو الخلايا ذات الصلة في معظم التراكيز المستخدمة. بالإضافة إلى ذلك، أظهر تحليل اللوحة الغربية انخفاضًا كبيرًا في التعبير عن بروتين الاندماج MFN2 في المجموعة المعالجة بـ DOX مقارنةً بمجموعة التحكم ($p^{***} < 0.001$) بينما ازداد التعبير عن بروتين الانقسام DRP1 في الخلايا المعالجة بـ DOX بالمقارنة مع مجموعة التحكم ($p^{**} < 0.01$) وتم تحقيق تطبيع كلا البروتينين عندما تم إعطاء 10 μ M ELAM قبل 48 ساعة من علاج DOX. في الاستنتاج، يمكن لـ ELAM أن يمارس دورًا مثيرًا للاهتمام في حماية القلب ضد السمية القلبية التي يسببها DOX من خلال استعادة التوازن بين عمليات الاندماج والانقسام في الميتوكوندريا.

الكلمات المفتاحية: دوكسوروبيسين، إيلامبريتايد، خط الخلايا H9c2، DRP1، MFN2.

Introduction

Doxorubicin (DOX) is a powerful, widely employed chemotherapeutic agent that belongs to a class of anticancer drugs known as anthracyclines (ANTs)⁽¹⁾. Unfortunately, the use of DOX in clinical settings is restricted by severe, permanent, and dose-dependent cardiac toxicity⁽²⁾. DOX-induced cardiotoxicity is a multifactorial process, in which several mechanisms are involved in the pathogenesis such as Topoisomerase II β poisoning, reactive oxygen species (ROS) generation, and mitochondrial impairment which is considered the major contributor⁽³⁻⁵⁾. Mitochondria are vital for all cell types and high energy-consuming cells are particularly sensitive to mitochondrial malfunction therefore, it is not surprising that primary mitochondrial dysfunction is linked to significant abnormalities in neurons and skeletal and cardiac muscles⁽⁶⁾.

Under healthy settings, mitochondria are kept in dynamic networks that are continuously elongating and dividing as a result of the balance between two opposing processes of fusion and fission resulting in the formation of a complicated set of connections known as mitochondrial dynamics⁽⁷⁻⁹⁾.

Mitochondrial fusion is the merging of the exterior and interior mitochondrial membranes of two mitochondria into one mitochondrion and it is orchestrated by nuclear-encoded dynamin-related GTPases known as mitofusins 1 and 2 (MFN1 and MFN2) located on the exterior membrane, and optic atrophy 1 (OPA1) on the interior membrane and intermembrane space^(7,10). In contrast, mitochondrial fission is characterized by the generation of two individual mitochondria via the division of a single mitochondrion⁽¹¹⁻¹³⁾. The major regulator of mitochondrial fission is the GTPase protein belonging to the dynamin family known as dynamin-related protein 1 (DRP1)^(14,15). Balanced mitochondrial dynamics are vital for cardiac homeostasis as it protects the myocardium from energy stress, therefore any disruption in this machinery appears to play a key role in the pathophysiology of numerous cardiovascular (CV) diseases⁽¹⁴⁾ such as DOX-induced cardiotoxicity⁽¹⁶⁾.

DOX-induced cardiotoxicity can potentially disrupt the expression of dynamically controlled proteins in mitochondria⁽¹⁷⁾. Du et al., (2019) stated that DOX increases mitochondrial fission in cardiomyocytes, as evidenced by increased phosphorylation of DRP1 and/or decreased phosphorylation of MFN2, and enhanced mitochondrial-dependent apoptosis⁽¹⁸⁾. These results revealed that DOX enhances mitochondrial fission while blocking mitochondrial fusion and enhancing the production of ROS⁽¹⁶⁾.

Because DOX has been demonstrated to change the levels of fusion and fission proteins, targeting these dynamic GTPases may be a feasible strategy for eliminating DOX-induced cardiomyopathy⁽¹⁶⁾. J. Huang et al., (2022) demonstrated that approaches aimed at enhancing mitochondrial function could be effective in ameliorating ANTs-induced cardiotoxicity. For example, the tetrapeptide elamipretide (ELAM) recovers cellular bioenergetics by specifically targeting the mitochondrial electron transport chain (ETC)⁽¹⁹⁾.

ELAM is an antioxidant peptide⁽²⁰⁾ that concentrates at the inner mitochondrial membrane (IMM)⁽²¹⁾ at which it selectively binds to mitochondrial cardiolipin (CL), resulting in the restoration of mitochondrial bioenergetics⁽²²⁻²⁴⁾.

ELAM has the best characterization and efficiency among other members of the Szeto-Schiller (SS) peptides family that are presently being investigated for treating mitochondrial dysfunction. Various studies have demonstrated that SS peptides have great efficacy in recovering mitochondrial function in a variety of diseases, including cardiomyopathy and heart failure (HF)^(25,26).

ELAM can restore mitochondrial activity and enhances left ventricular contractile capacity when administered as long term therapy in dogs with failing hearts⁽²⁷⁾. While there is no clear proof that ELAM can prevent ANTs-induced cardiomyopathy, the fact that mitochondrial dysfunction is a major contributor to ANTs' cardiotoxicity implies that ELAM could be a promising therapy⁽¹⁹⁾.

The objective of this study is to investigate the impact of DOX therapy on cardiac mitochondrial quality control, mainly fusion and fission processes, and, to explore the potential effect of ELAM treatment in the restoration of dynamic balance between these processes *in vitro* by utilizing H9c2 rat cardiomyoblasts cell line as a model.

Materials and Methods

Preparation of drug stock solution

1mM Stock solution of both DOX and ELAM (purchased from Hyper-Chem®, China) was prepared by dissolving the drug powder in DMSO then the resultant solution was filtered and stored at -40°C.

Cell culture and treatment

Rat H9c2 cells (supplied by Mustansiriyah University/ College of Pharmacy / Department of Pharmacology and Toxicology) were cultured in 75 cm² tissue culture flasks using Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/mL glucose, 1% Penicillin/Streptomycin solution 100X and 10% fetal bovine serum (Capricorn scientific, Germany) and kept in a humidified

incubator at a temperature of 37°C and CO₂ concentration of 5%^(28,29). The growth of the cells was observed daily under the microscope and was passaged when reaching 80-90% confluency⁽²⁹⁾. In a 96-well plate, the cells were seeded at a density of 5000 cells/well, and the plates were kept in a humidified incubator⁽³⁰⁾. When reaching 80-90% confluency, the drug treatments were added for 24, 48, and 72 hours. From the stock solutions that were previously prepared, different DOX (0, 0.01, 0.02, 0.04, 0.09, 0.18, 0.37, 0.75, 1.5, 3, and 6 µM) and ELAM (0, 0.001, 0.01, 0.1, 1, 10, 25, 50, 100, 250 and 500 µM) concentrations were prepared with fresh medium and added to the wells in three technical replicates after removing the old medium.

Measurement of cell viability

The viability of H9c2 cells was determined using MTT assay (purchased from HIMEDIA®, India). 20µL of MTT solution was added to each well after removing the old medium then, the plates were incubated for 4 hours in a humidified incubator. Next, MTT solution was discarded and 100µL of DMSO was added⁽³⁰⁾. The absorbance of the wells was measured via Bio-Tek® microplate reader at a wavelength of 490nm^(31,32). Data were collected from 3 independent experiments and the results were expressed as % cell viability of the control.

Assessment of the impact of ELAM pre- and co-treatments on DOX-induced H9c2 cells damage

The relevant cells were seeded at a density of 5000cells/well in 96 well plates with 100µL fresh medium, and the plates were incubated in a humidified incubator. When the cells became semi-confluent, they were divided into 3 groups: **control group**: cells incubated with only fresh medium for 72 hours, **pre-treatment group**: H9c2 cells were treated with ELAM 10 µM (obtained from the dose-response curve) for 24, 48, and 72 hours then DOX 0.3 µM (obtained from the dose-response curve) was added after discarding the old medium, and the cells were incubated for further 72 hours in a humidified incubator, and **Co-treatment group**: relevant cells exposed to DOX (0.3 µM) plus ELAM (10 µM) concurrently and incubated for 72 hours. Finally, cell viability was measured by MTT assay.

Western blot

Proteins from H9c2 cells that were exposed to DOX and ELAM treatments were extracted by adding RIPA lysis buffer (Elabscience®, USA). The cell lysate was centrifuged and then, quantified by a BCA assay kit (Elabscience®, USA). Next, equal amounts of proteins were separated by 8-10% SDS-PAGE (Elabscience®, USA). A semi-dry transfer system (Bio-Rad Laboratories, Singapore) was used to transport protein bands from gel to PVDF membranes which are then blocked in 5% skim milk in TBS-T for

1 hour at room temperature. After blocking, the membranes were incubated overnight with the primary antibodies against MFN2 (1:1000, Elabscience®, USA), DRP1 (1:1000, Elabscience®, USA), and GAPDH (1:1000, Elabscience®, USA) at 4° C under mild agitation. Next, the blots were washed 3 times/15 minutes with TBS-T buffer and incubated with species-specific secondary antibody conjugated with horseradish peroxidase (1:5000, Elabscience®, USA) for 1 hour at room temperature with gentle agitation. Then, the protein bands were visualized using a chemiluminescent detection kit (Elabscience®, USA) with ChemiDoc TM XRS+ imaging system (Bio-Rad Laboratories, France) and analyzed with ImageJ software. The experiment was performed in duplicates.

Statistical analysis

Data were expressed as mean ±SEM, and analyzed by GraphPad Prism 8.0.2 software (GraphPad Software Inc, USA). Analysis of Variance (one-way ANOVA) followed by Tukey's multiple comparison tests was used for comparison between experimental groups. Statistical significance was defined as p-value <0.05.

Results and Discussion

DOX-induced dose and time-dependent reduction in H9c2 cells viability

Figure (1) represents the dose-response curve of Dox. The viability of the relevant cells was significantly reduced as the DOX concentration increased, indicating that DOX suppresses H9c2 viability in a concentration-dependent manner. Additionally, the concentration-dependent inhibition of DOX on cellular viability became more apparent as exposure duration increased. For example, after 24 hours of exposure to DOX, the maximum DOX concentration (6 µM) caused 39% of cellular death, whereas, after 72 hours of exposure to the same concentration, this effect was increased to 78%. Thereafter, a 72-hour interval was considered for the calculation of the half-maximal inhibitory concentration (IC₅₀) and also for subsequent experiments. IC₅₀ of DOX was calculated using GraphPad Prism software by applying the nonlinear regression analysis and determined to be 0.3 µM.

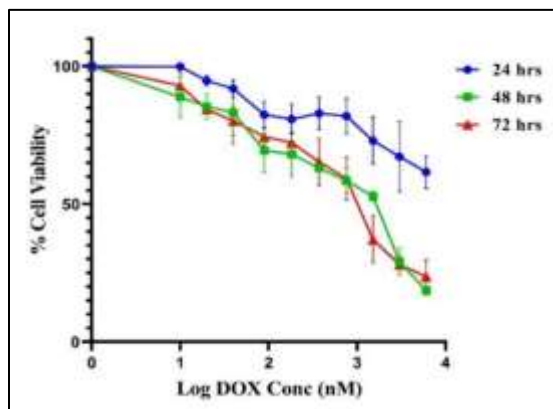


Figure 1. Dose-response curve for DOX. H9c2 cells were treated with Dox concentrations (0.01-6 μM) for 24,48 and 72 hours. Data presented as mean \pm SEM of 3 biological independent replicates (n=3).

ELAM produces no significant difference in the viability of H9c2 cells

Figure (2) shows that at low doses, ELAM slightly enhanced cellular viability which was determined by measuring the mitochondrial activity of the living cells via MTT assay. However, ELAM concentrations $\geq 50 \mu\text{M}$ significantly suppressed cellular viability in comparison to non-treated cells. The decline in cellular viability following a high dose of ELAM may arise from the fact that ELAM reduces the production of ROS⁽³³⁾ which are to a certain level necessary for many physiological activities within the cell such as defending against external insults and acting as redox messengers⁽³⁴⁾. For this research, a 10 μM concentration of ELAM was selected to be used for subsequent experiments.

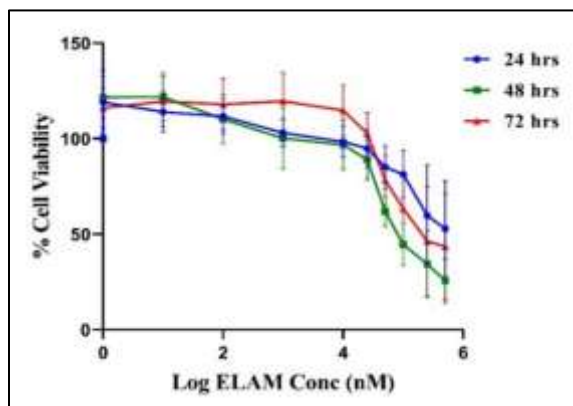


Figure 2. Dose-response curve for ELAM. H9c2 cells were treated with ELAM concentrations (0.001-500 μM) for 24,48 and 72 hours. Data presented as mean \pm SEM of 3 biological independent replicates (n=3).

ELAM protects H9c2 cells against DOX-induced cellular damage when administered as pre- and co-treatments

The effect of ELAM pre- and co-treatments on DOX-induced H9c2 cytotoxicity is shown in Figure (3). The results demonstrated that, in comparison to DOX therapy, both 48 and 72-hour pre- and the co-treatments with ELAM significantly increased H9c2 cell viability, with the exception of treatment prior to 24 hours of DOX, which demonstrated no significant difference. Thereafter, 48-hour pre-treatment with ELAM was selected to perform western blotting.

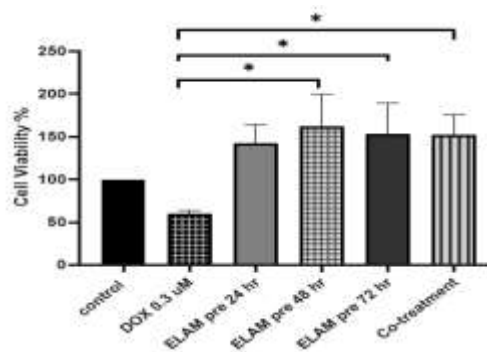


Figure 3. Effect of ELAM pre- and co-treatments on DOX-induced H9c2 cytotoxicity. H9c2 cells were treated with 10 μM ELAM for 24,48 and 72 hours prior to Dox treatment of 0.3 μM for a further 72 hours. In Co-treatment, both drugs were given concurrently for 72 hours. Significant compared to the DOX group ($p^* < 0.05$). Data presented as mean \pm SEM of 4 biologically independent experiments (n=4).

ELAM pre-treatment recovers DOX-induced downregulation of MFN2 expression

Figure (4) illustrated that DOX-induced significant reduction in MFN2 expression compared to the control ($p^{***} < 0.001$). In contrast, a significant increase in the expression of MFN2 compared to the DOX-treated group ($p^{****} < 0.0001$) was achieved when 10 μM ELAM was introduced 48 hours prior to DOX therapy. Several studies have revealed that DOX modifies the level of fusion/fission proteins in diverse ways but the suppression in MFN2 expression is thought to be the most notable phenotype among these investigations^(35,37). MFN2 is directly regulated by peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α)⁽³⁸⁾ which is a transcriptional co-activator that is normally overexpressed in the myocardium and plays a role in the pathology of cardiac failure⁽³⁹⁾. In cardiomyocytes treated with DOX, PGC-1 α expression, and activity are both suppressed which is thought to be a typical sign of heart dysfunction⁽⁴⁰⁾. PGC-1 α suppression is caused by

ANTs-induced topoisomerase II β inactivation which is considered the primary trigger of cardiotoxicity with these drugs⁽³⁹⁾. Ding et al., (2022) pointed out that restoration of MFN2 recovers mitochondrial fusion and thereby reduces mitochondrial dysfunction and cardiotoxicity induced by DOX⁽³⁶⁾. According to Bu et al., (2017) and Wang et al., (2021), Silent information regulator 1 (SIRT1) is the key upstream regulator of PGC-1 α that controls MFN2 expression^(41,42). SIRT1 activity is reduced in cardiomyocytes treated with DOX⁽⁴³⁾ since SIRT1 enzymatic activity is directly linked to the availability of nicotinamide adenine dinucleotide (NAD⁺) in cells⁽⁴⁴⁾. The cellular availability of NAD⁺ is strongly linked with

mitochondrial activity because NADH is oxidized to NAD⁺ in the mitochondria therefore, it is not surprising that different cell types have shown a decrease in NAD⁺ levels in response to treatment with ANTs⁽⁴³⁾. Zhao et al., (2017) revealed that ELAM is highly effective at raising the NAD⁺/NADH ratio and enhancing mitochondrial quality⁽⁴⁵⁾. The elevation in NAD⁺/NADH ratio results in the activation of SIRT1⁽⁴⁶⁾ which can protect the myocardium from oxidative stress by enhancing the antioxidant defense system⁽⁴⁷⁾. As reported earlier, SIRT1 is an important regulator of PGC-1 α , therefore, it is not surprising that ELAM produces a significant increase in PGC-1 α levels in dogs with induced heart failure receiving three months of therapy with ELAM⁽⁴⁸⁾. Since PGC-1 α controls MFN2 expression^(38,49) therefore, upregulation of PGC-1 α enhances the expression of mitochondrial fusion protein MFN2^(50,52).

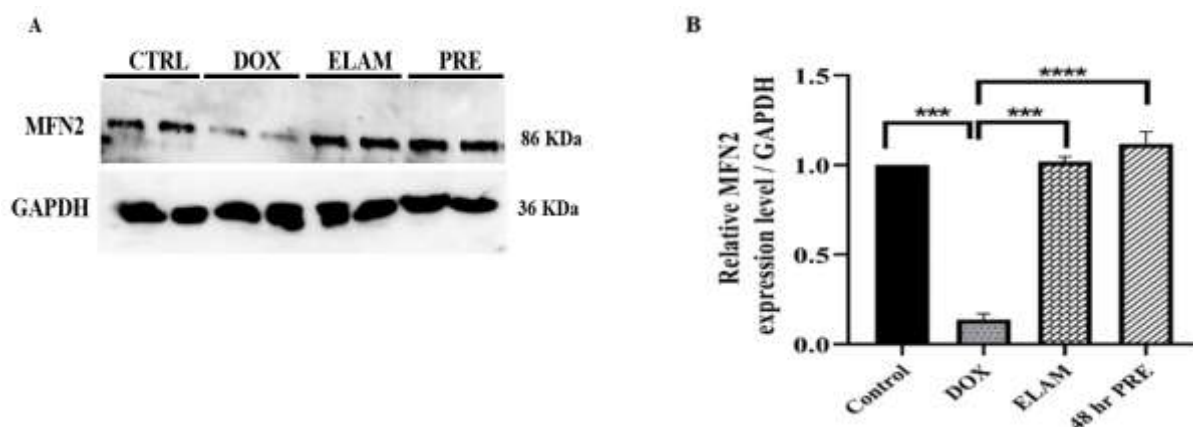


Figure 4. Effect of 48-hour ELAM pre-treatment on MFN2 protein expression in H9c2 cells treated with 0.3 μ M DOX for 72 hours. A) Western blot analysis of the cell lysate with GAPDH utilized as an internal control. B) Protein bands were quantified by ImageJ 1.53t software, standardized with GAPDH, and expressed as normalization of control. One-way ANOVA was applied to measure the significant difference between groups ($p^{***} < 0.001$, $p^{****} < 0.0001$).

ELAM pre-treatment alleviates DOX-induced upregulation of DRP1 expression

According to the findings depicted in Figure (5), DRP1 expression was significantly increased in the DOX-treated group compared to the control ($p^{**} < 0.01$). However, when 10 μ M ELAM was administered 48 hours before DOX therapy, there was a significant decline in the expression of DRP1 relative to the DOX-treated group ($p^{**} < 0.01$). As stated by Ding et al., (2022), DRP1 expression is induced by DOX therapy, which results in excessive mitochondrial fission⁽³⁶⁾. Under stressful circumstances, the dynamic balance in the mitochondria shifts towards fission to eliminate the severely damaged portions of mitochondrial network thereby leading to mitochondrial fragmentation and

eventually cell death^(53,54). Mitochondrial fission is initiated when DRP1 translocated from the cytosol into mitochondria⁽⁵⁵⁾ this requires DRP1 to be activated via phosphorylation at the Ser616 site which is promoted by upstream kinases, such as extracellular signal-regulated kinase 1/2 (ERK1/2) that is activated during DOX-induced cardiac damage^(54,56) as a result of oxidative stress induction and production of potentially fatal ROS⁽⁵⁷⁾ that acts as a mediator that activates ERK1/2 pathway^(56,58). The ERK pathway is the first cascade of the mitogen-activated protein kinase (MAPK) signaling pathway⁽⁵⁸⁾ which is considered the key upstream regulator of fission in mitochondria⁽⁵⁹⁾. ELAM-mediated suppression of mitochondrial fission may be related to the fact that ELAM reduces oxidative stress⁽⁶⁰⁾ and lowers the generation of harmful ROS⁽⁶¹⁻⁶³⁾ by binding to- and

stabilization of CL on the IMM⁽⁶¹⁾. Reduction in ROS production impairs the MAPK pathway⁽⁵⁷⁾ which as noted earlier, is responsible for controlling the

phosphorylation and activation of the fission-mediating protein DRP1⁽⁶⁴⁾.

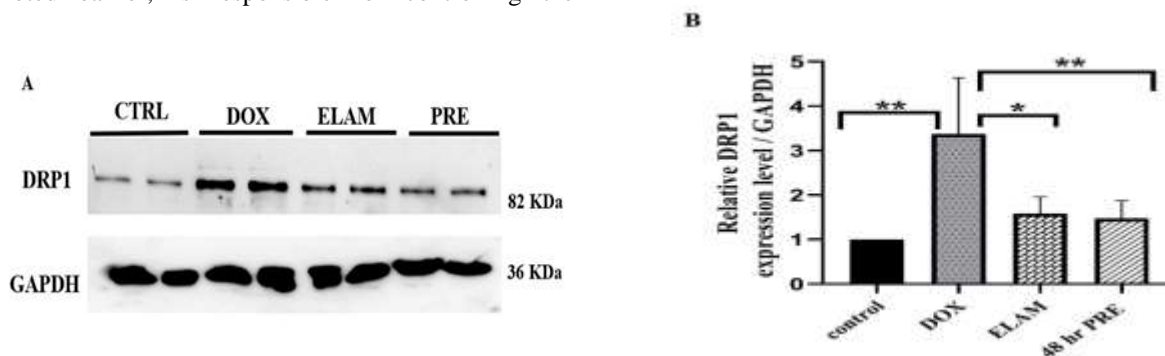


Figure 5. Effect of 48-hour ELAM pre-treatment on DRP1 expression in H9c2 cells treated with 0.3 μ M DOX for 72 hours. A) Western blot analysis of the cell lysate with GAPDH utilized as an internal control. B) Protein bands were quantified by ImageJ 1.53t software, standardized with GAPDH, and expressed as normalization of control. One-way ANOVA was applied to measure the significant differences between groups ($p^* < 0.05$, $p^{**} < 0.01$).

Conclusion

The ability of elamipretide to stabilize cardiac mitochondria and recovers mitochondrial quality control in H9c2 cardiomyoblasts offers a novel strategy for protection against doxorubicin-induced cardiac mitochondrial toxicity by restoring the dynamic balance between mitochondrial fusion and fission.

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Conflict of Interest

All authors declare that there is no conflict of interests.

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Ethics Statements

This article was approved by the ethical committee of the Pharmacology and Toxicology Department/College of Pharmacy/ Mustansiriyah University.

Authors Contribution

Inam S. Arif and Marwa A. Mahmoud designed the methodology. Marwa A. Mahmoud conducted the methodology, data analysis and writing the original manuscript. Inam S. Arif performed writing-editing and publication. Muthanna I. Al-Ezzi handled writing-review and publication.

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