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Mechanisms involved in mitoquinone-mediated protection of H9C2 cells against anti-cancer drug doxorubicin-induced cardiotoxicity

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Thesis

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INTRODUCTION

Doxorubicin (DOX) has been a staple chemotherapeutic agent for decades, treating adult and childhood malignancies. It is one of the most common anti-cancer drugs used in treating different types of cancers including: pediatric cancer, leukemia, breast cancer [1]. DOX's anti-cancer effect is exerted through DNA intercalation and inhibition of the topoisomerase II enzyme in fast-proliferating tumors [1]. DOX thus halts cancer metastasis, and ultimately leads to cancer cell death. However, its use has become limited due to its cardiotoxic effect. Specifically, DOX promotes cumulative dose-dependent cardiotoxicity, which affects the heart's functionality and can lead to irreversible damage [2]. One of the underlying mechanisms of this adverse effect is the accumulation of reactive oxygen species (ROS) in the mitochondria. Due to the nature of the mitochondria-dense cardiac cells, doxorubicin-induced oxidative stress is up to 10 times greater in the heart than in other tissues [3]. This leads to cardiac dysfunction and an increased incidence of mortality due to developing cardiomyopathy.

Unfortunately, among cancer survivors, complications from cardiac-related toxicity have become the second leading cause of death after secondary malignancy [4]. Specifically, cardiac events influenced by DOX are difficult to prevent when they can occur within 2 to 3 days of treatment or 6 to 10 years after its last administration [5]. The only FDA drug used in combination with DOX to prevent cardiotoxicity is dexrazoxane (DEX) [6]. However, DEX also inhibits a form of the topoisomerase II enzyme which may prevent DOX's anti-cancer ability and promote the early development of secondary malignancies in some cases [7]. Moreover, other drugs such as angiotensin inhibitors, and β-blockers have also been attempted and though they have shown some protective

action over Dox-induced cardiotoxicity they are not found to be highly successful in all cases [8]. DOX has shown the most success with treating cancers, and an improved method of drug delivery needs to be implemented so that patients can continue taking the drug without subsequent risk of cardiotoxic effect.

Clinical manifestation of DOX-Induced Cardiotoxicity

Cardiotoxicity can have acute and chronic manifestations. In acute cardiotoxicity, patients can develop arrhythmias, pericarditis-myocarditis syndrome, myocardial infarction, sudden cardiac death, congestive heart failure (CHF), and cardiomyopathy. Acute cardiotoxicity can occur within the first week of treatment for adults and children and is usually reversible when DOX is discontinued. Chronic cardiotoxicity has two subtypes: early-onset cardiotoxicity, which begins within a year of treatment, or late-onset chronic cardiotoxicity which can occur up to 30 years later. [9]. The risk of experiencing cardiac-related events persists even after 45 years [4]. This range makes preventing cardiotoxicity difficult.

Moreover, children and adult patients can show different cardiac pathological changes. For both subcategories of chronic cardiotoxicity, children develop restrictive cardiomyopathy, which can progress into dilated cardiomyopathy [10]. By contrast, patients treated in adulthood develop dilated cardiomyopathy in both the early-onset and late-onset progression of the cardiotoxicity [9]. In all manifestations of cardiotoxicity patients demonstrate decreased contractility, leading to decreased systolic and diastolic cardiac performance, leading to further complications, especially in children who often require heart transplants as adults [9]. Once chronic progression of cardiotoxicity has

developed, the damage is irreversible, and patients are subject to treatments that can only help manage their symptoms. Therefore, preventing cardiotoxic effects is vital in decreasing this irreversible damage.

One of the main risks in developing cardiotoxicity is reaching DOX cumulative doses of 500 mg/m^2 and above. In a study where 630 patients were followed while treated with doxorubicin, all recruited patients demonstrated normal left ventricular ejection fraction (LVEF) at the start of the study. By the end of the study, 149 experienced a DOX-induced cardiac event defined as at least a 20% reduction in LVEF. The severity and frequency of the cardiac events were closely related to the cumulative dose of DOX given. Starting at a cumulative dose of 150 mg/m^2 only 7% of patients experienced a cardiac event, however that increased to "9%, 18%, 38%, and 65% of patients at cumulative doses of 250 mg/m^2, 350 mg/m^2, 450 mg/ m^2, and 550 mg/m^2, respectively." [11]. A cumulative lifetime limit of 450 mg/m^2 is recommended to reduce the likelihood of developing cardiotoxicity. However, limiting DOX reduces patient options for effective cancer treatments.

Molecular mechanisms of DOX-induced Cardiotoxicity

Several mechanisms have been suggested to explain why DOX-induced cardiotoxicity occurs. One of the most widely accepted explanations for DOX's cardiotoxicity is its interactions with the mitochondria to promote the increase of ROS. If this effect is not counteracted by antioxidants it can lead to mitophagy and cell death [12]. Apart from this, DOX has also been linked to other mechanisms which include, altered iron regulation, disrupted calcium homeostasis, autophagy, and the release of nitric oxide,

inflammatory mediators, and apoptotic factors [1]. Our study focused on DOX mitochondrial cardiotoxicity which ultimately contributes to cardiomyopathy.

DOX is a lipophilic cation that is attracted to the highly negative mitochondrial membrane potential of -160 to -180 mV, compared to the cytoplasmic membrane potential of -60 mV [13]. Thus, mitochondria become the subcellular target for DOX-induced toxicity. Mitochondria are membrane-bound organelles that generate energy, in the form of ATP, to power the cell's metabolic functions. To maintain the high cellular demands of the heart, contractile cells (cardiomyocytes) require constant levels of ATP. This explains why 30% of cardiomyocyte cells' volume are solely comprised of mitochondria, making the heart the most susceptible to DOX accumulation [14].

Once DOX enters the mitochondrial matrix and its tetracycline ring interacts with the electron transport chain's complex I. At this complex DOX gets reduced into its reactive semiquinone free radical form. This compound then transfers an electron to mitochondrial O_2, generating superoxide anions (O_2^-). DOX repeats this process leading to an accumulation of mitochondrial superoxide anions. Accumulation of these anions lead to oxidative damage and mitochondrial dysfunction. Additionally, DOX further contributes to mitochondrial dysfunction via calcium (Ca^{2+}) upregulation, triggering the opening of the mitochondrial permeability transition pores (mPTPs). This disrupts the delicate electrochemical gradient that exists between the two membranes in the mitochondrial. A depolarized mitochondrial membrane often leads to triggered mitophagy and ultimately cell death. Finally, DOX has a high affinity for cardiolipin, a phospholipid found on the cardiomyocyte's mitochondria. It plays a vital role in

regulating the electron transport chain and ATP levels. When DOX attaches it forms a DOX-cardiolipin complex which inhibits complexes I and complex II thus inhibiting the effectiveness of the pathway, also leading to mitochondrial dysregulation [15]. Mitochondrial function is vital for the health of cells as the cells. Increased mitochondrial dysfunction along with decreased ATP levels significantly reduce cardiomyocytes' ability to sustain normal contractility and once the damage is done the cardiotoxic effect of DOX becomes irreversible [15].

Mitigation of Cardiotoxicity

There are currently no specific treatments available to mitigate DOX-induced cardiomyopathy. In addition to limiting the cumulative dose, other methods have been used in an attempt to minimize cardiotoxic risks. These methods include administering DOX concurrently with other anti-tumor drugs, using DOX analogs, alternating drug delivery, or using general antioxidants and iron chelators (i.e., DEX). However, the listed approaches have shown limited success [8]. DEX is an iron chelator that works by binding free iron which prevents the formation of a DOX-iron complex [16]. Binding of iron to DOX promotes iron cycling between Fe^{3+} and Fe^{2+} and increases ROS [15]. However, DEX is the only iron chelator to show protection against DOX-induced toxicity, suggesting an alternative mechanism for its protective effect [6]. Further research found that DEX also inhibits DOX's anti-cancer properties. Recall, DOX inhibits topoisomerase II within the cell and mitochondria. DEX inhibits this pathway by changing the configuration of topoisomerase II, preventing DOX from binding and

inhibiting its ability to kill cancer cells [15]. This makes the use of DEX, to limit DOX-induced toxicity, inappropriate during cancer treatment.

Antioxidants

Antioxidants work well to reduce increased levels of ROS. Common antioxidants such as vitamin A and C work to inhibit oxidants from reacting with each other. Yet, they do not accumulate in the mitochondria where they are most needed. Fortunately, there are also mitochondrial antioxidants, like mitoquinone (MitoQ), which are shown to be potentially favorable in attenuating this DOX-induced increase in ROS [17]. Unlike vitamin A and C, mitochondrial antioxidants accumulate in the mitochondria due to their positive charge and lipophilic feature. Though MitoQ shows promising cardioprotective features, its mechanisms have yet to be fully understood concerning its combined effect with DOX in cardiac cells [14].

Due to Mitoquinone's preference for accumulating in the mitochondria, this TPP+-conjugated antioxidant is more potent in reducing intracellular ROS accumulation than its lipophilic counterparts [18]. Similar to DOX, MitoQ also interacts with the electron transport chain's complex I, but instead of inhibiting the complex it has been shown to have positive affect on the substrate levels surrounding this complex, contrastingly to DOX, it improves oxidative phosphorylation at the mitochondrial level [19]. This is an important characteristic to note because it can provide competitive inhibition against DOX which is dependent on complex I for reduction. Additionally, previous studies have also shown that MitoQ has also an ability toward reducing the mitochondrial membrane potential [19]. The mitochondrial membrane potential is an

important factor the cells depend on in order to sustain appropriate energy levels when carrying out oxidative phosphorylation for ATP production. Many studies have demonstrated that a dysregulation of this potential may be harmful and lead to increased cell apoptosis but a reduction occurring in the presence of MitoQ does not show the same cytotoxic effects [13, 19, 20]. DOX depends on the mitochondrial membrane potential to accumulate within the mitochondria and if this potential is reduced then subsequent DOX accumulation can be hypothesized to also demonstrate a reduction. MitoQ's different characteristics show great potential toward inhibiting DOX-induced damage on the molecular level.

In order to understand the role MitoQ can play when given with DOX, first we must understand what benefits it has when given on its own. For example, in a cell model exploring the effect of Aβ peptides on N_2a cells, incubation with MitoQ led to partial protection against the effect of Aβ peptides which is known to lower cell viability and contribute to mitochondrial dysfunction [18]. Similarly, in a mouse animal model, MitoQ showed direct protection toward transgenic mice that overexpress human catalase localized to the mitochondria (mCAT) against the effect of mitochondrial ROS production [18]. These findings suggest that MitoQ could be beneficial for conditions and diseases characterized by dysfunctional mitochondria [18]. MitoQ was also shown to be effective in reducing ischemia-reperfusion injury when given for 8 weeks to hypertensive rats, and this was associated with a reduction of systolic blood pressure and cardiac hypertrophy [18]. These findings have led to further experimentation which explore MitoQ's benefits, safety, and efficacy.

MitoQ is an approved dietary antioxidant supplement that is well tolerated when given to healthy human adults and does not demonstrate significant adverse side effects when given alone [21]. A recent study in 2018 was the first human trial to show that 6 weeks of daily oral supplementation with MitoQ suppressed mitochondrial-derived oxidative stress and improved vascular endothelial function [22]. This is promising and suggests that it might protect cardiac tissue against DOX-induced oxidative stress. Very few studies have explored the effects of MitoQ as a protective agent against DOX induced cardiotoxicity. The most related study is one where healthy rats were treated with DOX, Mito-Q, and DOX plus Mito-Q (co-treatment) for 12 weeks [23]. The study found that DOX alone decreased left ventricular function, attenuated cytochrome c-oxidase, and altered healthy electron paramagnetic resonance in ex-vivo samples. All of these features are associated with DOX-induced damage which leads to cardiac dysfunction. The co-treatment group however showed significant improvements to restore these characteristics [23]. This study thus establishes the protective effect MitoQ has against DOX-induced cardiotoxicity, yet the mechanisms are poorly understood. Alternate methods of drug delivery such as MitoQ given as a pre-treatment have also not been thoroughly explored.

Previous Studies

On our lab, has looked into the differences between MitoQ given as a pre-treatment versus the benefits when given as a co-treatment with DOX. More specifically we also explored to see if there were differences in benefit when MitoQ was given as a pre-treatment 24 hours prior to DOX and compared that to the effects seen when given as

co-treatment with DOX in H9c2 cells. It was observed that both pre-treatment and co-treatment with MitoQ significantly reduced DOX-induced toxicity measured by intracellular dehydrogenase activity of viable cells and confirmed by calcein staining in H9C2 myoblasts. However, higher efficacy in cellular protection was shown to be significant when MitoQ was given as pre-treatment compared to MitoQ co-treatment (Figure 1). Two different mechanisms have been proposed to explain these findings: (1) accumulation of MitoQ in the mitochondria depends on mitochondrial membrane potential, which competes with DOX and thus prevents its accumulation; (2) MitoQ and DOX exert effects via the electron transport chain and cardiolipin, which may lead to interference when given at the same time [13]. However, more studies need to be done to fill this gap in understanding the mechanism behind the preferred pre-treatment potency.

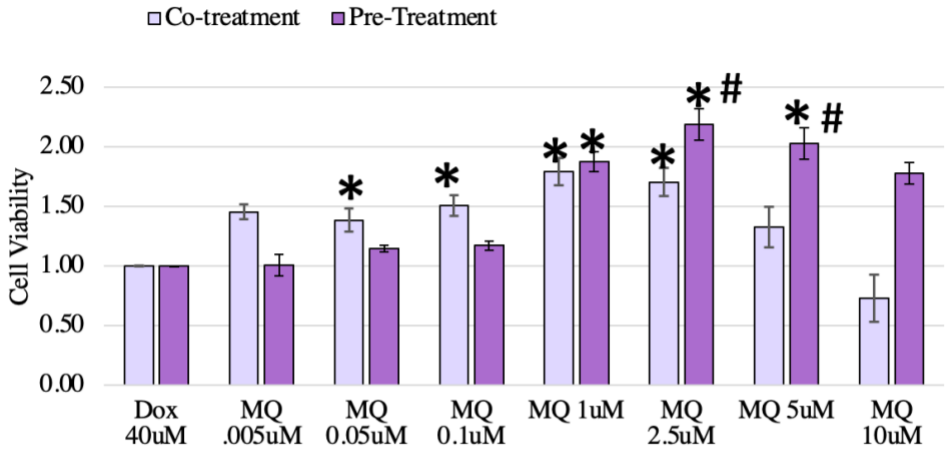

Figure 1. Dose-dependent effects of co-treatment and pre-treatment on cell viability by a calcein staining assay. (n= 9, * : $p < 0.05$ vs. control, # : $p < 0.05$ vs. Co-treatment) (Sacks et al., 2021)

Finally, antioxidant enzymes play an essential role in modulating ROS and reducing the damage done to cells via oxidative stress. Heme oxygenase-1 (HO-1) is an antioxidant considered the main protein associated with diseases resulting from oxidative and inflammatory insults [24]. First, heme oxygenase 1 is an inducible enzyme that regulates mitochondrial quality control in the heart. Specifically, in regards to influencing mitochondrial mechanisms, it has been shown to promote mitobiogenesis when overexpressed in cardiomyocytes [25]. Hull et al.'s study found that overexpression of heme oxygenase 1 prevented the increase of FIS-1, a protein expressed by dysfunctional mitochondrial triggering cell apoptosis. This demonstrates that heme oxygenase 1 may protect mitochondria by disrupting the positive feedback loop of mitochondrial injury and oxidative stress leading to apoptosis [25].

Additionally, increased expression of HO-1 is closely associated with the upregulation of superoxide dismutase 1 (SOD-1). Secondary antioxidant SOD-1 scavenges oxygen radicals that can be overproduced in the mitochondria's electron transport chain. It metabolizes superoxide radicals (O_2^-) to more stable molecules, molecular oxygen (O_2), and hydrogen peroxide (H_2O_2) [26]. In another study with diabetic rats, upregulation of HO-1 led to a robust increase in extracellular superoxide dismutase (EC-SOD) [27]. These proteins contributed to an increase in endothelial relaxation and a decrease in superoxide anions. Considering that superoxide anions are highly involved in the mitochondrial dysfunction associated with DOX-induced damage, it would be interesting to determine whether induction of these enzymes contributes to the protective effects of MitoQ. The goal of this project is to fill the gap regarding why pre-treatment with MitoQ is more effective than co-treatment against DOX-induced H9c2

cell damage. A better understanding of how pre-treatment with MitoQ protects cardiac cells from the toxic effects of DOX might allow expansion of the clinical utility of this chemotherapeutic agent.

Hypothesis, Aims, Expectations

Hypothesis

We hypothesized that pre-treating H9c2 cells with the mitochondrial antioxidant, Mitoquinone, influences more mechanisms of protection against Doxorubicin-induced cardiotoxicity as compared to when given as a co-treatment.

Specific Aim 1

We investigated several aspects of mitochondrial function, including the mitochondrial membrane potential, oxidative stress using MitoSOX, and mitochondrial DOX concentration via fluorescence measurements.

Specific Aim 2

We evaluated the expression of two important antioxidant enzymes: superoxide dismutase and heme oxygenase within treatment groups of MitoQ alone, MitoQ given as pre-treatment and co-treatment, and DOX alone.

Expectation

We expect the MitoQ pre-treatment to protect mitochondrial function by reducing DOX intracellular accumulation and thus related DOX-induced oxidative stress in cardiomyocytes. As seen in other cell types, MitoQ has been confirmed to show a

decrease in ROS, and testing it on H9C2 cells shows much promise as an effect has been demonstrated between a pre-treatment and co-treatment [13, 28]. We also expect an increase in antioxidant enzyme expression for heme oxygenase 1 and superoxide dismutase 1. These expectations will confirm mechanisms in which pre-treatment would be most effective against DOX-induced cardiotoxicity.

METHODS

H9C2 cells

Rat H9C2 cells, a clonal myoblast cell line derived from embryonic BD1X rat heart tissue, were obtained from the American Type Culture Collection (ATCC, CRL-1446). Cells were maintained in 75 cm^2 flasks and petri dishes in high glucose Dulbecco's modified Eagle's medium (DMEM) (Corning Life Sciences, Presque, Pennsylvania, USA), 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), and 10% fetal bovine serum (FBS) (Corning Life Sciences, Presque, Pennsylvania, USA). The cultures were incubated at 37°C in 5% CO_2 and medium. The medium was aspirated every 2-3 days, and cells were washed twice with PBS. The process was repeated until the cells reached 70%- 90% confluence for passaging. During passaging, cells were detached using trypsin/EDTA (Sigma Aldrich, St Louis, MO, USA). After collection, cells were resuspended in medium, and cells were counted using a 0.3% trypan blue kit (Sigma-Aldrich, St. Louis, MO). Cells were plated at a density of 2×10^4 cells per well in a 96-well plate. The remaining cell suspension was distributed into 150 mm cell culture dishes with 10 mL of medium. Seeded plates were treated after a 24 hr. incubation period, and dishes were treated once confluence reached 80%.

Experimental Groups

Doxorubicin or Mitoquinone alone

Cells in 96-well plates were treated with DOX alone (0.5–50 μM; M.W. =579.98g/mol, Sigma-Aldrich, St. Louis, MO) or MitoQ alone (0.005μM-10μM; M.W. =678.8 g/mol,

Cayman Chemical, Ann Arbor, Michigan). After cells were incubated for 24 hours, biochemical assays were conducted.

Co-treatment or Pre-treatment with MitoQ and DOX

To establish the co-treatment condition, cells were concurrently treated with MitoQ (0.005µM-10µM) and DOX (40µM). To establish the pre-treatment condition, H9c2 cells were pre-treated with MitoQ alone (0.005uM-10 µM), incubated for 24 hours before aspirating, and washed twice with PBS. After washing, the cells were given 100 µL of new medium and treated with DOX (40 µM). Biochemical assays were then performed after a 24 hr incubation period.

Biochemical Assays:

1. Cell Viability Analysis

Cell viability was evaluated using the Cell Counting Kit-8 (CCK), which is based on intracellular dehydrogenase activity. Dehydrogenases reduce WST-8 cells to form formazan which is directly proportional to the number of live cells. After completion of treatments, cells were washed twice with PBS and supplemented with fresh medium. Each well received 10 µL of CCK reagent and plates were incubated for 3-4 hours before absorbance was measured at 450 nm using the iMark™ Microplate Absorbance Reader (Bio-Rad)

2. Mitochondrial membrane potential (MMP) analysis

JC-10 (catalog #MAK159, SIGMA-ALDRICH) assay was used to observe differences in mitochondrial membrane potential ($\Delta\psi_m$). JC-10 is a cationic lipophilic dye that forms

red-fluorescent JC-10 aggregates (λex = 540/λem = 590 nm) that concentrate in the mitochondria of cells with polarized membranes. However, when the mitochondrial membrane potential is depolarized, the mitochondria cannot retain the dye, and JC-10 changes into its monomeric form, emitting a green fluorescence (λex = 490/λem = 525 nm) as it diffuses out of the mitochondria. JC-10 stock solution was diluted in buffer A to generate concentration of 1uM. Subsequently, the JC-10 or buffer A solution was added to cells which had the same treatments, respectively, and then plates were incubated at 37 °C for 45 minutes. Upon completion of the incubation period, the imaging buffer B solution was added to the cells. Fluorescence is measured by Fluoroskan Ascent CF Microplate Reader (Catalog # 21748, Cambridge Scientific) at respective wavelengths before adding JC-10 and after adding the buffer B solution. To remove interference of the fluorescence produced by DOX, the difference between the same treatment wells with JC-10 and without JC-10 were calculated. Lastly, the ratio between red fluorescence to green fluorescence was used to indicate MMP.

Mitochondrial Superoxide Production:

MitoSOX™ Red mitochondrial superoxide indicator (Catalog # M36008, Thermo Fisher Scientific) is a red fluorogenic dye used to visualize and measure the amount of superoxide present within the mitochondria of living cells. Mitochondrial superoxide is a byproduct that is generated during oxidative phosphorylation. Though usually a tightly regulated system, the mitochondria's electron transport chain allows for 1-3% leakage of mitochondrial oxygen that is not fully reduced. They quickly react with electrons to form superoxide anions, the predominant reactive oxygen species in the mitochondria. After

treatments, cells were washed twice with PBS, and 100- µl culture medium was added to each well with 100 µL of the 5 µM stock solution of MitoSOX. Cell plates were protected from light by covering with aluminum foil and then incubated for 10 minutes at 37˚C. Fluorescence was read via a Fluoroskan Microplate Reader at (510 nm excitation/590nm emission). MitoSOX fluorescence was first adjusted for DOX fluorescence by subtracting a background fluorescence reading of DOX alone (λex = 510/λem = 590 nm) taken prior to the addition of MitoSOX. Then the value was adjusted with viable cell number which was obtained from the CCK assay

Intracellular DOX measurement

After washing out all the drugs, a background fluorescence reading (λex = 510/λem = 590 nm) was taken from the cells which were treated with DOX alone or with MitoQ pre-treatment or co-treatment. The difference between DOX treated and non-treated viable cells indicated intracellular DOX accumulation.

Western blot

1. Protein Collection

After cells in the dishes reached 80%-90% confluence, they were prepared for the following conditions: non-treated control, MitoQ 2.5µM alone, DOX 40 µM alone, and co-treatment or pre-treatment of MitoQ 2.5µM and DOX as described previously. After cells were incubated for 24 hours, dishes were washed twice with PBS, and adherent cells were mechanically removed via a scraper. Cells were pipetted with 1X PBS solution into a 1.5 mL Eppendorf tube and centrifuged (5000rpm for 5 minutes at 4°C). The supernatant was aspirated, and the cell pellet was lysed with 30 µL of lysis buffer

containing Pierce RIPA Buffer (Catalog # UG286129, ThermoScientific), Halt Protease Inhibitor (Catalog #WG322531, ThermoScientific), and EDTA Solution (Catalog #WD323125, ThermoScientific). Upon completion of cell lysis, cells were centrifuged at 15000 rpm for 30 minutes for 4°C. The protein-rich supernatant was transferred to 0.5 ml Eppendorf tubes, and the pellets containing lipids, and cellular and nuclear membranes were discarded. Protein lysates were stored at -80 °C.

2. Protein Quantification

Protein was quantified using the PierceTM BCA Protein Assay Kit. The Bovine Serum Albumin (BSA) standard was diluted to various concentration levels from 0 to 2000 µg/mL. The samples and standards were added to a 96-well plate. Bradford Reagent was used to dye the proteins, and absorbance was measured at 540 nm (Pierce BCA Protein Assay Kit - Thermo Fisher Scientific, 2019). A standard curve of BSA concentration was made to compare with collected samples, and levels of protein concentration were determined.

3. Gel Electrophoresis

Protein samples (25-50 µg) were mixed with a loading buffer (NuPAGE LDS Sample Buffer 4X), then heated at 90-100 °C to denature the proteins and provide a negative charge. Samples were loaded into a precast polyacrylamide gel (NuPAGE™ 10%, Bis-Tris, 1.0 mm, Mini Protein Gels). The first well in the polyacrylamide gel was filled with 5 µL Bio-Rad Precision Plus Protein Dual-color Standards. After filling 1X NuPAGE MOPS SDS Running Buffer into the Bio-Rad Vertical Electrophoresis Cell, electrophoresis ran for one hour at a constant 150V.

4. Gel Transfer and Imaging

Proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane using the iBlot® Dry Blotting System. The membrane was first incubated by a 10x Blocking buffer for 30 minutes before adding the primary antibody overnight at a dilution of 1:1000. The preparation for the primary antibodies were as follows: 1. For the B-actin monoclonal antibody, blots were blocked with DI water and 5% milk for 30 minutes and incubated in mouse anti-actin antibody, 1:1000, in DI Water/5% milk overnight at 4 °C, 2. For the monoclonal HO-1, blots were incubated in rabbit anti-HO-1 antibodies, 1:1000, in DI Water/5% milk overnight at 4 °C, 3. For the monoclonal SOD-1, blots were incubated in rabbit anti-SOD-1 antibody, 1:1000 in DI water/5% milk overnight at 4 °C. After primary incubation, the membrane was rinsed with 10X FWBK Wash Buffer (Catalog # 37577, ThermoScientific) at a 1:10 ratio with deionized water, then incubated with the secondary antibody for 30 minutes at room temperature. B-actin blots were incubated in antibody dilution solution with secondary anti-mouse, 1:1000. Both HO-1 and SOD-1 were incubated with anti-rabbit secondary, 1:1000, and antibody dilution solution. Afterward, chemiluminescence reagents were used to visualize the bands with the Bio-Rad ChemiDoc MP Imaging System ("iBlot ® Dry Blotting System Instructions for using the iBlot ® Gel Transfer Device to perform dry blotting of proteins from mini- or midi-gels with iBlot ® Gel," 2011). To prepare membranes for multiple probing, a stripping buffer was used to remove previously bonded primary and secondary antibodies. The membrane was then blocked for 30 minutes using 5% milk prior to re-probing.

Statistics

All experiments were performed in triplicate and repeated at least three times. The data was expressed by mean ± SE. The data were analyzed using ANOVA followed by the Student Newman-Keuls Test. P-value of < 0.05 was considered statistically significant.

RESULTS

DOX-induced cytotoxicity correlates with DOX intracellular accumulation

The effects of DOX (500 nm-50 μM, *n* = 3) on H9c2 cell viability is shown in Figure 2A and demonstrate dose-dependent reductions. Compared to non-treated control cells, treatment with DOX doses ranging from 40 μM to 50 μM significantly reduced cell viability to 0.30 ± 0.17 and 0.21 ± 0.17 respectively (both $p < 0.05$, see Fig 2A). Additionally, DOX demonstrated a dose-dependent increase in intracellular accumulation (see Figure 2B). DOX 20 μM, 40 μM, and 50 μM demonstrated a significant increase in DOX intracellular accumulation to 4.43 ± 0.65, 8.45 ± 1.94, 17.08 ± 3.20-fold when compared to the non-treated control, respectively (n = 5, $p < 0.05$, see Fig 2B).

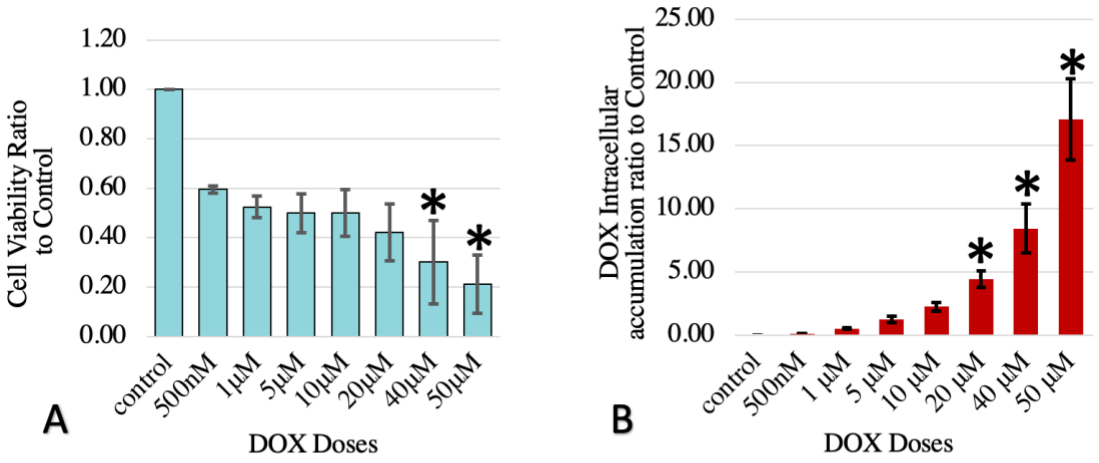

Figure 2A. DOX dose-dependently reduced cell viability as measured by a calcein staining assay. (n=3, * : p < 0.05 vs. control). Figure 2B. DOX exerted a significant dose-dependent increase in intracellular accumulation. (n=5, * : p < 0.05 vs. control).

DOX increased mitochondrial superoxide levels and decreased MMP

We further explored the effect of DOX on the level of mitochondrial superoxide anions and MMP (see Figure 3A-B). We found that DOX induced a dose-dependent

increase in mitochondrial superoxide anions compared to the non-treated control. In particular, the lower doses of DOX (5 μM and 10 μM) both significantly increased mitochondrial superoxide levels to 1.37 ± 0.08 and 0.09-fold (n=3, $p < 0.05$ vs. control). Additionally, the higher doses of DOX (20 μM, 40 μM, and 50 μM) also significantly increased mitochondrial superoxide levels to 2.04 ± 0.11, 3.30 ± 0.17, and 5.45 ± 0.12-fold, when compared to non-treated control, respectively (n = 3, $p < 0.0001$). This showed a dose-dependent increase of DOX intracellular accumulation. Moreover, DOX also reduced the MMP. The reduction reached a plateau at 1 μM, and higher doses of DOX (5 μM – 50 μM) showed a significant reduction of the MMP to 16-18% of the non-treated control (n=3, $p<0.05$; see Fig 3B).

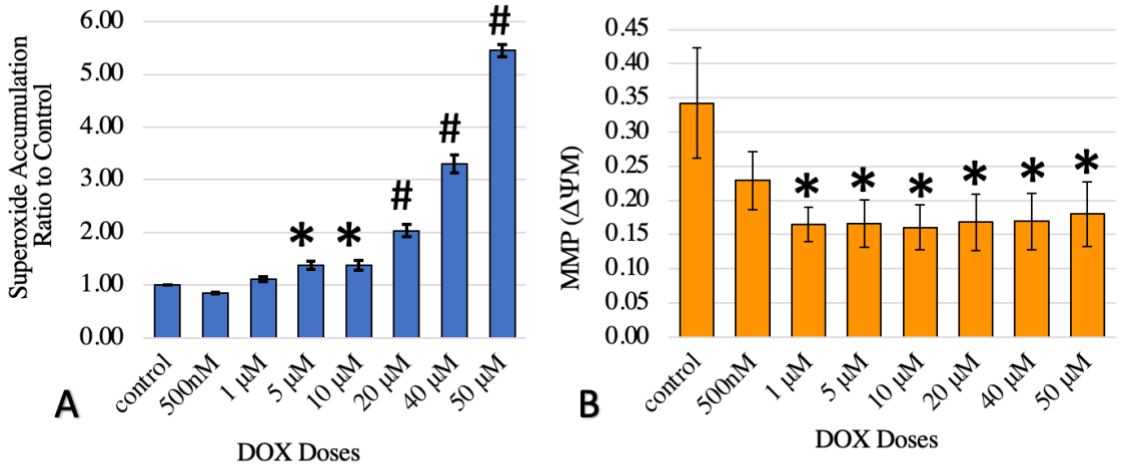

Figure 3A. Dose-dependent effects of DOX on mitochondrial superoxide levels by MitoSOX Assay (n=3, * : $p < 0.05$ vs. control, # : $p < 0.0001$ vs. control). Figure 3B. Effects of DOX on mitochondrial membrane potential by JC-10 assay. (n=3, * : $p < 0.05$ vs. control)

MitoQ's effects on cell viability, mitochondrial superoxide levels, and MMP

The effects of MitoQ (0.005 µM – 10 µM, n = 3) on H9c2 cell viability are shown in Figure 4A. MitoQ alone slightly increased cell viability, but the effects were insignificant compared to the non-treated control. Furthermore, MitoQ demonstrated a reduction in mitochondrial superoxide anion levels in most doses (0.005 µM- 5 µM, see Fig 4B). MitoQ (1 µM) showed the maximal reduction to 0.52 ± 0.01 when compared to non-treated control (n=4, $p < 0.05$; see Fig. 4B). By contrast, MitoQ (10 µM) significantly increased superoxide levels to 1.38 ± 0.12, when compared to non-treated control (n=4, $p < 0.05$, Fig. 4B).

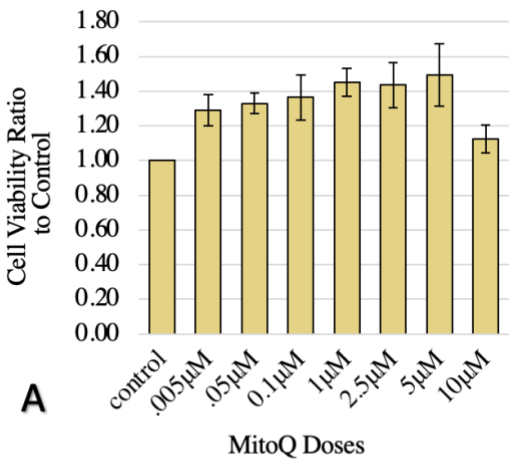

Figure 4A. Effects of MitoQ on cell viability by a calcein staining assay. (n=4)

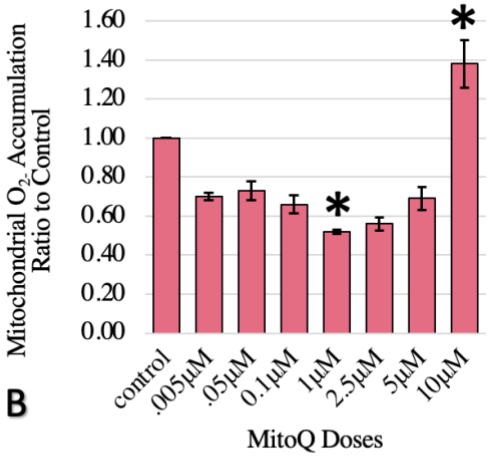

Figure 4B. Effects of MitoQ on mitochondrial superoxide anions levels. (n=4, * : $p < 0.05$ vs. control)

Moreover, we explored MitoQ's effect on the MMP shown in Figure 4C. Compared to non-treated control, lower doses of MitoQ (0.005 -0.1 µM) maintained a similar MMP as non-treated control. However, intermediate and higher doses of DOX (1-10 µM) significantly reduced the MMP to 0.11- 0.15 when compared to non-treated control (n=3, $p<0.05$; see Fig 4C).

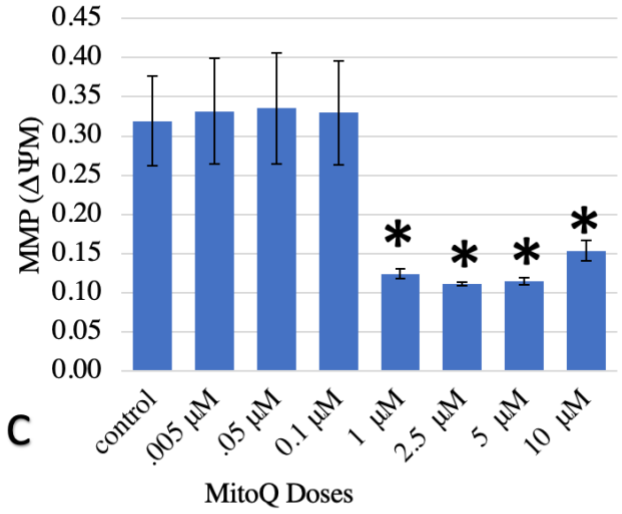

Figure 4C. Effects of MitoQ on mitochondrial membrane potential.
(n=4, * : $p < 0.05$ vs. control)

Co-treatment and Pre-treatment decreased intracellular DOX accumulation

We explored the effects of co-treatment and pre-treatment on the level of intracellular DOX accumulation. Compared to DOX 40 μM, MitoQ (0.005-5 μM), co-treatment showed a significant reduction in intracellular DOX accumulation in a dose-dependent manner (see Fig 5A). MitoQ (2.5 μM) exhibited the maximal reduction of intracellular DOX to 0.44 ± 0.10 when compared to DOX 40 μM ($n=3$, $p<0.05$). By contrast, MitoQ (10 μM) significantly increased intracellular DOX accumulation to 1.59 ± 0.25 when compared to DOX 40 μM ($p < 0.05$). Similarly, MitoQ, when given as pre-treatment, also demonstrated a dose-dependent reduction in intracellular DOX accumulation compared to the positive control (DOX 40 μM, see Fig 5A). MitoQ (0.05 μM) started to show a significant reduction, which was 10 times higher than the dose (0.005 μM) when given as co-treatment. By contrast, pre-treating with MitoQ (2.5 μM) exhibited a maximal reduction of intracellular DOX to 0.22 ± 0.08 compared to DOX 40 μM. The effect was

lower than the maximal effect of MitoQ (2.5 μM) when given as co-treatment. Additionally, the 10 μM dose of MitoQ pre-treatment did not significantly reduce intracellular DOX levels (n=3, 0.84 ± 0.25) relative to DOX 40 μM. However, DOX levels were significantly lower than the effects of 10 μM MitoQ as co-treatment ($p < 0.05$, see Fig 5A).

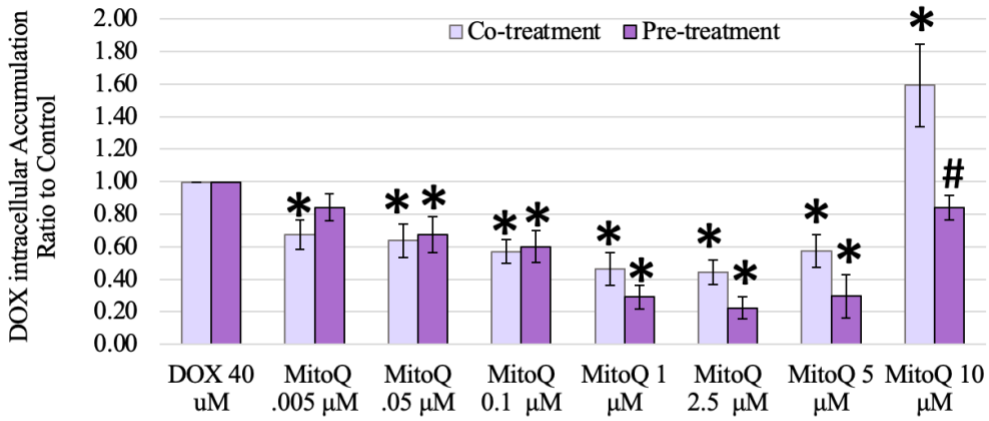

Figure 5A. Dose-dependent effects of co-treatment and pre-treatment on DOX Intracellular accumulation. (n=6, * : $p < 0.05$ vs. DOX 40 μM, # : $p < 0.05$ vs. Co-treatment)

Pre-treatment reduced mitochondrial superoxide levels

The effect of co-treatment and pre-treatment on mitochondrial superoxide levels is illustrated in Figure 5B. We found that MitoQ as a co-treatment (n = 4) slightly reduced mitochondrial superoxide levels, although the effects were insignificant compared to DOX 40 μM (see Fig 5B). On the other hand, MitoQ as pre-treatment showed biphasic effects on DOX-induced mitochondrial superoxide levels. At the lower doses of MitoQ (0.005 μM and 0.1 μM), treated cells demonstrated a slight but significant increase (1.39 ± 0.07 vs. control, n = 4, $p < 0.05$) in superoxide levels when comparing to DOX 40 μM

and/or co-treatment at the same dose (n=4, $p < 0.05$). Contrastingly, all the higher doses of MitoQ (1-10 μM) significantly reduced superoxide levels. MitoQ (2.5 μM) showed the maximal reduction to 0.53 ± 0.07 when compared to DOX 40 μM (n = 4, $p < 0.05$).

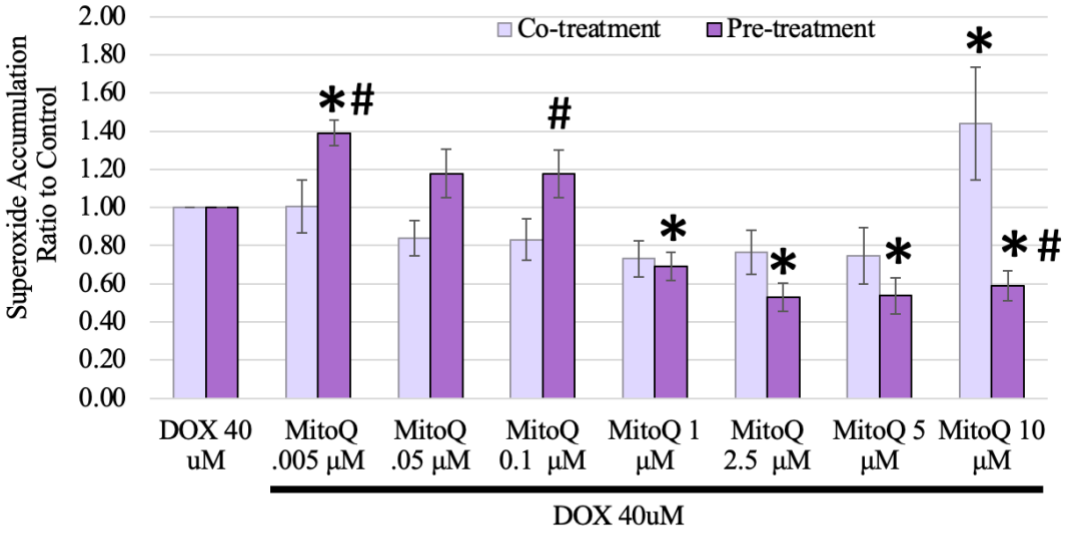

Figure 5B. Dose-dependent effects of co-treatment and pre-treatment on mitochondrial superoxide anions levels. (n=4, * : $p < 0.05$ vs. DOX 40 μM, # : $p < 0.05$ vs. Co-treatment)

Co-treatment and Pre-treatment reduced MMP

The effects of MitoQ co-treatment and pre-treatment on mitochondrial membrane potential are illustrated in Fig 5C. Compared to DOX 40 μM, both MitoQ co-treatment and pre-treatment showed similar results on MMP. Cells treated with the lower doses of MitoQ (0.005 -0.1 μM) and the highest dose (10 μM) exhibited similar MMP as DOX 40 μM. By contrast, the intermediate and higher doses (1-10 μM) reduced MMP as compared to DOX 40 μM alone, but not to a significant level.

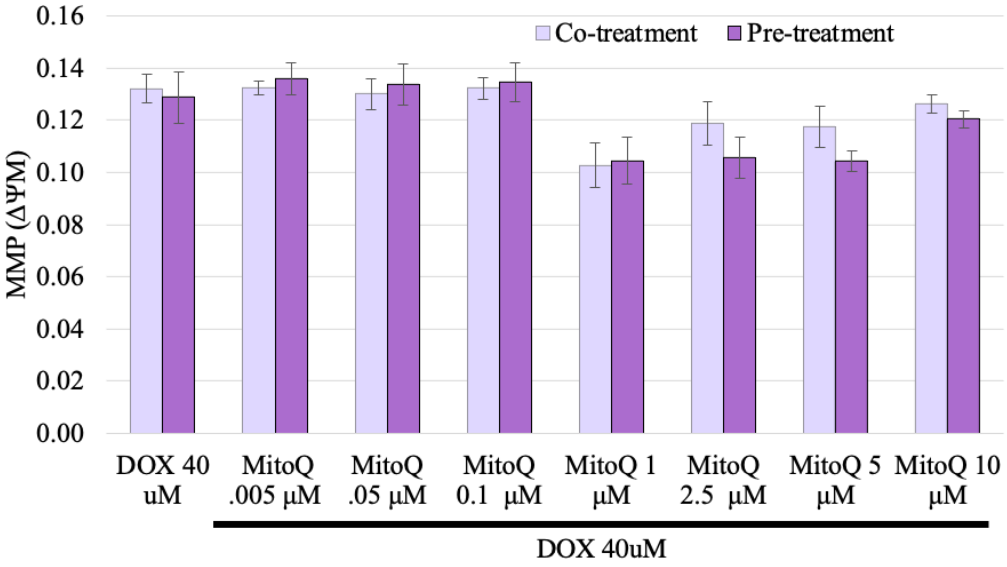

Figure 5C. Effects of co-treatment and pre-treatment on mitochondrial membrane potential (MMP). (n=4, vs. DOX 40 µM)

Effects of MitoQ, DOX, MitoQ co-treatment, and pre-treatment on HO-1 and SOD-1

The representative western blot results of MitoQ, DOX, MitoQ co-treatment, and pre-treatment on the expression of antioxidant enzymes HO-1 and SOD-1 are illustrated in Figure 6. MitoQ showed similar expression of HO-1 and SOD-1 as the non-treated control. By contrast, DOX 40uM showed lower expression of both antioxidant enzymes when compared to the non-treated control. Additionally, MitoQ pre-treatment exhibited increased expression of both antioxidant enzymes compared to co-treatment.

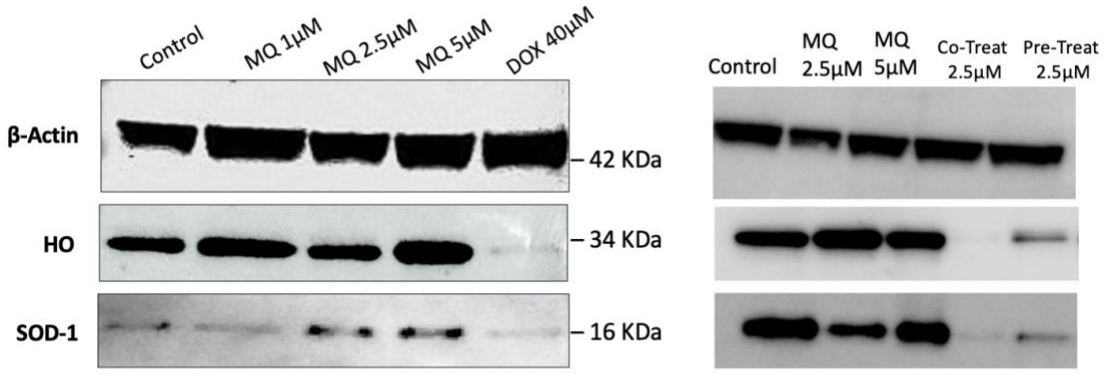

Figure 6. On left, Dose-dependent effects of MitoQ (1- 5 μM) and DOX (40 μM) on HO and SOD-1 expression. One right, Dose dependent effect of MitoQ (2.5- 5 μM) , and effect of Co-treatment (MitoQ 2.5 + DOX 40) and Pre-treatment (MitoQ 2.5, 24 hours prior + DOX 40) on HO and SOD-1 expression. β-Actin was probed as internal control for both.

DISCUSSION

Dox-Induced H9c2 cell damage

DOX, a class of anthracyclines, has been a widely prescribed anti-cancer treatment against various types of solid tumors. However, its use is limited due to its cardiotoxic effect. Our study showed that DOX (0.5-50 μM) dose-dependently reduced H9c2 cell viability and increased mitochondrial superoxide anions after 24 hours of incubation. Our findings are supported by a study by Zhang et al. and Upadhayay et al., demonstrating DOX's dose-dependent effects on cell damage and elevated ROS following treatment [2, 29]. More specifically, in Upadhayay's study, H9c2 cells treated with DOX (5 μM) showed a 50% reduction in H9c2 cell viability, which is similar to what we found [29]. Even though they seeded their wells with $1x10^5$ cells, which differs from the $2x10^4$ that we used, our experiments are almost identical in that we measured the differences based on cell density compared to the non-treated control.

Doxorubicin intracellularly accumulates in the cell, but more specifically, it targets the mitochondria [23]. Considering cardiac cells are dense in mitochondria and highly dependent on mitochondrial pathways for energy, mitochondrial dysfunction becomes detrimental to the overall functionality of the heart [31]. This leads to an increased risk of congestive heart failure and mortality. As a cation, doxorubicin is attracted to the -150-180 mV mitochondrial membrane potential, and its lipophilic features allow it to easily permeate the mitochondria via passive diffusion. More specifically, doxorubicin can accumulate in the mitochondria 100 times more than in the plasma, ~50-100uM compared to 0.5-1uM, respectively [23]. Additionally, when it is

intravenously infused, the distribution half-life of the drug is 3-5 minutes while the terminal half-life is 24-36 hrs, demonstrating that it takes the body much more time to process the drug out of the system, leaving the cells more prone to injury when accumulating within non-cancerous sites [31]. Our study demonstrated DOX dose-dependently accumulated within the cells and showed the most significant accumulation among the highest doses (20-50 μM). Our data is supported by Sardão et al.'s study, which observed doxorubicin's intracellular accumulation on H9c2 cells via epifluorescence microscopy [32]. Even though they more specifically measured for nuclear accumulation of doxorubicin, they also mentioned that they could not exclude mitochondrial accumulation.

ROS is the main contributor toward DOX'S proposed mechanisms of action, leading to DOX-induced damage. More correlated explicitly to the mitochondria, DOX induces an increase in superoxide anions. Superoxide anion formation can be attributed to several proposed mechanisms. For example, doxorubicin attaches to cardiolipin, an inner mitochondrial membrane protein that plays a key role in modulating enzymes for the energy-producing electron transport chain. The DOX-cardiolipin complex causes inhibition of the ETC's Complex I and II, disrupting the pathway and promoting ROS production [15].

Additionally, DOX can interact directly with complex I. It is reduced to the reactive semiquinone free radical, donating an electron to O_2, creating the superoxide anion (O_2^-), thus increasing ROS levels. The semiquinone becomes oxidized back to its original quinone state to repeat the cycle in what is referred to as redox cycling. In our

study, DOX (5-50 μM) significantly and dose-dependently increased levels of superoxide anions which is consistent with the previously explained mechanism.

In addition to increased levels of ROS, DOX causes the disruption of intracellular Ca^{2+} accumulation, inhibition of TOP II-B and anti-apoptotic proteins, and induction of pro-apoptotic pathways in cardiomyocytes [2]. All of these pathways can trigger intrinsic apoptosis in cardiomyocytes, as demonstrated in our data. DOX 40 μM and 50 μM showed the most significant reduction in cellular viability, measured by intracellular dehydrogenase activity of viable cells and confirmed by calcein staining. This is in agreement with what we observed in our lab's previous studies [13].

As previously mentioned, DOX can disrupt Ca^{2+} homeostasis and contribute to Ca^{2+} overload, triggering the mitochondrial permeability transition pore (mPTP) opening. An important consequence of the opening of the mPTP is that it allows H^+ ions to disperse freely, thus depolarizing the membrane potential and leading to decreased production of ATP [33]. Our study demonstrated that DOX (1-50 μM) significantly reduced the mitochondrial membrane potential in a dose-dependent manner compared to the non-treated control. Similarly, Upadhayay and company also observed DOX-induced membrane potential depolarization in H9c2 cells. They looked further to see if the depolarization affected cytochrome C levels. Due to the Ca^{2+} imbalance and the opening of the mPTP, the mitochondria become more permeable to cytochrome-c, an important indicator for cell apoptosis and necrosis [15, 29]. Using western blot to evaluate the expression difference in H9c2 cells, Upadhayay's found DOX treatment (5 μM) upregulated cytochrome-C after 24 hours of incubation, thus resulting in an increased

level of cellular death, also demonstrated by our observed effect with DOX on cell viability. To summarize, our data showed that DOX exerted a dose-dependent increase in mitochondrial superoxide anions and DOX intracellular accumulation, leading to lower levels of cell viability with increasing doses.

MitoQ reduces mitochondrial superoxide levels and MMP

MitoQ is one of the most extensively studied mitochondrial antioxidants and is now in phase II of human trials [34]. Currently, MitoQ is available over counter as an anti-oxidant supplement. This study suggested that MitoQ alone did not significantly reduce cell viability. Compared to non-treated control, it demonstrated a slight increase in cell viability in the middle dose ranges (0.005 – 5 μM).

Mitoquinone is a ubiquinone derivative conjugated to triphenylphosphonium (TPP) and lipophilic in nature. Unlike other ubiquinone analogs, it selectively accumulates in the mitochondria several hundred folds more than in the cytoplasm [17, 35]. Within the mitochondria, MitoQ's ubiquinone moiety is reduced to ubiquinol by complex II, forming a potent antioxidant. In this form, it constantly gets recycled by components of the ETC [35]. Our data showed that MitoQ significantly reduced mitochondrial superoxide anion levels in most doses (0.005 μM- 5 μM). However, MitoQ (10 μM) significantly increased superoxide levels when compared to non-treated control. Fink et al's study suggested that the pro-oxidant feature of MitoQ depends on fuel selectively in vascular endothelial cells [19]. They let bovine aortic endothelial cells exposed to differing concentrations of complex I substrates, and MitoQ was evaluated. Results demonstrated that even though pro-oxidant effects were observed, alongside

decreased membrane potential, cytotoxic effects were not observed, which is similar to what we with respective to non-significant changes made to cell viability. However, further research is required to elucidate its safe dose range and potential toxicity.

MMP is not a direct indicator of cytotoxicity when comparing MitoQ to DOX

Our data indicated that MitoQ significantly reduced MMP. However, cell viability was not impacted with reduced MMP after administration of MitoQ. This is a different scenario when compared to DOX-induced cell damage. DOX not only reduced MMP but also induced higher mitochondrial superoxide production. Both disturbances and other proposed mechanisms (e.g., cardiolipin damage) determined cell death. It requires more attention that higher doses of MitoQ can be cytotoxic [20, 36, 37]. Mendez et al. found that MitoQ (10 μM) is cytotoxic to platelets [37]. Similarly, Ng MRAV, et al. report that guinea pigs with co-treatment of MitoQ (20 mg/kg) and gentamycin (130 mg/kg) showed severe multiple organ damage [20]. Therefore, the safe profile of MitoQ warrants further evaluation.

MitoQ pre-treatment reduced intracellular DOX accumulation and mitochondrial superoxide levels and upregulated antioxidant enzymes

In the clinical setting, heart pre-conditioning by transient ischemia/reperfusion episodes is a strategy that helps the heart to develop resilience to harsher conditions such as a prolonged reperfusion injury. This strategy attempts to mitigate DOX-induced cardiotoxicity. Maulik et. al. demonstrated that preconditioning via hypoxia/reoxygenation helped to attenuate the DOX-induced cardiotoxic effect against cardiac myocytes [38]. Similarly, Galan-Arriola et al.'s study found that remote ischemic

preconditioning before DOX treatment significantly preserved left ventricular ejection fraction, mitochondrial morphology, and DNA copies in pigs' hearts [39]. However, in our study, using ischemic preconditioning, we pretreated cells with the mitochondrial-targeted antioxidant, MitoQ, 24 hours before DOX (40 μM) or concurrently with DOX. We also compared the effects of pre-treatment to those of co-treatment.

Our previous study showed that both co-treatment and pre-treatment demonstrated an increased level of cell viability when compared to DOX 40 uM. However, co-treatment and pre-treatment differed in the range of doses that offered the most potent protection. Co-treatment was more protective at the lower range of MitoQ doses (0.05- 2.5 μM) and pre-treatment offered better overall protection among the higher doses of MitoQ (1-5 μM). Overall, pre-treatment showed a more potent and higher efficacy of protection than co-treatment.

In this study, we found that co-treatment started to show significantly lower levels of DOX accumulation of at the lower doses of MitoQ (0.005-0.1 μM). By contrast, pre-treatment demonstrated significant reductions among the higher doses of MitoQ (1-10 μM) accompanied with significantly higher reduction in DOX intracellular accumulation. As suggested in the literature, MitoQ relies on Complex I and II to be recycled between its oxidant and reduced form [40]. It is equally important to note that DOX depends on complex I to transform to its reactive semiquinone form [12]. These interactions may offer insight into why pre-treatment showed higher efficacy than co-treatment. Less DOX accumulation would induce less superoxide production and less cell damage. In consequence, we found that pre-treatment (1-10 μM) demonstrated significantly lower

levels of superoxide accumulation than co-treatment. However, co-treatment (at the MitoQ 10 μM dose) demonstrated a dose-dependent significant increase in both DOX intracellular accumulation and superoxide anion levels (when compared to DOX 40 μM). This coincides with the reduced level of cell viability demonstrated with co-treatment at the same dose. MitoQ itself at higher dose (e.g., 10 μM) may become pro-oxidant and potentiate DOX-induced oxidative stress.

Moreover, the reduced MMP also contributed to DOX accumulation in mitochondrial after MitoQ was given as pre-treatment or co-treatment. We found that cells treated with DOX with or without MitoQ had significant reduction of MMP when compared to DOX alone. However, the degrees of MMP reduction were the similar as MitoQ alone. Although MMP reduction can reduce ATP production, it may initiate some protective mechanisms, such as activation of AMPK, mitophagy, and/or reduction of superoxide production [12]. These affects can provide further protection against DOX-induced damage on the cellular level.

In addition to MitoQ's antioxidant ability, Hu et al.'s study in 2018 indicated that MitoQ pre-treatment activated Nrf_2 signaling, which enhanced antioxidant capacity to protect mitochondrial DNA in an intestinal ischemia/reperfusion model [42]. However, the specific role Nrf2 has during cardioprotective needs to be further explored.

Our preliminary data found that MitoQ pre-treatment showed higher expression of heme oxygenase 1 and superoxide dismutase 1, which might aid in quenching reactive oxygen species. For example, heme oxygenase 1 transgenic mice models reveal that overexpression of the antioxidant promoted a reduced infarct size following

ischemia/reperfusion injury due to its overexpression and similar studies are discussed regarding the antioxidant's ability in providing protection against oxidative stress [24]. Similarly, superoxide dismutase 1 and related enzymes have been shown to control a variety of ROS and reactive nitrogen species thus limiting the potential toxicity of these molecules and offering protection against oxidative stress [26]. Our study demonstrated that MitoQ, given as a pre-treatment, upregulated both antioxidant enzymes more than co-treatment. This difference was also demonstrated when compared to DOX 40 µM. Higher levels of antioxidant enzymes can better protect cells against oxidative stress [27]. However, more experiments need to be done to confirm this finding.

LIMITATION AND FUTURE STUDIES

We acknowledge that this study was performed on a rat H9c2 cardiomyoblast cell line instead of primary cultured cardiomyocytes. However, the literature suggests that H9c2 cells and primary cardiomyocytes are very similar with regard to mitochondrial biogenesis, function, and energy metabolism [42]. Additionally, we would like to validate further the effects of pre-treatment of mitochondrial-targeted antioxidants in a Dox-induced cardiotoxicity animal model and collect more samples to demonstrate a clearer Western Blot. Additionally, within the co-treatment and pre-treatment conditions, we need to evaluate Mitoquinone's protective effect against the anti-cancer properties of DOX. In the literature, it has been demonstrated that MitoQ was 30 times more cytotoxic to breast cancer cell lines than to healthy mammary epithelial cells [43]. It also increased DOX's anti-cancer effect while mitigating Dox-induced cardiotoxicity [43]. Finally, given that MitoQ may compete with DOX to demonstrate its impact, it would be interesting to see if pre-treatment or co-treatment would provide better anti-cancer

effects. We also hope in our attempts to understand the relationship of MitoQ with DOX that, we may provide insight on how patients can take antioxidants to prevent DOX-induced cardiotoxic effects.

CONCLUSION

In conclusion, DOX demonstrated a significant dose-dependent increase in mitochondrial superoxide levels, a depolarization of the MMP, and an increase in its intracellular accumulation, which promoted the dose-dependent decrease in cell viability for H9c2 cells. DOX alone may also reduce the expression of antioxidant enzymes, heme oxygenase 1 and superoxide dismutase 1. Contrastingly, the present study demonstrated that MitoQ alone did not significantly reduced cell viability. It was found to have promoted a reduction in mitochondrial superoxide anion levels, and a reduction in MMP. Though the latter is usually associated with cytotoxic effects, cell viability was not significantly affected with respect to the depolarized MMP. Moreover, pre-treating H9c2 cells with MitoQ (1 uM- 10 uM) showed a higher reduction of DOX intracellular accumulation and mitochondrial superoxide levels when compared to the co-treatment condition. Additionally, pre-treatment also demonstrated an increased expression of heme oxygenase 1 and superoxide dismutase 1, which may also promote more anti-oxidant effects to protect cells against DOX-induced damage. Thus, MitoQ pre-treatment can produce the best level of cardioprotection against the DOX induced damage. The relationships demonstrated by our experiments can lead to the clinical applications of providing patients with a preferred methods of treatment when supplementing DOX anti-cancer treatments with a mitochondrial antioxidant like MitoQ. For the future direction of our studies, we hope to repeat western blots to develop clearer images and draw more

conclusive statements regarding the expression of antioxidant enzymes. Finally, we would like to explore if there is any interference with MitoQ and DOX in breast cancer cells.

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APPENDIX