Study on Doxorubicin Induced Cardiotoxicity in Adult Zebrafish Model Using High Frequency Echocardiography

使用高頻心臟超音波於成年斑馬魚模型研究化療藥物 Doxorubicin 誘發之心毒性

1. Abstract：

Zebrafish as an excellent non-mammalian vertebrate model for studying human cardiovascular disease, have a limited access to accurate assessment of cardiac function due to its opaqueness in the adulthood. Traditional method used for detecting the adult zebrafish heart is the electrocardiogram (ECG). [1, 2] Nevertheless, the ECG signal is obtained by inserting needles into the fish body, which might lead to injuries and death to experiment animals. Echocardiography, with its doppler properties of the wave, can counter this problem by penetrating through the tissue and visualizing the heart structure and measuring blood flow velocity non-invasively. [3, 4, 5]

In this paper, I aim to investigate the related myocardial functions of DIC model in the adult zebrafish.by using high frequency echocardiography in order to extend our understanding of the side effects induced by chemotherapy.

The ultrasound-based architecture was set up step by step in the paper by using a 18MHz transducer. Then, after 4 weeks treatment of the DIC model, the DOX-treated group reached half of the deaths and shows significant decrease in heart rate. The E/A ratio remains unchanged, which shows no diastolic dysfunction. However, the worsening of global cardiac function of the DOX-treated group was characterized by high MPI ratios comparing to DMSO group at 28dpi (2.345 ± 1.294 and 0.7793 ± 0.2911, $P < 0.05$).

The findings of this proposal can be more in line with the 3R principle, which can reduce animal suffering in experiments. Moreover, the findings can provide a state-of-art platform to extend our understanding of the side effects induced by cancer drugs and have great potential in the field of cardiovascular measurement in adult zebrafish.

2. Content：

2.1. Background/Motivation:

There are many ways to investigate cardiac response in zebrafish, including electrophysiology (ECG), cryosectioning, and flow cytometry. However, their invasive property might lead experimental animal to death. In contrast, echocardiography imaging has no such issues and can present deep penetration depth non-invasively, which can measure the cardiac function in vivo.

The echocardiography techniques are particularly challenging in the adult zebrafish model due to its small size of heart.[3] Parameters and fixation skills were tried out to set up the high frequency echocardiography. The construction allows visualization of adult zebrafish heart and quantification of vital cardiac parameters, including heart rate, E/A ratio and MPI.[4, 5]

Doxorubicin (DOX) is a commonly used anticancer agent in treating malignant tumors, including breast cancers, soft tissue sarcomas, and leukemia. Moreover, multiple injections of DOX are used clinically in the chemotherapy. However, previous research shows that the induced toxicity and the cumulative dose would increase the risk of heart failure highly.[6, 7] Thus, I plan to investigate the DIC model in the adult zebrafish.by using high frequency echocardiography in order to extend our understanding of the side effects induced by chemotherapy.

2.2. Purpose:

I plan to verify the feasibility of the high frequency echocardiography and investigate the related cardiac functions of DIC model in the adult zebrafish in order to extend our understanding of the side effects induced by chemotherapy.

2.3. Method:

Three steps were needed to meet the experiment goal. First, I will establish a high-frequency ultrasound-based technique with a 18MHz transducer to detect the adult zebrafish. Second, I will generate a doxorubicin induced cardiotoxicity (DIC) model in adult zebrafish by multiple injections intraperitoneally. Finally, I will quantify important functional parameters of the zebrafish heart by the pulse wave (PW) doppler mode when coupled with the use of the other two modes of echocardiography, B-mode and color doppler mode.

My research methods would include doxorubicin induced cardiotoxicity (DIC) model generating, and echocardiography imaging.

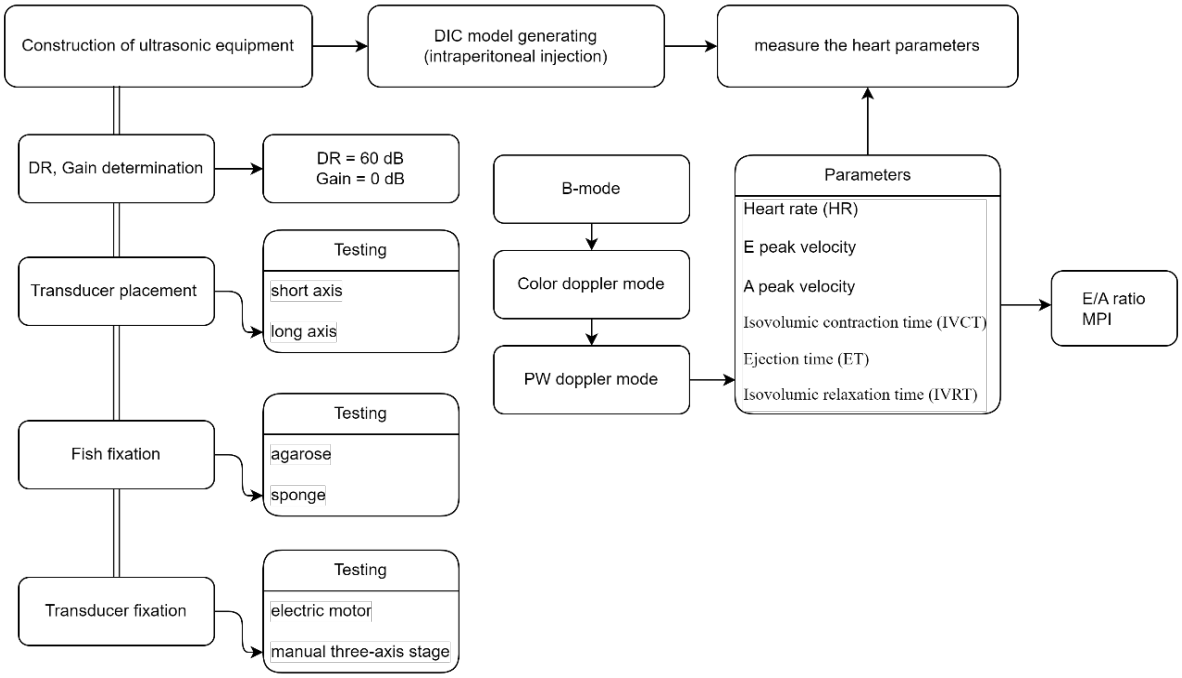

Fig.1 The flow chart of the experiment.

2.3.1. System setup of the experiment

In order to measure the blood flow of the fish heart, it is necessary to first establish an ultrasonic measurement device. After trying different ultrasonic parameters, frequency was set at 16.5Mhz, dynamic range (DR) at 60db and gain set at 0db to do the following measurements. As to the transducer displacement, fish fixation tools, and transducer fixation tools, the transducer was placed at the long axis parallel to the zebrafish midline by a manual three-axis stage and the fish was fixed with a damp sponge.

After anesthetizing, the adult zebrafish was clamped in a damp sponge with its abdomen side up in a tank. The Y-shaped incision in front of the sponge allows the gills of the zebrafish to move so as to keep it breathing. Due to the fact that sound waves cannot penetrate through air, the tank will be filled with tricaine and the transducer will be immersed into the solution approximately 1cm.

A 18Mhz ultrasonic transducer was used to assess the B-mode image, color doppler images and the PW doppler images at a frequency of 16.5MHz. The blood flow of the atrioventricular valve of the zebrafish heart was recorded. Through the measured heart rate, E/A ratio and MPI values, the changes in myocardial function are judged to enhance the understanding of the cardiotoxicity of cancer drugs in adult zebrafish model.

2.3.2. Experiment protocol

Both male and female zebrafish were randomly separated into control group and DOX-treated group. The control group was treated with 0.1% DMSO while the DOX-treated group was treated with DOX 20mg/kg. Doses of DOX was calculated based on the weight of the fish to ensure DOX-treated group had the same concentration of 0.1% DMSO as the control group. (Table 2-1, Table 2-2) The chemical was administrated for two times in total, while the next administration was carried out at 17 dpi (days post injection). After the first IP injection, the echocardiography signal was measured at 2, 4 wpi (weeks post injection) to investigate the heart function in the DIC model. The mortality was observed daily.

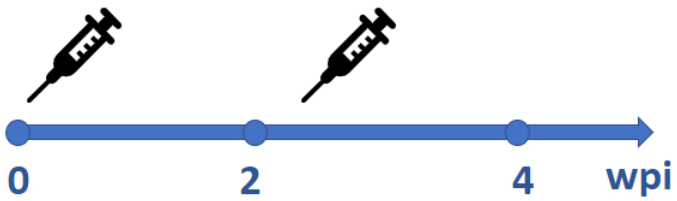

Fig.2 The **Experiment protocol**

2.3.3. Zebrafish anesthetization

Zebrafish were anesthetized by immersing them in the anesthetic solution until no further movement response. The anesthetic solution was made by combining 70 ppm Tricaine methane sulfonate (MS-222) (CAS no. 886-86-2) (Sigma-Aldrich, St. Louis, MO, USA) with 70 ppm isoflurane (SKU: 1001936060) (Baxter, Deerfield, IL, USA).

2.3.4. Adult zebrafish intraperitoneal (IP) injection

The adult zebrafish was first anesthetized and placed in a damp sponge with the abdomen side up to prepare for the IP injection. Before each injection process, the Hamilton syringe was washed with 75% ethanol and 1X PBS sequentially. Then, the needle was inserted into the midline between the pelvic fins with a 45° angle and penetrate 1-2 mm at into zebrafish body cavity to inject 4 µl chemical solution. The process of IP injection was followed by the protocol in the previous study.[8]

2.3.5. Cardiac parameters – E/A ratio and MPI

E/A ratio, calculated by E wave peak velocity and A wave peak velocity, is a marker of ventricular diastolic function. E wave velocity represent the early ventricular diastole and A wave represent the late ventricular diastole. The E/A ratio of zebrafish is less than 1, which is different from human (>1), represented that the atrial contraction is the major cause of ventricular filling. On the other hand, MPI, derive by

IVCT, IVRT and ET, is an integrated measure of systolic and diastolic function, represent the overall heart function. Increases in E/A ratios and MPI values indicating ventricular diastolic function and increases a worsening of cardiac function, respectively.[4, 5] E/A ratio and MPI are calculated as follows,

$$E/A\ ratio = \frac{\text{E wave peak velocity}}{\text{A wave peak velocity}}$$

$$\text{MPI} = \frac{IVCT + IVRT}{ET}$$

2.4. Results

2.4.1. Echocardiography images of B-mode, Color doppler mode and Pulsed-wave Doppler mode

From the B-mode images of the long-axis of the adult zebrafish model, we can clearly distinguish the ventricle, atrium and bulbus arteriosus (BA). After initially locating the zebrafish heart, we used the Color doppler mode to find the best section. Through the colors, the position of the atrium and ventricle can be confirmed, while the section with the strongest blood flow signal in the zebrafish heart can be located by the area size of the color, which lead as to find the position of the AV valve. Finally, we used the pulsed-wave Doppler mode to detect the adult zebrafish ventricular inflow, which allows the following assessment of cardiac functional parameters, including E/A ratio and MPI. From the images of PW doppler mode, we can get E-wave, A-wave, Isovolumic contraction time (IVCT), ejection time (ET) and Isovolumic relaxation time (IVRT) (Fig 3).

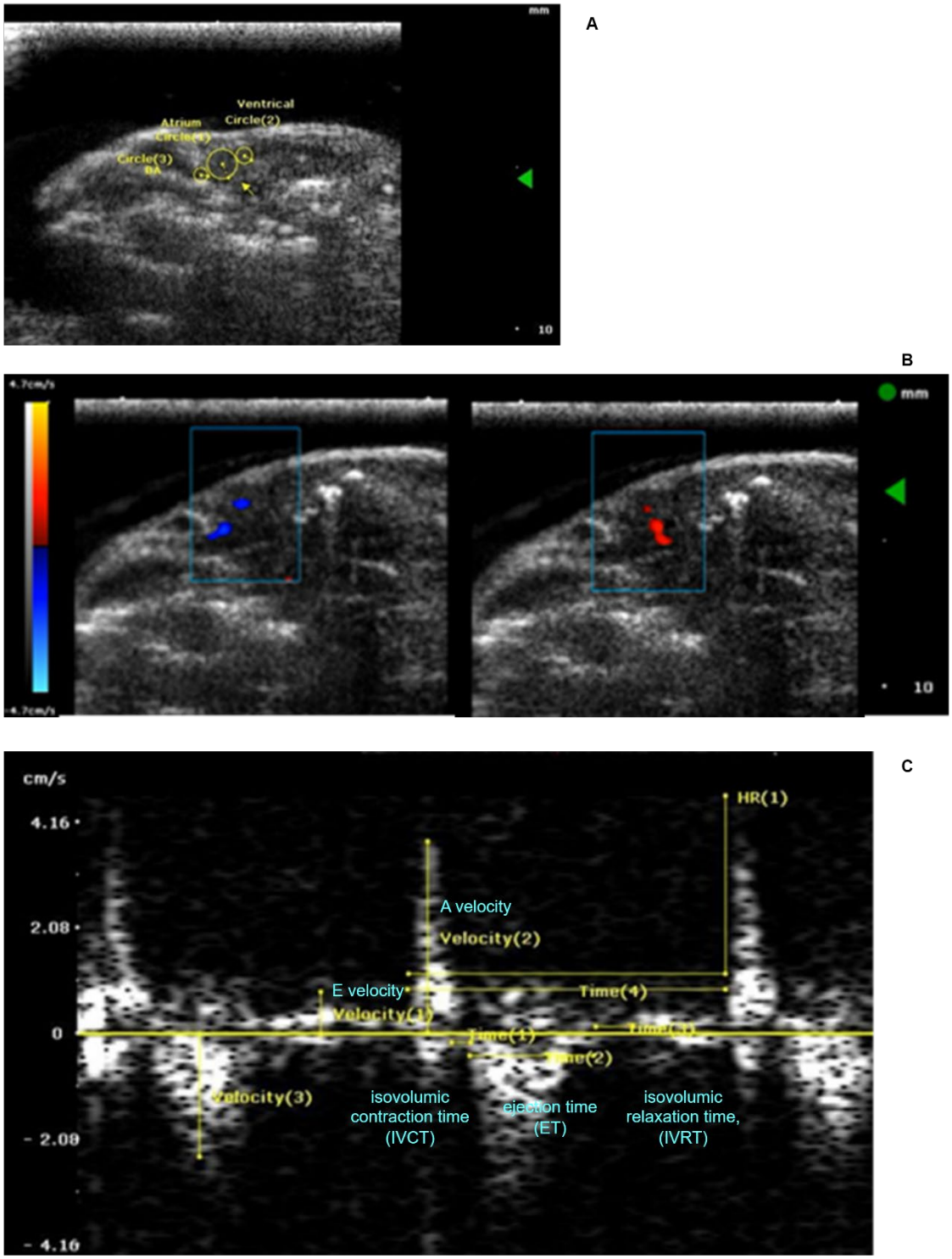

Fig.3 Echocardiography images of the three modes. (A) B-mode (B) Color doppler mode (C) Pulsed-wave Doppler mode

2.4.2. Measurements of the Doxorubicin-induced cardiotoxicity model in adult zebrafish the by pulsed-wave Doppler mode.

Following the 4 weeks of doxorubicin treatment, the survival rate of DOX 20 mg/kg and DMSO were 50% and 75%, respectively. Additionally, there was a significance decrease in the heart rate in the DOX20 group (137.3 ± 21.05 bpm) comparing to the DMSO group (203.5±57.39 bpm) at 28dpi Also, there is a rise in E/A ratios from 14dpi to 28dpi in the DOX-treated fish (0.2949±0.1123bpm and 0.454±0.4439bpm). However, the E/A ratio in DOX20 shows no significance increase indicating no diastolic dysfunction. Furthermore, The MPI ratios at 28dpi reached a dramatically higher rate comparing to the DMSO group (2.345±1.294 and 0.7793±0.2911, $P < 0.05$), and also shows a significance increase from 14dpi to 28dpi (0.8331±0.7125 and 2.345±1.294, $P < 0.05$), indicating the worsening of global cardiac function. (Fig.4).The result was similar to previous studies.[5]

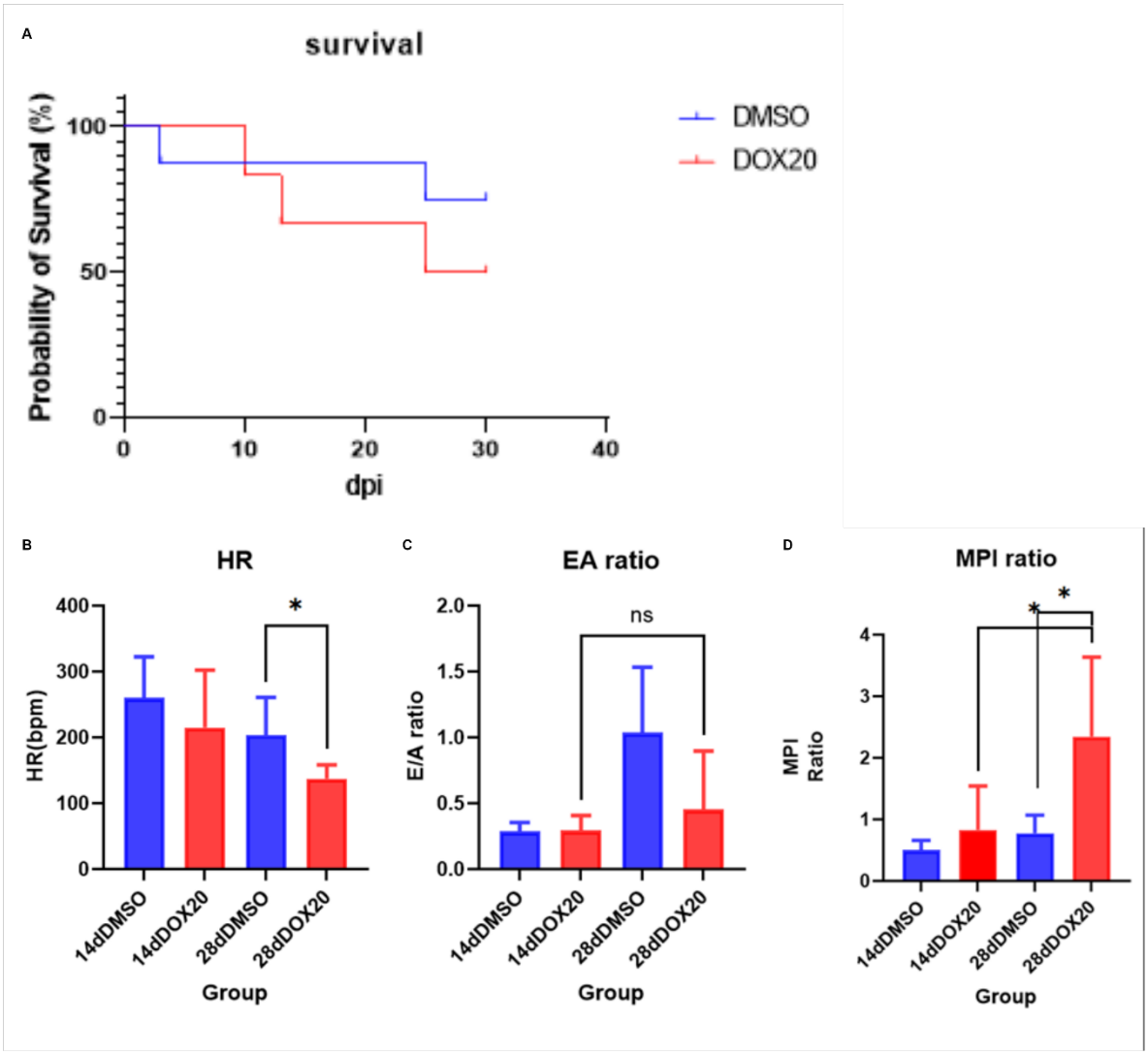

Fig. 4 Survival rates and parameters measured by PW doppler mode of adult zebrafish after injected with DOX 20 mg/kg and DMSO intraperitoneally (i.p.) for two times

within 4 weeks. (A) Survival rates (B) Heart rate (C) **E/A wave ratios (D) MPI.** Data are shown as mean ± SD. N = 1. n = 8-12 / per group. * indicates $p < 0.05$, as calculated by paired t-test.

2.5. Conclusion

The ultrasound-based technology with a high frequency transducer (18Mhz) is successfully constructed to detect the adult zebrafish heart. Besides, the zebrafish DIC model was also successfully generated by intraperitoneal (IP) injection, which lead as to the further measurement of the related myocardial functions in wild-type adult zebrafish DIC model.

Moreover, after 4 weeks of doxorubicin treatment, lower heart rate and higher MPI values were measured in the DIC model of the adult zebrafish compared to the control group, while E/A ratio remains the same. The result indicates that the DOX of dosage 20 mg/kg, though not causing diastolic dysfunction at 28dpi, could result in global cardiac function worsen after injected intraperitoneally (i.p.) for two times within 28 days.

3. Reference

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4. 心得感想：

測量的過程讓我體驗到做動物實驗的困難，從一開始找不到心臟的位置，到後來雖然能快速確認心臟位置，卻仍難以找到想要的波型，有時少了 E 波，有時找不到 IVCT 位置，一隻魚量測了九小時，最後也死了。前幾次實驗，多數魚隻在檢測後就

死了，經過不斷的調整與練習，現在死亡率有大幅度的降低。然而，欲得到的波型也很難快速得到，目前平均一小時測量三隻魚。前一次成功完成了八周的實驗，但數據趨勢混亂，難以進行分析。期望經過多次的練習後，量測能夠愈來愈順利，並完成趨勢相同的三重複。