Video Article

A Doxorubicin-induced Cardiomyopathy Model in Adult Zebrafish

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URL: https://www.jove.com/video/57567
DOI: doi:10.3791/57567

Keywords: Medicine, Issue 136, Doxorubicin, cardiomyopathy, acute phase, chronic phase, zebrafish, intraperitoneal injection, disease model

Date Published: 6/7/2018

Citation: Ma, X., Ding, Y., Wang, Y., Xu, X. A Doxorubicin-induced Cardiomyopathy Model in Adult Zebrafish. *J. Vis. Exp.* (136), e57567, doi:10.3791/57567 (2018).

Abstract

The genetically accessible adult zebrafish (*Danio rerio*) has been increasingly used as a vertebrate model for understanding human diseases such as cardiomyopathy. Because of its convenience and amenability to high throughput genetic manipulations, the generation of acquired cardiomyopathy models, such as the doxorubicin-induced cardiomyopathy (DIC) model in adult zebrafish, is opening the doors to new research avenues, including discovering cardiomyopathy modifiers via forward genetic screening. Different from the embryonic zebrafish DIC model, both initial acute and later chronic phases of cardiomyopathy can be determined in the adult zebrafish DIC model, enabling the study of stage-dependent signaling mechanisms and therapeutic strategies. However, variable results can be obtained with the current model, even in the hands of experienced investigators. To facilitate future implementation of the DIC model, we present a detailed protocol on how to generate this DIC model in adult zebrafish and describe two alternative ways of intraperitoneal (IP) injection. We further discuss options on how to reduce variations to obtain reliable results and provide suggestions on how to appropriately interpret the results.

Video Link

The video component of this article can be found at https://www.jove.com/video/57567/

Introduction

Doxorubicin (DOX), also named Adriamycin, has been developed as an anti-neoplastic drug since the 1960s[1,2]. It is now still actively used as an important chemotherapeutic drug for a broad spectrum of tumors. However, clinical application of DOX was hampered by its dose-dependent toxicities, especially cardiotoxicity characterized by variable symptoms ranging from asymptomatic electrocardiographic changes to pericarditis and decompensated cardiomyopathy[1,2]. To date, at least three major hypotheses have been raised to explain DIC, including activated reactive oxygen species (ROS)[1,3,4,5], inhibition of topoisomerase II-β (TOP2β)[6,7], and modulation of intracellular calcium release[1,8,9]. Accumulating evidence also suggests genetic predisposition as a pivotal risk factor for DIC[10,11,12,13]. Gene identities related to these DIC predispositions, however, remain largely unknown. Dexrazoxane is the only adjuvant agent approved by the US Food and Drug Administration (FDA) to treat DIC, but with limited implementation[14,15,16], underscoring the need to identify additional therapeutic strategies. Animal models of DIC are therefore explored for these purposes. Owing to their accessibility and simplicity, mechanistic studies on DIC models could potentially have broader impacts on other types of cardiomyopathies: common pathogenesis might be shared among cardiomyopathies of different etiologies, especially at later pathological stages[17,18,19,20].

In addition to rodent models of DIC, zebrafish DIC models with higher throughput have been developed to facilitate the discovery of both new genetic factors and therapeutics. An embryonic DIC model has been established in the transparent zebrafish embryos for screening therapeutic compounds[21]. Given that the cardiomyopathies are adult onset diseases with a progressive pathogenesis, adult zebrafish cardiomyopathy models have been developed[22,23,24,25,26]. We generated the first acquired model for cardiomyopathy resulting from chronic anemia[24], followed by DIC as the second acquired cardiomyopathy model in adult zebrafish[23]. We found that injection of a single bolus of DOX into adult zebrafish induces cardiotoxicity that consists of an acute phase roughly within 1 week post-injection (wpi), followed by a chronic phase of cardiomyopathy up to 6 months post-injection. While haploinsufficiency of the mechanistic target of rapamycin*(mtor)* ameliorates cardiomyopathy at the chronic phase, it exaggerates fish mortality at the acute phase, underscoring the value of the adult DIC model to discern stage-dependent mechanisms[23]. We further demonstrated that the adult DIC model can be used to stress a collection of zebrafish insertional cardiac (ZIC) mutants that are being generated via a transposon-based insertional mutagenesis approach[27]. A pilot screen identified 3 known cardiomyopathy genes as well as DnaJ (Hsp40) homolog, subfamily B, and member 6b (*dnajb6b*) as new DIC susceptibility genes[28]. Therefore, the generation of the adult DIC model in zebrafish led to a new methodology that systematically enables identification of genetic modifiers for DIC, which complements the existing genome-wide association study (GWAS) and quantitative trait locus (QTL) analysis.

During the generation and implementation of the adult zebrafish DIC model, we noticed significant variations among different researchers and/or even among different injections performed by the same investigator. The longitudinal nature of the model imposes challenges to registering results from different investigators and to the sequential troubleshooting process. To facilitate the use of this simple cardiomyopathy-inducing stress method by the research community, we describe our protocol in detail, present two types of IP injection, and discuss considerations to reduce variations among different researchers.

Protocol

All procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Academies Press. 2011), and they were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

1. Adult Zebrafish Preparation

1. Set up sufficient breeding pairs in crossing tanks to acquire at least twice as many as the total fish needed for DOX injection. If comparing fish with different genetic backgrounds, breed all fish within the same week to ensure age-matched controls.
2. Collect the fish embryos the next morning, transfer them to 100 mm Petri dishes, and keep them in a 28.5 °C incubator. Keep embryos at a low density (<100 embryos/Petri dish).
3. Refresh the embryo water daily to avoid sex imbalance, and manually remove dead eggs in a timely fashion using a transfer pipette.
4. Put the same number of embryos in each tank (for example, 60 embryos/3 L medium tank initially) to ensure density-matched controls.
5. Start paramecia feeding at 4 days post-fertilization (dpf).
6. Inspect the fish daily during the juvenile stage. Adjust the fish number as needed to ensure similar fish density.
7. When fish reach 4 weeks of age, transfer up to 20 fish into each new 3 L medium tank for further growth. Start to feed the fish with live hatched brine shrimp.

2. Preparation and Storage of DOX Stock Solution

NOTE: DOX can be purchased from various bio-companies. The compound is usually acquired as a powder in dark brown containers.

1. Thoroughly dissolve the DOX powder in de-ionized water to ensure no clumps are visible, with a final concentration of 5 mg/mL as the stock solution. Aliquot 1 mL of DOX stock into each 1.5 mL safe-lock tube. Wrap the 1.5 mL tubes with aluminum foil paper to protect the DOX from light exposure.
   NOTE: Perform this step in a chemical hood.
2. Keep the DOX stock solution at 4 °C for storage. For long-term storage (>4 weeks) of DOX stock solution, perform the optional Section 3 described below.

3. Quality Control of DOX Using Zebrafish Embryos (Optional)

NOTE: DOX is both moisture- and light-sensitive, so it can lose its drug efficacy for modeling DIC after extended storage. For DOX purchased from different companies, or even different batches from the same company, it is useful to calibrate its drug efficacy using wild-type (WT) zebrafish embryos before conducting the experiments on adult fish. This method is derived from a reported zebrafish embryonic DIC model[21].

1. Collect WT zebrafish embryos from at least 2 pairs of fish. Dechorionate embryos at 24 h post-fertilization (hpf) manually by using a syringe with a micro needle. Alternatively, treat the embryo with proteinase K at final concentration of 10 µg/mL for 10-15 min in a 30 °C incubator. Refresh the embryo water after dechorionation. Remove dead embryos and maintain at least 36 embryos of each batch.
2. Dilute the DOX stock solution in fresh embryo water to a final concentration of 100 µM. The volume of solution is 100 µL for every 3 embryos. Mix the diluted DOX solution by vortex. The final diluted solution should be a light, red color.
3. Add 100 µL/well of diluted DOX solution of a clean 96-well transparent plate.
4. Take 3 dechorionated embryos with a plastic transfer pipette, and keep the embryos close to the end of the pipette tip. Put the pipette tip into each well with DOX solution. Let the end of the tip touch the solution and allow the embryos to swim into the well.
   NOTE: Avoid manually pushing the embryos, which will add more water into the well and dilute the DOX solution.
5. Refresh the DOX solution at 48 hpf. By this time, observe the wells under a microscope with 10X magnification to identify dead embryos (cessation of heartbeat) or embryos with edema. Count and remove any dead embryos in a timely fashion, otherwise the remaining embryos exposed to the solution with dead embryos can die quickly as well.
6. Check the embryos at 72 hpf and count them. The DOX treatment is considered "good drug efficacy" if >25% death (cessation of heartbeat) can be observed in both batches of embryos.

4. Pre-injection Preparation

NOTE: Fish of 8 weeks to 6 months of age are used for DOX injection. The body weights (BWs) of a matured Wild Indian Karyotype (WIK) fish to be injected can range from 0.2-0.5 g.

1. Fast the fish for 24 h before injection.
2. Anesthetize the fish with embryo water containing 0.16 mg/mL tricaine. Use a clean filter paper to dry the water from both sides of the body. Measure the BW of each fish on a scale. Group fish within 10% difference in BW together for later injection.
   NOTE: To minimize the workload at this step, fish within 10% difference in BW are considered the same size; therefore, prepare one DOX working solution according to their mean BW.
3. Plan to inject each adult fish with 5 µL of solution. Calculate the DOX working concentration according to the fish numbers and BWs.

NOTE: To study chronic cardiotoxicity up to 6 months, use DOX at a dose of 20 mg/kg. To study acute cardiotoxicity of DOX, the DOX dose can be increased up to 50 mg/kg of BW.

4. Dilute the DOX stock in 1x Hank's balanced salt solution (HBSS) for corresponding working concentrations. Vortex to mix the solution. Briefly spin down to collect the solution.

5. DOX Injection in Adult Fish

1. Place a clean 100 mm Petri dish with a sponge inside it, under a dissection microscope, then adjust the focus. Cut the sponge to make a cavity of about 4 cm in length to hold a fish. Make a longer cavity for a larger fish.
2. Prepare a 34 G needle with a 10 µL micro-syringe. Rinse the needle with 1x HBSS buffer to remove any bubbles and blocks from the syringe and tubing.
3. Anesthetize the adult fish in embryo water containing 0.16 mg/mL tricaine for 2 min.
   NOTE: Prolonged anesthetization over 5 min followed by DOX injection can easily cause fish death.
4. Soak the sponge in embryo water with tricaine, and transfer the fish onto the sponge for injection.
5. **Perform IP DOX injection by either of the two methods described below.**
   1. Classic IP injection[29]
      1. Position the fish with the abdomen up in the cavity of the sponge. Quickly insert the needle, with a 45° angle to the fish body into the midline between the pelvic fins, and penetrate approximately 1-2 mm. Release all DOX solution slowly. Wait 5 s before pulling out the needle. Check the DOX delivery by a visible red color in the fish belly.

   2. Alternative IP injection
      1. Place the fish laterally onto the sponge with the anterior to the right. Gently stabilize the fish using a blunt end forceps with the left hand, and hold the micro-syringe with the right hand.
      2. Position the needle below the lateral line above the pelvic fin, with the bevel facing up. Pointing at the 7 o'clock position at a 45° angle, insert the needle 3-4 mm to the fish cavity located between the pelvic and the anal fins, and then slowly depress the plunger. Check the DOX delivery by a visible red color in the fish belly.

6. Quickly transfer the injected fish to a clean crossing tank filled with fresh system water to allow the fish to recover. Rinse the needle once with 1x HBSS buffer between injections.

6. Post-injection Fish Management

1. After injection, return the fish to the system with running circulation. If possible, maintain DOX-treated fish separately from the main system to avoid cross-contamination among different tanks that share the circulation.
2. Fast the injected fish for another 24 h for recovery. Observe the fish daily during the first week. Remove the dead fish in a timely fashion to avoid infection to the other fish.
   NOTE: Fish deaths within the first 24 h are likely due to physical lesions caused by the injection.
3. Further maintain the DOX-stressed fish for longitudinal observations. Remove dead fish in time to avoid infections to other fish in the tank.
   NOTE: Fish numbers are documented to generate a survival curve.
4. Use different experimental assays to phenotype the DOX-stressed fish, such as echocardiography[30], cardiac function reporter transgenic line[23], swimming challenge[26], and quantification of other pathological remodeling markers[23].

Representative Results

Here, two methods to perform IP injection to model DIC in adult zebrafish are presented. While using the classic, established IP injection method[29], it was noted that the injected DOX solution (red color) could sometimes ooze out from the location where the needle penetrated. The alternative IP injection uses a different location for needle penetration that is 3-4 mm away from the peritoneum where the DOX is released (**Figure 1A**), which effectively prevents the leakage (**Figure 1B**, **1C**). Successful delivery of DOX into the peritoneum for both methods is evidenced by rapid distribution of the red color throughout the fish belly, which is visible on the opposite side of the injection locus.

Injection of DOX dosed at 50 mg/kg using the alternative IP method leads to severe toxicity, where the majority of fish die within one week (**Figure 2**). By contrast, injection of DOX dosed at 20 mg/kg using the alternative IP method causes almost no fish death during the first 2 weeks and ~10% fish death at 4 wpi (**Figure 3A**). Injection of DOX dosed at 20 mg/kg using the classic IP method exhibits ~30% fish death at 4 wpi (**Figure 3C**). Fish injected with either method exhibit a similar ~20% fish death from 4 wpi to 10 wpi (**Figure 3B, D**).

We have leveraged the *casper; Tg(cmlc2:nusDsRed)* fish to assess the progression of cardiac dysfunction in the DIC model (**Figure 4A**)[23]. The transparent body enables documentation of a red heart at both systolic (**Figure 4B**) and diastolic (**Figure 4C**) stages under a florescent microscope. After injection of 20 mg/kg DOX using the alternative IP method, ventricular function decline can be detected starting from 4 wpi (**Figure 4D**).

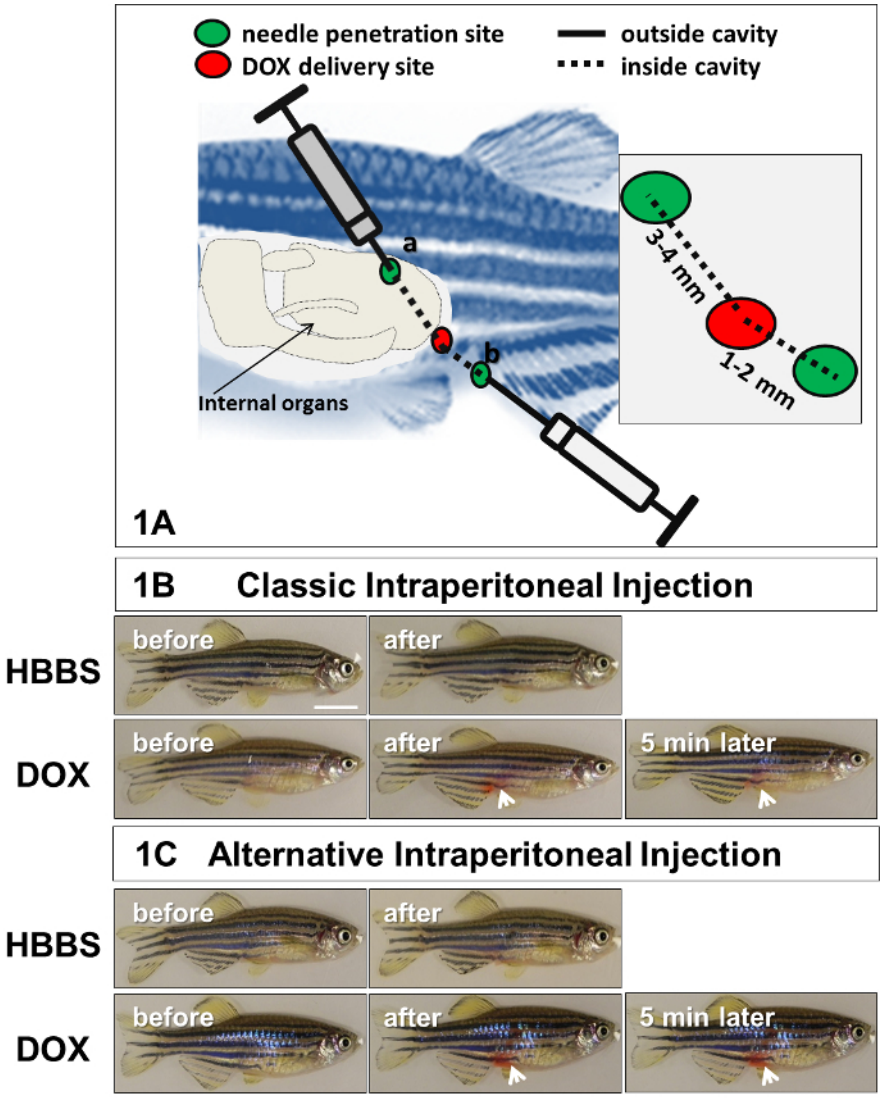

Figure 1: Injection routes. (**1A**) Schematics of the classic IP injection (b) and alternative IP injection (a) methods. The red circle indicates the common DOX releasing site for the two injection methods. Green circles indicate the needle penetration sites. The distances are estimated based on an adult WIK fish weighing approximately 0.3 g. (**1B**, **1C**) Representative results indicating a successful DOX delivery using two intraperitoneal injection methods. Redness inside the adult fish belly can be noted immediately after the injection. Injected fish were checked again after 5 min recovery in a fresh system water. HBSS: 1x Hank's balanced salt solution. WT WIK zebrafish were employed. Scale bar: 5 mm. Please click here to view a larger version of this figure.

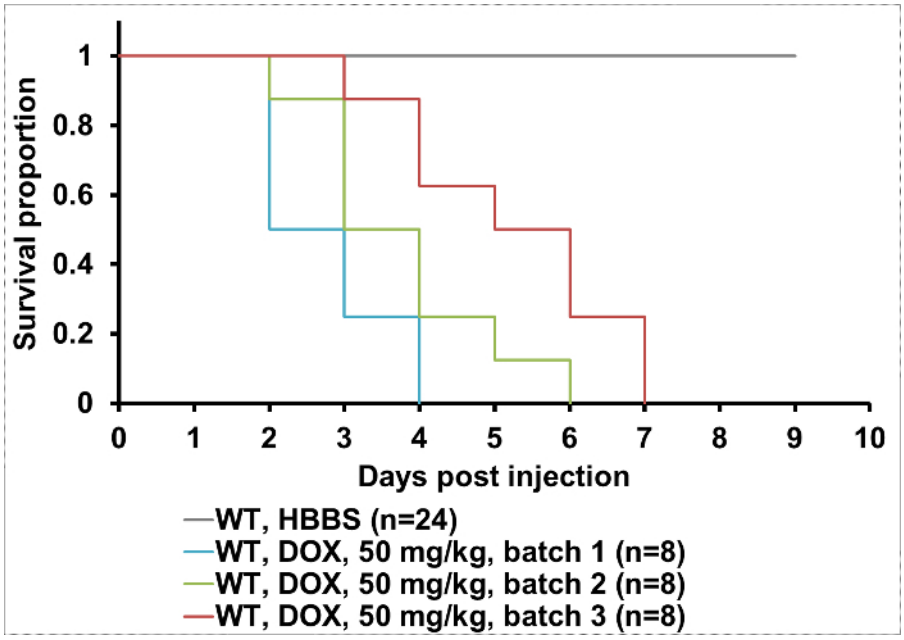

Figure 2: Representative survival curves of adult zebrafish following DOX stress with 50 mg/kg DOX injection. Shown are 3 sets of DOX injections in different batches of WT adult fish at 3-6 months of age. In total, n = 24 fish were employed in the 1x HBSS control group, and n = 8 fish were employed in each batch injected with DOX. No difference in fish survivals were noted between the two injection methods. WT WIK zebrafish were employed. Please click here to view a larger version of this figure.

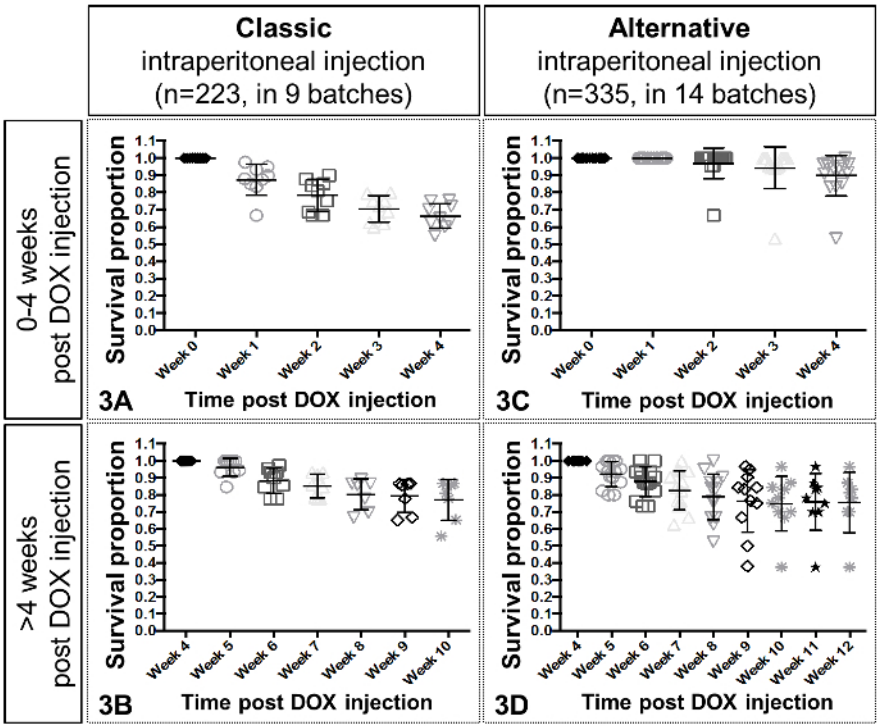

Figure 3: Representative survival curves of adult zebrafish following DOX stress with 20 mg/kg DOX injection and the comparison between two intraperitoneal injection methods. (**3A**) Fish survival within 0-4 weeks following DOX delivery by classic intraperitoneal injection. Percent fish number at week 0 was regarded as 100%. (**3B**) Fish survival after 4 weeks post DOX delivery by classic intraperitoneal injection. Percent fish number at week 4 was regarded as 100%. (**3C**) Fish survival within 0-4 weeks following DOX delivery by alternative intraperitoneal injection. Percent fish number at week 0 was regarded as 100%. (**3D**) Fish survival after 4 weeks following DOX delivery by alternative intraperitoneal injection. Percent fish number at week 4 was regarded as 100%. Data shown in (**3A**) and (**3B**) are 9 different batches of DOX injection from a total 223 injected fish at 3-6 months of age. Data shown in (**3C**) and (**3D**) are 14 different batches of DOX injection from a total 335 injected fish at 2-6 months of age. The numbers of live fish are recorded weekly. Error bars represent standard deviation in percentage of survival among different batches at each week. WT WIK zebrafish were employed. Please click here to view a larger version of this figure.

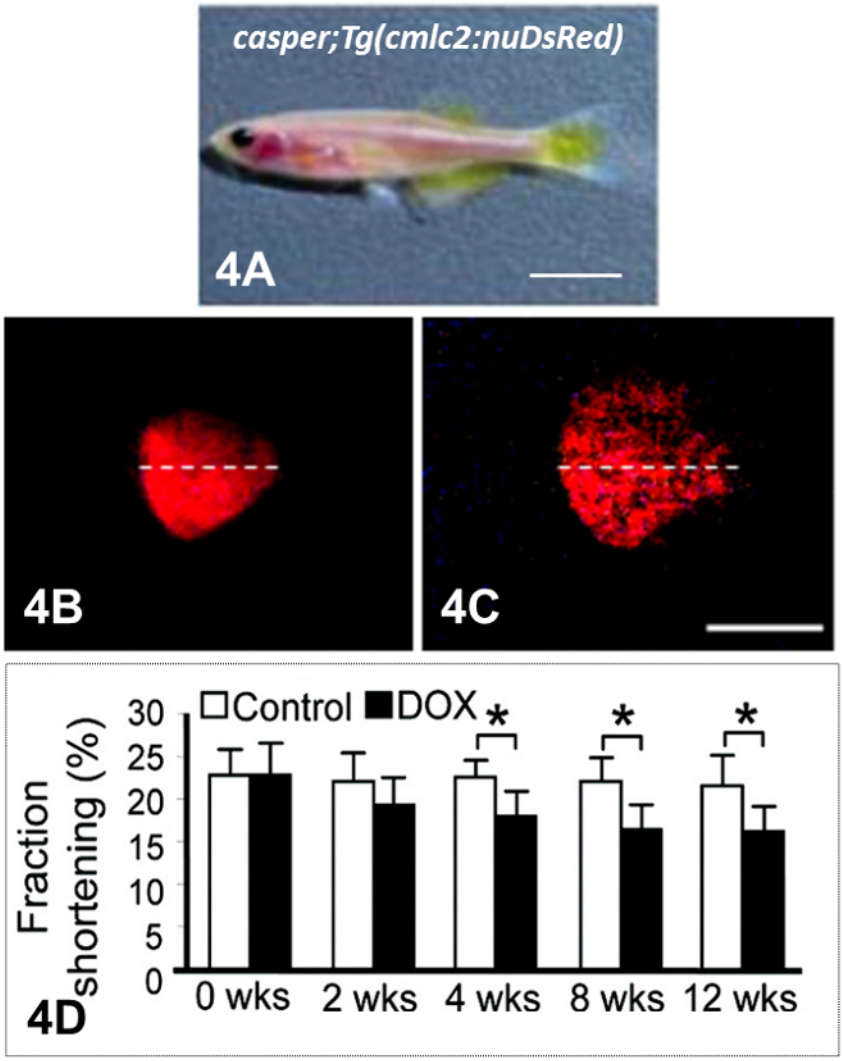

Figure 4: Representative cardiac function assessment following DOX stress using *casper; Tg(cmlc2:nusDsRed)* fish. (**4A**) A photograph of an adult *casper; Tg(cmlc2:nusDsRed)* fish. Scale bar = 1 cm. (**4B**) Representative image of a red ventricle at the end systolic stage of a matured *casper; Tg(cmlc2:nusDsRed)* fish. Dash line represents end systolic diameter (ESD). (**4C**) Representative image of a red ventricle at the end diastolic stage of a matured *casper; Tg(cmlc2:nusDsRed)* fish. Images in (**4B**) and (**4C**) were extracted from movies of a beating heart captured with a fluorescent dissection microscope using 6.3x magnification. Dash line represents end diastolic diameter (EDD). Scale bar = 1 mm. (**4D**) Representative cardiac function measured using *casper;Tg(cmlc2:nuDsRed)* adult zebrafish after 20 mg/kg DOX injection. EDD and ESD were measured as shown in (**4B**) and (**4C**) for individual fish, and ventricular fraction shortening is calculated by the formula (EDD − ESD)/EDD. Significantly decreased ventricular fraction shortening was detected at 4 weeks and thereafter. The alternative intraperitoneal method was used. Values are shown as the mean ± standard error. n ≥ 3 in each group. The Student *t* test was used for comparison of two groups. **p* <0.05. These figures have been modified from Ding *et al.*[23] Please click here to view a larger version of this figure.

Discussion

To model a progressive DIC, the dose of 20 mg/kg DOX was determined experimentally as the highest dose that does not cause significant fish death during 1 wpi but still results in fish death and the reduction of cardiac function after 4 wpi (**Figure 3** and **Figure 4C**). This dose is comparable to those frequently used in rodent DIC models (15-25 mg/kg) and to the limit cumulative dose in humans (550 mg/m^2, which is equivalent to 15 mg/kg)[4,7,31,32,33]. Higher doses of DOX, such as 50 mg/kg, show significant fish death during 1 wpi, so they can be used to study only acute cardiac toxic responses to DOX[23,28].

During the implementation of this DIC model, we noticed that it was difficult for new investigators to reproduce the same results initially. Even for experienced investigators, about a 20% variation of mortality in the chronic phase can still be found among different injections (**Figure 3**), indicating uncharacterized biological confounding factors in the current model. Despite a less than perfect model, we still believe that this current DIC model is sufficient to make sound discovery because of the following evidence. First, after several practices, the results from the DIC model can be later registered among most, if not all, investigators. Second, based on this DIC model, we identified 4 meaningful genetic modifiers. Existing evidence from literature reports supported 3 of them as cardiomyopathy genes[34,35,36,37]. The 4th one is *DnaJ homolog subfamily B member* 6 (*DNAJB6*), which has been proven to be a new cardiomyopathy gene supported by a human genetic study[28]. Thus, we conclude that

results based on intra-experimental groups using stringently age-matched controls are still highly repeatable, albeit the DIC model in its current form is not reliable for inter-experimental comparison.

The confounding factors that contribute to the inconsistency observed in our current DIC model likely include the following: (1) Aging and gender-difference have been regarded as crucial risk factors for cardiovascular diseases including DIC[38,39,40]. While both hypothesized cofounders remain to be tested in our model specifically, we did notice that aged fish tend to be more sensitive to DOX toxicity (data not shown). The inter-batch variations observed using both IP methods (**Figure 3**) are also likely contributed to these two cofounders. (2) Different from larger animal models, the size of an adult zebrafish is small. Thus, local damage caused by DOX injection can be more severe and variable. (3) Death of injected fish might result from DOX toxicity in other organs (*e.g.*, renal toxicity), in addition to the heart. While more carefully designed experiments are needed to address each of the aforementioned confounding factors, previous work suggests that the following cautions will help reduce phenotypic variations: First, it is critical to guarantee the efficacy of DOX. DOX powder should always be kept in a dry and dark area, and solutions should be handled with care to reduce exposure to light. It is recommended that DOX working solutions be prepared freshly each time before fish injection. We typically do not use stock DOX solutions after 4 weeks in storage. When there is a doubt, perform the optional Section 3 in this protocol using the quick embryonic DIC model to calibrate the drug efficacy of each DOX batch. Second, adult fish synchronization is pivotal. The same fish strain needs to be raised at the same density, to ensure similar body size. We then pre-select fish with similar BWs for dose calculation. It is recommended to maintain at least twice as many fish in total for this pre-selection process. All fish are fasted for 24 h before the pre-selection process. Third, it is recommended to always using fish of similar age, and to sex the fish before DOX injection because of their different growth rates and potentially different cardiac responses to DOX.

We noticed that the majority of inconsistent conclusions could be ascribed to a questionable control group. Therefore, we recommend investigators new to the DIC model to practice DOX injections before conducting real experiments. When 20 mg/kg DOX is injected using the alternative IP injection method, a good injection technique can be indicated by a nearly zero fish death during the 1 wpi of DOX and a relatively consistent mortality rate at 2-3 months post-DOX injection. The ultimate evidence for successful modeling is the reduction of the cardiac-functional index, which could be quantified via echocardiography[25,30], by using a *casper; Tg(cmlc2:nusDsRed)* transgenic line, or by using a newly developed *ex vivo*-based cardiac function assay (data not shown).

In addition to the IP injection, other drug delivery routes such as retro-orbital injection[41], oral feeding[42], and water incubation are also frequently used in adult zebrafish. We did not adopt the retro-orbital injection approach, despite its direct drug releasing into the circulation system, due to the lack of a method to validate successful drug delivery, as the redness of DOX can be easily masked by potential bleeding. We tried an oral delivery protocol through embedding DOX with absorbable gluten[42], which was mixed with meals to feed adult fish. Unfortunately, with up to the accumulative dose of 150 mg/kg used within a 4-week feeding period, we did not observe any severe cardiotoxicity, suggesting the ineffectiveness of oral DOX delivery. Alternatively, an oral gavage technique could be further explored[43]. Moreover, the incubation protocol of soaking fish in a solution containing DOX is also a potential delivery route that could be tested in the future.

We acknowledge that one of the major limitations of the current DIC model is the single bolus injection method, which could result in high toxicity and local damage to the internal organs. This approach was designed to reduce workloads and to increase throughput, so that genetic screening can be conducted at a high throughput in adult fish[28]. In the future, models with multiple injections of DOX at lower doses should be pursued, which will better recapitulate the DIC observed in cancer patients treated with chemotherapy.

Disclosures

The authors declare no conflicts of interests.

Acknowledgements

This work was supported in part by a Scientist Development Grant from the American Heart Association (14SDG18160021 to YD), the US NIH R01 grants HL 81753 and HL 107304 to XX, and the Mayo Foundation to XX.

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