Chapter 5

A model of doxorubicin induced cardiotoxicity in hPSC-CMs.

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Introduction

Both cancer and cardiovascular disease are leading causes of morbidity and mortality worldwide.[1] The rise of new cancer therapeutics, together with improvements in prevention, early detection of tumors, and enhanced treatment schedules, have resulted in a dramatic increase in the number of cancer survivors (~15 500 000 in the United States alone).[2] However, about 1 in every 10 cancer survivors now experience cardiovascular complications related to their cancer treatments.[3] Cardiotoxicity has been observed both within the first year after cancer treatment and up to decades afterwards. These therapeutics induce cardiotoxicity due to direct, non-selective damage to the myocardium. Doxorubicin cardiomyopathy is similar to dilated cardiomyopathy, with an enlarged heart and reduced left ventricular ejection fraction and contractile function. Doxorubicin cardiomyopathy is therefore treated with the standard pharmacological treatment of dilated cardiomyopathy.[4] Early detection of doxorubicin cardiomyopathy is crucial as subsequent initiation of heart failure treatment can lead to partial or full recovery of cardiac function.[5]

To understand the molecular mechanics of DOX-induced cardiotoxicity (DIC), several studies have exposed rodents and rodent-derived CMs to DOX and demonstrated cardiotoxicity [6-8] A single injection of high dose DOX in male Wistar rats was found to induce cardiomyocyte apoptosis for at least 7 days following the injection. With repeated DOX injections during a 7-day period there were transient further increases in the apoptosis rate of cardiomyocytes, however no additive effect was found.[6] Another study exposed neonatal rat CMs to 0.1 μM DOX. This dose closely mimics the dose that the in vivo myocardium is exposed to during cancer treatment.[8] In response to this low dose of DOX, there was increased oxidative stress in the neonatal rat CMs. Recently, it was reported that a single dose of DOX also decreases the contractile function of mouse hearts, akin to the phenotype observed in patients.[7] In recent years, several groups have also studied DIC in hPSC-CM models. [9-12] They demonstrated an increasing apoptotic response with increasing doses of DOX.

One limitation of both the rodent and hPSC-CM based DIC-models reported thus far has been either their use of supraclinical doses (i.e. 10-50 times higher) or the lack of repeated doses of DOX. [13] None of the studies has utilized multiple low doses of DOX, even though repeated exposure to low doses of DOX would most closely mimic the exposure of the in vivo myocardium during standard cancer treatment. Clinical studies have indicated that the cumulative dose of DOX received during cancer treatment determines the fraction of patients subsequently developing doxorubicin cardiomyopathy. Although the reported incidence of doxorubicin cardiomyopathy differs based on the definition used for the diagnosis, a large retrospective analysis of > 4000 patients who had received DOX indicated that about 2% went on to develop doxorubicin cardiomyopathy.[14] At higher doses the incidence has been indicated to be much higher, rising to 18% at a dose of 551-600 mg/m² and 36% of patients when the dose exceeds 600 mg/m².[15] Next to the cumulative dose, the speed of infusion also determines the risk of significant cardiotoxicity, with slower infusion speeds over long periods causing less cardiotoxicity.[16] Furthermore, it has previously been speculated that the chronic nature of DIC results from the accumulation of dysfunctional cardiac cells with repeated exposures to DOX. According to this theory, after exposure to DOX, part of the cells in
the myocardium reach a threshold at which repair mechanisms are no longer able to fully offset the damage caused by DOX, and these cells become dysfunctional to varying degrees. With each repeated exposure to DOX, new cardiac cells reach this threshold towards dysfunction and the function of other cells which are already dysfunctional deteriorate further.\[2\]
The aim of the present study was to model DIC in a human setup. We hypothesized that two repeated exposures to low doses of 0.1 μM DOX would produce a distinctly different cardiotoxic response in hPSC-CMs than both a single low dose of 0.1 μM DOX, a single medium dose of 0.2 μM DOX, and a single high dose of 5 μM DOX. Figure 1 displays these three different DOX dosing regimens. To characterize the cardiotoxic response of hPSC-CMs to these DOX treatment, we performed extensive phenotyping of the contractile function using the BASIC method. Furthermore, we assessed the sarcomeric integrity, viability, oxidative stress, and metabolic function of hPSC-CMs in response to DOX treatment.

![Figure 1 | Overview of This Study. Shown are an overview of the doxorubicin (DOX) treatment schedule and assays performed in this study.](image)

**Materials and Methods**

**Cell Culture and Cardiac Differentiation**

HUES9 human embryonic stem (hES) cells (Harvard Stem Cell Institute) were maintained in Essential 8 medium (A1517001; Thermo Fisher Scientific, Waltham, MA, USA) on a Geltrex-coated surface (A1413301; Thermo Fisher Scientific) and medium was refreshed daily. Cells were incubated under controlled conditions at 37 °C, with 5% CO₂ and 100% atmospheric humidity. Differentiation to cardiomyocytes was achieved as described previously.\[17\] Briefly, hES cells were dissociated with 1x TrypLE (12604-021; Thermo Fisher Scientific) for 4min and plated as single cells in Essential 8 medium containing 5 μM Y26732 (S1049, Selleck Chemicals, Houston, TX, USA); Essential 8 medium (without Y26732) was refreshed daily. Once cultures reached 80% confluency, cells were washed with phosphate buffered saline (PBS) and differentiation was initiated (day 0) by culturing cells in RPMI1640 medium (21875-034, Thermo Fisher Scientific) supplemented with 1x B27 minus insulin
A Model of Doxorubicin Induced Cardiotoxicity in hPSC-CMs

(Thermo Fisher Scientific) and 6 μM CHIR99021 (13122, Cayman Chemical, Ann Arbor, MI, USA). At day 2, cells were washed with PBS and medium was refreshed with RPMI1640 supplemented with 1x B27 minus insulin and 2 μM Wnt-C59 (5148, Tocris Bioscience, Bristol, UK). From day 4, medium was changed to CDM3 medium as described by Burridge et al. and was refreshed every other day as cardiomyocyte maintenance medium.[18] This resulted in cultures with > 90% spontaneously contracting cardiomyocytes at day 8-10. To further enrich these cultures, starting from day 12, differentiated cardiomyocytes were cultured in glucose-free RPMI1640-based (11879, Thermo Fisher Scientific) CDM3 medium supplemented with 5mM sodium DL-lactate (CDM3L; L4263, Sigma-Aldrich, St Louis, MO, USA) for 6-10days.[18] This resulted in > 99% pure spontaneously beating cardiomyocytes. Experiments were started between day 20-30.

Luciferase Viability Assay
Cell viability assays were performed using RealTime Glo MT Cell Viability Assay (Promega), a non-lytic NanoLuc Luciferase reaction occurs in the culture medium. NanoLuc Luciferase and MT Cell Viability Substrate are added to cell culture media. The MT Cell Viability Substrate then diffuses into cells where it is reduced to form a NanoLuc Substrate, which exits the cell and is used rapidly by NanoLuc Luciferase in the media, which signals the presence of metabolically active cells. Assay was performed according to the manufacturer’s instruction. Briefly, cardiomyocytes were seeded and cultured in sterile 96-well plates, 65,000 cells/well, and further subjected to DOX treatment. At the indicated time points, 100 μl of RealTime-Glo™ reagent was added to the cells. After a 15-min incubation at 37 °C, the luminescence was recorded in a Synergy H1 microplate reader (Biotek) with an integration time of 1 s per well.

Reactive Oxygen Species Assay
Total ROS production was quantified using CellROX Orange assay (Thermo Fisher Scientific). The intensity of CellROX Orange fluorescence is proportional to the level of free radical oxidation. CMs were loaded with 5 μM CellROX Orange in culture medium and stained in the dark for 60 min at 37 °C. Stained cells were washed once with PBS, dissociated with TrypLE and suspended in PBS. Cells were centrifuged for 5 min at 2000x g and suspended in 600 μl of PBS. 100 μl of cell suspension were transferred to a black 96 well plate (5 wells per condition) and analyzed immediately on a Synergy H1 microplate reader (Biotek) using 540/570 nm excitation/emission wavelength. For each condition the signal intensity was corrected to the number of cells as counted manually with a Fuchs-Rosenthal hemocytometer.

Immunocytochemistry
CM were washed twice with ice-cold PBS and fixed in 4% paraformaldehyde (PFA) for 5 min on ice, permeabilized with ice-cold PBS + 0.3% Triton X100 for 5 min and then washed twice with PBS. Cells were incubated for 1 hour in blocking solution: 3% BSA (Serva), 2% goat serum (Sigma Aldrich), 0.1% Tween 80 (Sigma-Aldrich) in PBS. Primary antibodies were diluted in blocking solution and incubated with the cells for 1 hour at room temperature. All used primary and secondary antibodies are listed in Supplementary Table 1. Coverslips were mounted using Vectashield mounting medium.
with DAPI (Vector Laboratories). 4 to 7 random pictures were taken with 20x magnification on Leica AF-6000 microscope and 150-200 cells/per condition were analyzed.

Generation of Cardiac Microconstructs
The production of micropatterned substrates for subsequent seeding of hESC-CMs was performed as described previously.[19] Briefly, 35 mm Fluorodishes (FD35-100, World Precision Instruments) were coated with 125 µl Sylgard 527 (Dow Corning) to achieve 5 kPa substrates. PDMS stamps were created with a micropattern of rectangles with dimensions of 240 by 60 µm and spacing of 100 µm between rectangles. All materials were UV-sterilised for 15 min before starting the microcontact printing procedure. Microcontact printing of Matrigel on these flexible substrates was performed in a two-step transfer process. Matrigel dissolved 1:10 in DMEM-F12 (Thermo Fisher Scientific) was transferred from the PDMS stamps to polyvinyl alcohol (PVA) films for 20 min by placing them in contact. The Matrigel was then transferred to the flexible PDMS substrates by placing the micropatterned Matrigel-coated side of the PVA film in conformal contact with the substrates for 30 min. The PVA films were then dissolved by washing 3 times with 4 ml PBS for 5 min, leaving the micropatterned Matrigel pattern on the flexible PDMS substrates. Differentiated cardiomyocytes were dissociated with TrypLE and resuspended in CDM3 medium supplemented with 1x B27 minus insulin (Thermo Fisher Scientific). The cells were then seeded onto the coated Fluorodishes at a density of 50,000 cells/cm².

Cell Classification
The pictures taken as described in the immunocytochemistry section were analyzed using Fiji according to the method previously described by Birket et al.[20] Briefly, hESC-CMs were classified as Class I when the surface area contained more than 50% sarcomeric anti-parallel banding. Class II hESC-CMs contained less than 50% sarcomeric anti-parallel banding. And Class III hESC-CMs contained no significant anti-parallel banding but rather a speckled staining pattern of sarcomeric proteins.

Contraction Analysis
5-7 days after seeding hESC-CMs on micropatterned substrates, DOX treatment was initiated. Cardiac microconstructs were imaged at the appropriate time points using a DeltaVision microscope (GE). Cells were left to acclimatize for 10 min in a climate-controlled chamber at 37 °C with 5% CO₂ prior to imaging. Time lapse images were acquired during 15 s at 50 frames per second. Cardiac microconstructs were randomly chosen for movie acquisition and coordinates of the microscope stage were saved to take time lapse images of the same cardiac microconstructs before and after DOX treatment. Subsequently, the average contractility of the cardiac microconstructs for all contractions during 10-20 s was analysed using the BASiC method as described previously.[21]

Seahorse Mitochondrial Flux Analysis
Differentiated cardiomyocytes were seeded in 24-wells Seahorse assay plates at a density of 100,000 cells/well. Mitochondrial function was determined by means of a Mito Stress test. Briefly, one hour
prior to the assay, medium was replaced by XF assay medium (102365-100, Agilent) supplemented with 10 mM glucose and 1 mM sodium pyruvate and cells were incubated at 37 °C without CO₂. After three baseline measurements, the ATP synthase inhibitor oligomycin (1 μM; 75351, Sigma-Aldrich) was injected, followed by subsequent injection of the uncoupler FCCP (0.5 μM; C2920, Sigma-Aldrich), and complex I and III inhibitors rotenone (1 μM; R8875, Sigma-Aldrich) and antimycin A (1 μM; A8674, Sigma-Aldrich) respectively. Cellular respiration was measured on a Seahorse XF24-3 Analyzer. Oxygen consumption rate (OCR) was normalized for total protein in each well. ATP synthase-linked (ATP-linked) respiration was calculated as the fraction of basal OCR minus the inhibited OCR after oligomycin addition (OCRbasal - OCRoligomycin; i.e. respiration dedicated to the production of ATP). Respiratory reserve was calculated as the capacity of cells to induce OCR beyond basal respiration (OCRFCCP-OCRbasal).

Statistical Analysis

Values are displayed as averages of at least three independently performed experiments. For all graphs: each dot represents results of biologically independent experiments. Differences between two groups were assessed by Student’s t-test, while comparisons between three or more groups were assessed by one-way ANOVA followed by Bonferroni post-hoc test. (Prism v6, GraphPad Software). For qPCR, analysis was performed using GenEx software (MultiD Analyses AB). The following indications of significance were used throughout the manuscript: * p < 0.05, ** p < 0.01.

Results

Cardiac microconstructs (CMCs) were created by microcontact printing Matrigel on soft PDMS substrates in an array of 240 by 60 μm rectangles as described in Chapter 3. Subsequent seeding of a > 95% pure hESC-CM population on these substrates produced aligned and integrated cardiac microconstructs (Figure 2A). Immunofluorescent staining of CMCs for alpha-actinin and nuclei demonstrated the purity of hESC-CMs within the CMCs. Additionally, the advanced integration of sarcomeric structures between hESC-CMs within the CMC is visible (Figure 2B). Further analysis showed that there are an average of 5.7 (standard deviation (SD), 3.1) nuclei present within each CMC (Figure 2C).

Next, we examined the effect of the DOX treatments described in Figure 1 on the contractility of the hESC-CM CMCs using the BASiC method as described in Chapter 2. Videos of the same CMCs were obtained at day 0 and day 7 of the treatment schedule to assess the difference in fractional shortening (FS) and force generation. The increase in FS was 0.45 ± 0.42% in the control group, comparable to the increase in FS after a single low dose of DOX of 0.5 ± 1.15% and the non-significant decrease of 0.35 ± 0.67% in FS after a single medium dose of DOX. Conversely, two low doses of DOX and a single high dose of DOX both did significantly reduce FS, respectively by 1.51 ± 0.44% (p < 0.01) and 2.63 ± 0.34% (p < 0.0001), as compared to control (Figure 3A). A similar pattern was observed in the effect of DOX treatment on force production of CMCs. The total force produced by each CMC was 2.28 ± 0.17 μN and remained similar at 2.49 ± 0.23 μN on day 7, resulting in a non-significant
force increase of $0.21 \pm 0.22 \text{ μN (p = 0.36)}$ over the 7-day treatment period. Compared to control, a single low dose and a single medium dose of DOX did not decrease the force generation. Two low doses and a single high dose did significantly reduce the force generated by CMCs by respectively $0.88 \pm 0.21 \text{ μN (p < 0.001)}$ and $1.78 \pm 0.15 \text{ μN (p < 0.0001); Figure 3B}$.

To investigate whether changes in sarcomeric integrity are involved in causing the impaired contractility after DOX treatments, monolayers of hESC-CMs were co-stained for α-actinin, troponin T and DAPI (Figure 4A). Subsequently, the sarcomeric integrity of hESC-CMs was classified by a previously described method.[20] Class I hESC-CMs contain more than 50% surface area with sarcomeric banding, Class II hESC-CMs contain less than 50% surface area with sarcomeric banding, and Class III hESC-CMs contain no discernible sarcomeric bands but more of a spot-like sarcomeric protein pattern. The control group consisted of 73% Class I hESC-CMs. A single low dose of DOX did not impact the sarcomeric structure of hESC-CMs. Conversely, the sarcomeric structure was severely
disrupted in response to two low doses of DOX, with a decrease of Class I hESC-CMs to 5% of total and a sharp increase in the number of Class III CMs to 43% compared to only 2% Class III hESC-CMs in the control group (p < 0.0001). A single high dose of DOX also induced a sharp decrease to 22% of Class I hESC-CMs (p < 0.0001; Figure 4B).

Figure 3 | Effect of Doxorubicin (DOX) on hESC-CM Contractile Function. DOX treatment at a single low dose and single medium dose did not affect the fractional shortening of CMCs. Conversely, the fractional shortening of CMCs decreased after two low doses of DOX and a single high dose of DOX (A) A similar pattern was observed in the force generation of CMCs in response to the various DOX treatments. ** p<0.01, *** p < 0.001, **** p < 0.0001.

To further characterize the response of hESC-CM monolayers to DOX treatment, we assayed the production of reactive oxygen species (ROS; Figure 5). In comparison to control, all DOX treatment groups except for the single low dose had significantly increased ROS production (p < 0.01). There was no significant difference in ROS production between the single low dose, two low doses, and single medium dose DOX groups. However, the ROS production was significantly higher in the hESC-CMs exposed to a single high dose of DOX than in the other DOX treatment groups (p < 0.0001).

Next, we sought to more specifically quantify the effect of DOX treatments on the viability of hESC-CMs. To this end, we analyzed the cell density and used a Real-Time Glo luciferase assay that quantifies the amount of luminescence based on the reducing potential of a pro-substrate as a proxy for live cell number. When hESC-CMs were exposed to DOX, we found that only with a single high dose of DOX the cell density was significantly reduced (p < 0.0001; Figure 6A-B). Notably, in the luciferase assay the luminescence of hESC-CMs exposed to two low doses of DOX was significantly reduced by 35% (p < 0.0001) even though the cell density was not reduced. Conversely, the single medium dose DOX group received the same cumulative dose as the two low doses DOX group but did not show reduced luminescence as compared to control. Instead, a significant difference between the two low doses DOX group and the single medium dose DOX group was observed (p < 0.0001). hESC-CMs
exposed to a single low dose and a single medium dose of DOX had a luminescence level similar to control (Figure 6C).

**Figure 4** | Effect of Doxorubicin (DOX) on Sarcomeric Structure and Reactive Oxygen Species (ROS).
Immunocytochemistry staining for α-actinin (green), DAPI (blue), and troponin T (red) of hESC-CMs after various DOX treatments, demonstrating a disrupted sarcomeric structure particularly after treatment with two low doses of DOX (A) The sarcomeric integrity of individual hESC-CMs was classified. A significant decrease in the sarcomeric integrity in response to two low doses of DOX and a single high dose of DOX is demonstrated (B) **** p < 0.0001.

**Figure 5** | Effect of Doxorubicin (DOX) on Reactive Oxygen Species (ROS) Generation. Reactive oxygen species were measured using a CellRox Orange Assay, showing increased ROS with two low doses and a single medium dose of DOX, as compared to control. A higher increase in ROS production is observed with a single high dose DOX treatment, as compared to control and the other DOX treatments. ** p<0.01, *** p < 0.001, **** p < 0.0001.
Because we observed changes in ROS production and viability in response to DOX treatments, we opted to further investigate the mitochondrial function of hESC-CMs after exposure to DOX using the Seahorse Mito Stress test to measure the oxygen consumption rate. Interestingly, after treatment of hESC-CMs with two low doses of DOX, both the basal respiration rate and the respiratory reserve were markedly reduced (Figure 7A–C). Consequently, mitochondrial ATP production was also dramatically decreased after exposure to two low doses of DOX (Figure 7C). Overall, the bioenergetic profile of hESC-CMs after treatment with two low doses of DOX closely resembled the results obtained after exposing hESC-CMs to a single high dose of DOX. In contrast, hESC-CMs exposed to a single low dose of DOX had a mitochondrial function similar to untreated control cells.

Figure 6 | Effect of Doxorubicin (DOX) on Viability. Brightfield images displaying monolayers at D7 of the various DOX treatments, clearly demonstrating reduced cell density after a single high dose of DOX (A) Quantification of the cell density after DOX treatments (B) Quantification of viability data with Real-Time Glo assay (C) luminescence units (LU) are shown. **** p < 0.0001.
Figure 7 | Effect of Doxorubicin on hESC-CM Mitochondrial Function. Representative traces for control cardiomyocytes and cardiomyocytes treated with doxorubicin (DOX) according to the treatment schedule in a Seahorse Mito Stress test (A) Basal respiration rate and spare respiratory capacity are shown (B) Mitochondrial membrane proton leak and ATP production are shown (C) OCR, oxygen consumption rate. * p < 0.05.

Discussion

Here, using a hESC-CM based model for DIC, we have demonstrated the differential responses to single doses of DOX in low, medium, and high concentrations, and repeated low doses of DOX. We showed that the fractional shortening and force generation of CMCs is lowered substantially by repeated low doses of DOX, but not by a single low dose or single medium dose of DOX. We then further investigated the cause of these differential effects of DOX on contractility and found that while a single low dose of DOX did not affect sarcomeric structure and viability, repeated low doses of DOX did severely impair the sarcomeric structure and viability. Importantly, this could not be explained by a cumulative dose difference between a single low dose of DOX and repeated low doses of DOX. A single medium dose of DOX containing the same cumulative dose as repeated low doses of DOX did not affect the viability or contractile function of these cells, similarly to a single low dose of DOX instead of repeated doses of DOX. ROS production was increased in response to all DOX treatments except for the single low dose of DOX but much more pronounced in the single high dose of DOX group. Finally, further investigation of the metabolic profile with the SeaHorse Mito
Stress test showed that repeated low doses of DOX induced a heavily compromised metabolic profile similar to that found after a single high dose of DOX, while a single low dose of DOX was similar to control. Together, these results explain the functional impairment of the cardiac microconstructs in response to DOX. Notably, these results also shed light on the origin of the significant difference in contractile impairment between a single dose of DOX and repeated doses of DOX. Interestingly, repeated doses of DOX induced far more cardiotoxicity than a single dose of DOX. After repeated doses of DOX we observed significantly more sarcomeric disintegration, significantly reduced viability, reduced mitochondrial function and consequently reduced contractile function of hESC-CMs, as compared to a single dose of DOX. This is likely due to an accumulation of damage caused by the repeated injuries in response to repeated DOX exposure. Our data indicates that upon repeated exposure to DOX hESC-CMs eventually reach a threshold after which repair mechanisms are no longer able to sustain normal mitochondrial function, leading to sarcomeric disintegration and subsequently impaired contractile function. In the experiments performed we observed some degree of intercell and interconstruct variability in the response to DOX treatment. It is therefore likely that with each exposure to DOX, new hESC-CMs reach the threshold towards dysfunction while the function of already dysfunctional hESC-CMs deteriorates even further.

Notably, repeated low doses of DOX more closely resemble the in vivo situation during cancer treatment of patients, whom are typically treated with DOX between six to eight times with cardiac tissue exposure at a similar low dose of 0.1 μM as the one used in this study. Our data demonstrates the importance of replicating this clinically used dosing regimen with repeated exposures to DOX. A clear limitation to other studies is that either supraclinical doses of DOX were used or only a single dose of DOX was administered.[5-7,11-14] The data presented here using a DOX treatment that more closely resembles the treatment of cancer patients, suggests that the primary driver of DOX induced cardiomyopathy is impaired contractile function of CMs due to mitochondrial dysfunction and sarcomeric disintegration rather than loss of CMs.

Exceptions to the increased cardiotoxicity upon repeated exposure to DOX were the cell density and ROS production. However, ROS production is likely a transient response to DOX treatment and subsides after the damaging stimulus is removed. Therefore, any measurements of ROS production provide a snapshot of current ROS production rather than being a reflection of accumulated ROS production. In this light, the fact that there was no difference observed in ROS production between the groups receiving a single low dose and repeated low doses of DOX is due to the transient nature of ROS production. Conversely, the effects of DOX on viability, metabolic activity and contractile function are more lasting and thus provide a better measure of the accumulated injuries caused by DOX treatment.

As expected, a high dose of DOX induced large amounts of ROS production, leading to cell death. In the remaining cells there was severely impaired mitochondrial and contractile function. This data is in accordance with a previously published report by Maejima et al., who found that a dose of 1 μM DOX induced oxidative stress and apoptosis of neonatal rat CMs, but a lower dose of 0.1 μM no longer induced apoptosis although oxidative stress was still present.[7] Importantly, our model for DIC demonstrated heavily reduced fractional shortening and force generation of CMCs in both the repeated low doses and a single high dose of DOX groups. This
is in accordance with the impaired contractility found in a mouse model of DIC.[6] Moreover, the fractional shortening of CMCs is a proxy for ejection fraction, which is markedly reduced in patients affected by the cardiotoxicity of DOX whom go on to develop a DOX induced cardiomyopathy.[4] A limitation to the current study is that we were not able to fully recapitulate the clinical dosing regimen. During cancer treatment, patients typically receive six to eight doses of DOX over a period spanning several months. In replicating this dosing regimen we only used two doses over the period of 1 week because this was a more practical approach. If a dosing regimen was used that fully resembles the clinical situation, this study would be also better suited to study the long-term effects of exposure to DOX. However, we were still able to determine the importance of repeated dosing versus a single dose of DOX.

To summarize, we have developed a human model of DIC by studying the effects of DOX on hESC-CMs in single doses at various concentrations and with repeated clinical dose exposure. Both high dose and repeated dose exposure to DOX induced a phenotype of DIC with severely impaired mitochondrial function and contractility. However, only after a single high dose of DOX did we observe significant amounts of cell death. The repeated low dose exposure most closely resembles the dosing regimen of DOX received by patients during cancer treatment. Therefore, we conclude that impaired mitochondrial dysfunction and subsequent sarcomeric disintegration, but not cell death, might play pivotal roles in causing DOX induced cardiomyopathy. However, further studies are needed to solidify these findings. For example, a study using an even more extensive dosing regimen that fully replicates the dosing regimen used in cancer treatment of patients would be interesting to explore.
References

## Supplementary Material

**Supplementary Table 1 | An Overview of the Primary and Secondary Antibodies Used in this Study.**

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