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Importance of Mitochondrial Homeostasis in Heart Disease and Its Prevention

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Importance of Mitochondrial Homeostasis in Heart Disease and Its Prevention

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in

Physiological Science

by

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ABSTRACT OF THE THESIS

p38 MAP Kinase Regulates Remodeling of Heart via IRE1α During Early Postnatal Development

by

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Master of Science in Physiological Science

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Professor Yibin Wang, Co-Chair

Professor Rachelle Hope Watson, Co-Chair

Heart disease has complicated etiology, however, in most of the cases studied there has been bioenergetic impairment observed which is associated with unmet cellular energy demands, mtDNA damage and other abnormalities linked to mitochondrial function. Thus, we studied whether mitochondrial replacement plays a role during heart disease, and whether suppressing mitochondrial damage can prevent or possibly reverse heart failure. In these efforts, we used the cardioprotective drugs DRZ and HNG and the combined therapy of these to study their effects against DOX-induced cardiomyopathy. DRZ and HNG are known to have protective effects in mitochondria through different pathways, but we observed that the combination therapy of the two had even greater protective effect, completely restoring heart function. Next, we studied mitochondrial stability in a TAC-induced cardiomyopathy and during the recovery. We observed that at the very start of functional recovery, there are changes occurring in the expression of

genes involved in mitochondrial homeostasis. We conclude that pathways involved in mitochondrial clearance and replacement may be involved in the functional recovery of the heart.

The thesis of Rozeta Avetisyan is approved.

Xinshu Xiao

Yibin Wang, Committee Co-Chair

Rachelle Hope Watson, Committee Co-Chair

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1. INTRODUCTION:

According to the 2015 Heart Disease and Stroke Statistics Update compiled annually by the American Heart Association, the Centers for Disease Control and Prevention, the National Institutes of Health heart failure (HF) is the leading cause of mortality globally; in U.S. alone about every second someone dies of HF (1). Development of heart failure is a long and complex process including changes in cardiac structure and function, and involving dynamic interactions between pathological stresses and intrinsic molecular pathways (2).

Most of the human cardiomyopathies for which the energetics have been studied have been accompanied by bioenergetic impairment associated with unmet cellular energy demands (3). These findings point in the direction of mitochondria, since these are the main organelles in the cells responsible for production and allocation of ATP. Furthermore, HF patients have important symptoms such as skeletal muscle abnormalities, renal dysfunction and insulin resistance all of which are known to be linked with mitochondrial dysfunctions (4, 5, 6).

Additionally, mtDNA mutations have been linked to many cardiomyopathies (10). Due to the fact that mtDNA is close to the sites of mitochondrial ROS generation and has poor repair mechanisms, it is especially vulnerable to oxidative stress (3). mtDNA damage is reported to be a significant mechanism involved in cardiomyopathy induced by anthracyclin also known as doxorubicin (DOX) use for cancer treatment (11, 12). More recently, Top2B gene was found to be critical to decreased mitochondrial bioenergetics associated with mtDNA damage in DOX induced cardiomyopathy, further supporting the role of mtDNA damage in the pathogenetic process (34). However, drug dexrazoxane (DRZ) has been suggested to antagonize doxorubicin-

induced DNA damage in H9C2 cardiomyocytes through its interference with DNA topoisomerase II (35). These findings led to the hypothesis that mitochondria are affected during heart disease by accumulated mutations. Furthermore, mitochondrial quality control mechanisms are important for functional recovery. Due to the fact that mitochondria are central in the regulation of cellular metabolism and viability, these have been identified as important targets for interventions to treat many diseases already, including Type II Diabetes Mellitus (19), and familial Parkinson's Disease (20).

In order to study this postulation, we first tested if Humanin/DRZ cound mediate cardio-protection against DOX induced cardiomyopathy. Both HNG and DRZ are known to have cardioprotective qualities which are via mitochondria (18). This experiment will be an important pre-clinical study for Dox induced cardiomyopathy and will help determine if mitochondrial defects can be reversed and prevented therapeutically and what would be the underlying mechanisms.

Of course, the role of mitochondria is not limited to energy supply. Many reports indicate that maintenance of the mitochondrial homeostasis in many cell types, including in dopaminergic neurons, whose malfunction causes the motor deficits in Parkinson's Disease (PD), is very important (7). A very important quality control pathway to help maintain this homeostasis is mitophagy which is the clearance of mitochondria with decreased membrane potential via autophagosomes (8). More specifically, this mitophagy is induced by translocation of Parkin to depolarized mitochondria (9) which also prevents mitochondrial fusion through mediating the degradation of MFN1 and MFN2. This suggests that depolarized mitochondria can be

differentiate from their healthy counterparts by looking at Mfn1 and Mfn2 expression levels (7). At the same time previous findings showed that Mfn1 and Mfn2 are critical in interceding mitochondrial remodeling during the dramatic transitions in the bioenergetics which happen in the initial stages postnatal cardiac development (14). Dr. Gerald Dorn's laboratory postulated that since the cellular ATP biosynthetic pathways highly ordered it would be unlikely for mitochondria to be able to adjust their profile fast enough to be able to flexibly fuel the cell during the substrate availability changes. Their work recently reported that in fetal to mature heart transition, cardiomyocyte mitochondria undergo PINK1-Mfn2-Parkin–mediated mitophagy and replacement (16). The mitochondria in a cell at a particular time seem to be optimized for that given metabolic setting. Once these conditions change, like during the perinatal period, these organelles are replaced with their counterparts more "fit" for the new milieu. Since substantial bioenergetics changes occur during heart disease as well (3), and improved cardiomyocyte bioenergetics are observed with Left ventricular assist device (LVAD) therapy in congestive heart failure (CHF) (17), this raised the question of whether similar mechanism of mitochondrial replacement is playing a role during heart disease and whether suppressing mitochondrial damage can prevent or even reverse heart failure. In order to test that, we need to establish an animal model to investigate possible mechanisms involved in functional recovery after removal of the pathological stressor and whether mitochondrial reprogramming is a necessary step. Accordingly, my thesis work has two parts:

First, we aim to test if HNG/DRZ have synergistic cardioprotection against DOX induced cardiomyopathy which should be associated with mitochondrial quality control mechanisms (Figure 9, A.). In the second part of the study we aim to establish TAC and de-TAC induced

pathological hypertrophy and reverse remodeling, and try to elucidate roles of mitochondrial reprogramming in de-banding induced functional recovery (Figure 9, B.).

2. MATERIALS AND METHODS:

Ethics Statement:

Mouse experiments were performed ensuing recommendations the University of California, Los Angeles Institutional Animal Care and Use Committee recommendations. Dosages of isoflurane used to achieve adequate sedation during echocardiography were used with consideration to minimize the effects of inhaled isoflurane on heart rate and function during echocardiogram. Weekly body weight (BW) and health assessment were performed for all mice throughout the duration of the study. All mice were euthanized with intraperitoneal (i.p.) injection of pentobarbital (200mg/kg) or exsanguination. The heart tissue was harvested for mRNA, protein analysis, TUNEL assay for apoptosis, immunohistochemistry.

Animal Injections and TAC:

Wild-type 10-week old male C57BLK/6 mice were randomly divided into eight groups and according to the group were i.p. injections of saline, HNG (5 mg/kg/day), Doxorubicin (DOX 3 mg/kg/week), and Dexrazoxane (DRZ 60mg/kg/week) based on previous studies and publications (Levi Z et al., Reproduction 2015; 150:357-366) for a duration of 10 weeks as follows:

Group 1. 10 mice received daily i.p. injection of saline as control.

Group 2. 10 mice received 5mg/kg of HNG i.p. daily injection.

Group 3. 10 mice received 3mg/kg of Doxorubicin (DOX) i.p. weekly injection.

Group 4. 10 mice received 60mg/kg of DRZ i.p. weekly injection.

Group 5. 10 mice received DOX+HNG treatment.

Group 6. 10 mice received DRZ+HNG treatment.

Group 7. 10 mice received DOX+DRZ treatment.

Group 8. 10 mice received DOX+DRZ+HNG treatment.

The mice in the reverse-remodeling part of the study underwent trans-aortic constriction surgery, which was performed according to procedure described before (44).

Echocardiography:

Minimum doses (under 1.5%) of isoflurane were used achieve adequate sedation for the procedure in order to minimize the effects of inhaled isoflurane on HR and function. Echocardiography measurements were performed according to peer-reviewed publication guidelines (21).

The measurements were performed by a single operator according to the standard protocol detailed below using the Vevo 2100 ultrasound system (VisualSonics, Inc., Toronto, ON, Canada).

A parasternal long-axis B-mode image was obtained by positioning the probe parallel to the long-axis of the left ventricle (LV) with the ultrasound beam running perpendicular to the LV. Afterwards, the probe was rotated at 90° to obtain a parasternal short-axis view of the LV. The papillary muscle was used as a landmark to ensure reproducible and similar images between the animals. At this position an M-mode short video clip was stored to document LV dimensions for further analysis.

For the part of the project associated with banding and de-banding procedures all mice including controls had aortic valve peak velocity measurements taken. Blood flow was assessed using PW Doppler-mode, by positioning the Doppler sample volume parallel to flow direction, which was assisted by Color Doppler-mode from a suprasternal view to measure AoV peak velocity (AoVPV).

Echocardiographic parameters for ejection fraction (EF), mages were analyzed using the Vevo 2100 cardiac analysis package by a single observer (JJW).

The distribution of the data was analyzed (Fig.2). Since based on the histogram and QQ Plot the distribution of EF was non-Gaussian and bimodal, the non-parametric statistical methods were used to further analyze the data. Main effect box-plots were created to further investigate the effects of time and treatment in mice studied (Fig.3). These plots hinted that the main effect on EF comes from treatment rather than time lapse. To further prove this point, the control group was analyzed (Fig. 4) separately using One-Way Repeated Measures Non-Parametric ANOVA. The F statistic obtained was 0.003424801 with p-value = 0.1549. Assuming no significant interaction of time, Two-Way Repeated Measures Non-Parametric ANOVA was performed on fold change at TAC Time-point (FC= (TAC-Baseline)/Baseline) and at final time-point (FC= (Final-Baseline)/Baseline). The F statistics for TAC FC, Treatment, and TAC FC * Treatment were -4.858796, -4.355424, -4.355424, respectively. The P-values will also be calculated.

RNA extraction and Quantitative real-time PCR (qPCR) analysis

mRNA from frozen LV tissue was isolated by homogenizing the tissue in TriZol reagent. It was later purified using iso-propanol and ethanol. RNA quality was checked via spectrophotometry with all RNA 230/280 ratio at 2.0 ± 0.15. cDNA library was created and the gene expression

changes were checked using BioRad qPCR kit and apparatus. The raw expression levels for each gene were normalized to the expression of 18S gene.

Tissue processing and staining

LV tissue was processed in 8% formalin for 2 days for preservation of ultrastructure. These samples were then washed with distilled water and sent to UCLA Department of Pathology and Laboratory Medicine for paraffin embedding and staining using Masson's Trichrome for fibrosis estimation. Slides from embedded tissue were also used for TUNEL staining according to previously established protocols (45).

Statistical analysis

Data distribution was first analyzed for possible Gaussian distribution. No Gaussian distribution was found in any of the data. Thus, non-parametric statistical testing was performed. Graph and table generation as well as statistical calculations were performed either using R software or Prism 7.

Results:

Part I. HNG/DRZ mediated cardioprotection against DOX induced cardiomyopathy:

i. Functional impact of HNG/DRZ treatment in DOX induced cardiomyopathy:

The initial and final echocardiographic parameters of the mice undergoing chronic treatment with DOX, with or without HNG, DRZ or various combinations were analyzed. Based on echo parameters, we found DOX treatment induced a progressive cardiomyopathy characterized by loss of cardiac mass, contractile function, induction of apoptosis and interstitial fibrosis (Figure

1). HNG and DRZ treatment individually exerted modest protection DOX-induced cardiac dysfunction and pathology. However, combined treatment of HNG with DRZ significantly preserved cardiac function based on EF and left ventricular posterior wall thickening reduced by DOX treatment. Furthermore, this combination treatment prevented other characteristics of HF. Body weight loss induced by DOX treatment was prevented and cardiac muscle loss measured as in LV mass (normalized to body weight) was significantly attenuated.

ii. HNG/DRZ treatment in DOX induced cardiomyocyte death:

Since it is known that DOX has its cardiotoxic effects by inducing double-stranded breaks, generation of reactive oxygen species which ultimately lead to activation of apoptosis (34), TUNEL staining was performed. Combination treatment attenuated cardiomyocyte apoptosis induced by DOX treatment Figure 2) . To further look into these cell death mechanisms, gene expression of Trp53, BAX and BAD was checked. Even though the expression changes of those genes did not reach statistical significance (Figure 3 B), we observed a trend consistent with cell death measurement. In vertebrate cells, apoptosis can proceed through either the intrinsic or the extrinsic pathways (22). The intrinsic pathway involves the mitochondrial outer membrane permeabilization which is pushed forward by pro-apoptotic effectors such as BAX and effector relay molecules such as BAD. The extrinsic apoptotic pathway which can be initiated via TNF has a cross-talk point with the intrinsic pathway. Interestingly, in DOX+HNG+DRZ treated mice while there is a downward trend in BAX and BAD expression, TNF expression seems to be increased, though not showing statistical significance. However, TNF expression with increased NF-kB has been shown to trigger cell survival (23). Thus, expression of NF-kB in DOX+HNG+DRZ treated mice should be checked.

iii. HNG/DRZ treatment in DOX induced cardiac fibrosis:

Fibrosis estimation using trichrome staining of tissue sections and measured under light microscopy revealed accumulation of interstitial fibrosis following DOX treatment. The tempering in fibrosis levels was statistically significant even though the total increased fibrosis due to DOX did not have high effect size (Figure 4D).

Part II Reverse Remodeling in Pressure-Overloaded Mouse Heart

One report found improved respiratory capacity in mitochondria with long-term left ventricular assist device therapy implying the possibility to for the reversal of cardiomyocyte metabolic dysfunction seen in heart failure (24). In order to study if functional recovery in heart is associated with and dependent upon mitigation of mitochondrial abnormalities, we aim to characterize an experimental model of TAC/dTAC in mice.

i. Esablishment of TAC/dTAC mediated induction and reversal of cardiac remodeling

Transaortic constriction (TAC) in mice causes pressure overload and causes development of cardiac hypertrophy and cardiac dysfunction—indications of HF (23, 27, 28, 29). TAC mimics human aortic stenosis and is a widely used method to create mouse cardiac hypertrophy and heart failure models (30). This model was utilized to mimic heart failure effect. Heart failure progression was monitored via echocardiography (echo). Peak blood velocity at aortic valve after TAC surgery increased from normal 700-900 to about 2400-3500 (Figure 5). Significant decrease in EF was found in these mice after about one-month period (Figure 6). After the desired ejection fraction (EF) was observed, the TAC was removed for one group of the mice

and the mice were further monitored via echo. At the end of the experiment, there were three groups of mice: mice which had no surgical intervention, and were only monitored via echocardiography (C), mice which had TAC without de-banding (B) and mice which had TAC and subsequent de- banding once EF was low enough to signify heart failure (DB). The DB animals were monitored via echo the day after the de-banding was performed. As the constriction was removed and the aortic velocity decreased, significant EF recovery was obtained (Figure 6). TAC and control animals were monitored via echo at the same time, which was noted as final echo. After final echo all animals were sacrificed; and heart tissue was collected for weight and molecular analysis.

ii. Molecular signature associated with TAC/dTAC induced cardiac remodeling and reversal: The expression of molecular markers of HF were assessed for all mice. While both ANF and BNP expression were significantly increased in B mice, only BNP expression was significantly lowered in DB group. ANF expression, though not significant, was only modestly decreased in DB animals (Figure 7). Expression changes of Cox2, PGC-1α, MFN1, MFN2 and Top2B were not significant, but showed interesting patterns. COX2 expression which reflects mitochondrial content associated with level of mitophagy (31) was increased in B mice and decreased back to normal in DB. PGC-1α expression which is linked with increased mitochondrial biogenesis (33) was higher in B tissue but even higher in DB mouse hearts. It has been shown that TopIIβ, doxorubicin decreases the transcription of genes involved in the regulation of mitochondrial biogenesis and function (36). Inhibition of Top2β was what lead to to defective mitochondrial biogenesis and metabolic failure (36). TopIIB expression in DB tissue was found to be similar to control, while in B tissue it was much lower. However, the decreased Top2β expression did not

result in decreased PGC-1α trend in B mice, but quite the opposite. This may mean that there are different pathways affecting PGC-1α and thus mitochondrial biogenesis.

iii. Correlation between EF and hypertrophy:

There was strong correlation observed between the final EF and the total heart weight, left ventricle (LV) weight and left atrium (LA) weight associated with binding and de-banding (each normalized by animal body weight) (Table 1). These findings support the hypothesis that the changes observed in the functionality of the heart could be related to the improvement in the actual tissue itself.

4. DISCUSSION:

Doxorubicin effects on cardiac dysfunction inducing double-stranded breaks, generation of reactive oxygen species, disruption of mitochondrial biogenesis, and activation of apoptosis, are shown to be via Top2B gene (34, 36). The cardiotoxicity protective effects of HNG+DRZ seem to be due to their beneficial effects on mitochondria. HNG enhances the cardiac protection of Dexrazoxane against DOX-induced cardiac dysfunction. HNG has been shown to have cardioprotective effects through activation of antioxidant defense mechanisms leading to preservation of mitochondrial structure and attenuation of oxidative stress (18). Similarly, DRZ acts by preventing DOX-induced mitochondrial DNA (mtDNA) copy number decrease and reactive oxygen species (ROS) generation mitochondria (36). Our finding suggests a combination therapy of both HNG and DRZ may offer synergistic benefits against DOX induced cardiomyopathy.

In reverse-remodeling model Cox2 and PGC-1α expression changes are not statistically significant. The reason for this could be small sample size and highly variable gene expression. Since as discussed earlier there was variation in how fast the mice developed heart failure. This could mean different mechanisms of action. In order to better understand this possibility the expression analysis will be repeated with a higher sample size. This is important because the gene expression pattern suggests that there may be differences in mitophagy. Recently it has been shown that Cox2 expression is decreased by increased mitophagy (31). Cox2 expression is in parallel with Mfn2 expression, and the latter has been shown to be a key element in mitophagy during mitochondrial replacement in the transition of heart from aerobic to anaerobic respiration (16). Furthermore, PGC-1α is increased most in DB mice. This may mean that mitochondrial replacement is affected in B mice since even though there is increased mitochondrial biogenesis, mitophagy is decreased allowing for the increased cell death (32).

With most of the heart tissue weight and gene expression analysis obtained at the final stage of the study, the changes between C, B and DB groups showed very interesting patterns which can hint on underlying mechanistic pathways involved. However, these changes are not statistically significant. Considering that heart failure is a complex condition which highly depends on environmental stressors, correlation between EF changes due to TAC and dTAC procedures were correlated with the findings (Table1). As expected, the changes in weight and gene expression are highly correlated with changes in EF. This shows that there might be underlying multicollinearity in the study due to variances in EF (43). In current efforts we aim to decrease or eliminate these EF variances in order to be able to clearly see the underlying molecular physiology. Furthermore, we aim to study at the effects of de-banding after a longer period of time.

Current therapies such as β-Blockers, ivabradine, and antagonism of the renin–angiotensin–aldosterone system (RAAS) all act to lower myocardial energy requests and moderate or prevent further adverse cardiac alteration. Most standard pharmacological methods to treat HF focus on lessening workload on the failing heart rather than focusing on rebalancing energy supply and energy demand in the heart (3). Even though patient survival is enhanced, these treatments cannot improve the poor quality of life (3). Furthermore, the prevalence of failure rate in the recent phase III trials in patients with HF has been argued to be due to the fact that they focus on effects secondary after the myocardium itself is altered (3, 38). The consensus is that full cardiac functional recovery is unlikely unless therapies are aimed at the aberrations that lead to worsening cardiac function in the first place (3, 38, 39). These key abnormalities have been shown to be in the signalling pathways, myofibrillar function, mitochondrial energetics, calcium handling etc. and may be well interconnected (41). The myocardium of the failing heart has been described to be dysfunctional but viable [40]. We argue that studying myocardium during the reverse-remodeling stages and in dissection of cardio-protective mechanisms has high potential to provide deeper insight into these key biological processes governing heart health and disease progression. The equilibrium between cardiac energy requirement and allocation of energy is shifted in heart failure (3). However, when external stressors are removed or attenuated, such as surgical correction of aortic stenosis or implement of ventricular assist devices (VAD) as bridge therapy for end-stage heart failure, the underlying pathology, including cardiac hypertrophy and dysfunction can be reversed (42). These changes have also been associated with improved mitochondrial respiration and overall function (24,25). Although much of our current studies focus on the molecular events involved in the pathogenic triggering of heart failure, relatively very little knowledge is known about the underlying mechanisms involved in the “reverse

remodeling" in diseased heart. In conclusion, our results indicate the importance of mitochondria as therapeutic targets for heart disease treatment. These organelles directly influence cell injury and death (3) and should be thoroughly studied in both pathological conditions and when these conditions are prevented or reversed.

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5. TABLES AND FIGURES:

Figure 1

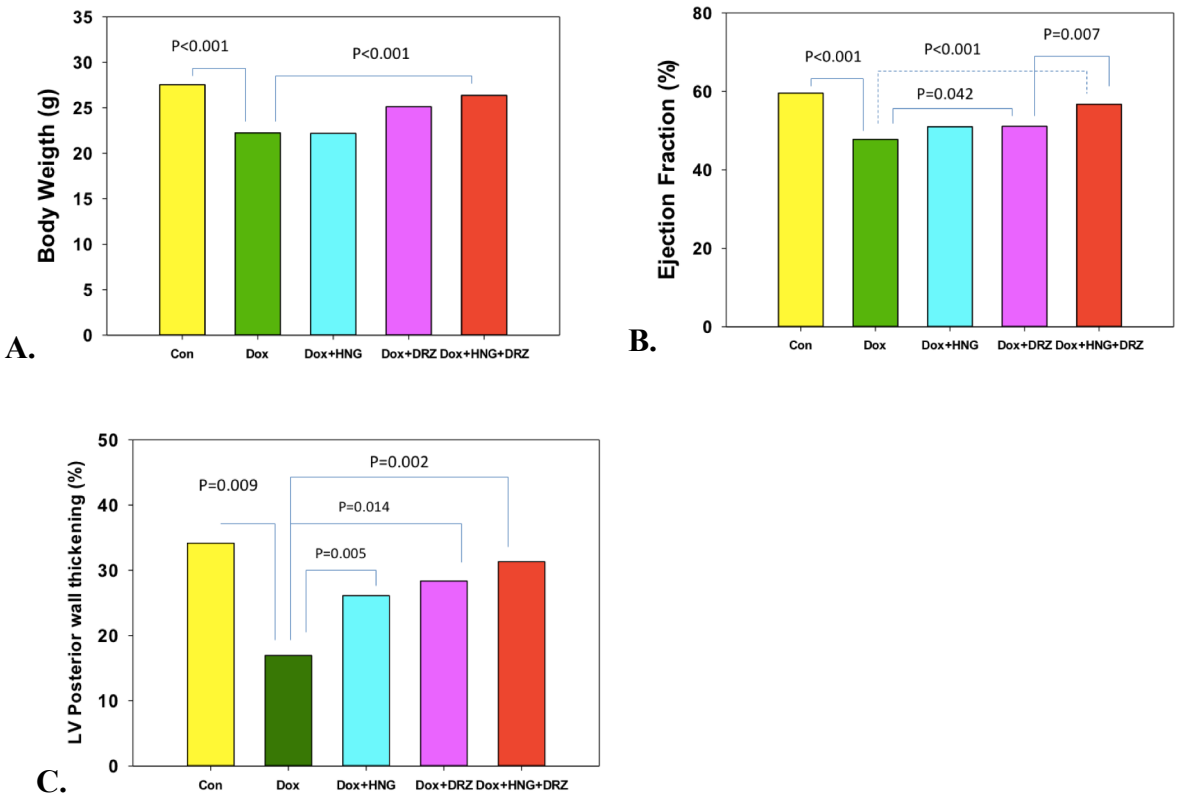

Figure 1: DRZ and HNG Combination Treatment Synergistically Prevents Cardiotoxicity. A. Final body weight of the animals. B. EF measured at the final stage. C. LV Posterior wall thickening measured at the end of the treatment.

Figure 2

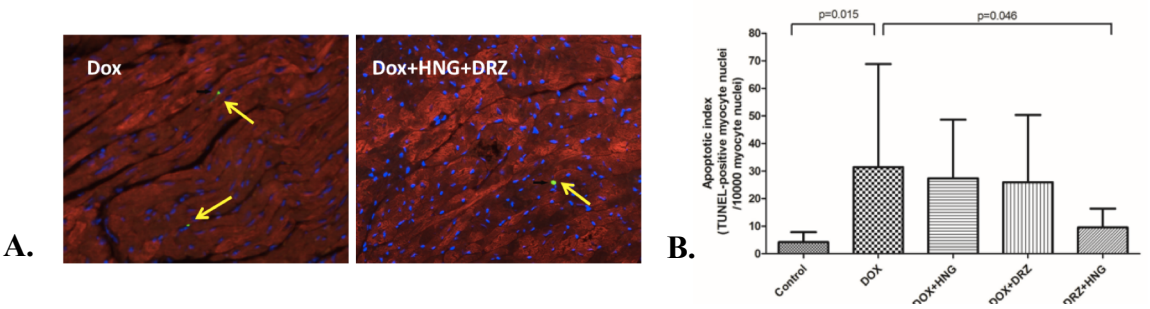

Figure 2: Combined treatment of HNG with DRZ (combination treatment) attenuated cardiomyocyte apoptosis induced by DOX treatment. A. Representative images of TUNEL staining on heart tissue obtained from mice treated with DOX (left) and mice treated with DOX+HNG+DRZ (right). The yellow arrows point to areas undergoing apoptosis. B. Quantification of apoptosis in control animals, DOX-treated animals, DOX+HNG treated animals, DOX+DRZ treated animals and animals with combination treatment of DOX+HNG+DRZ.

Figure 3

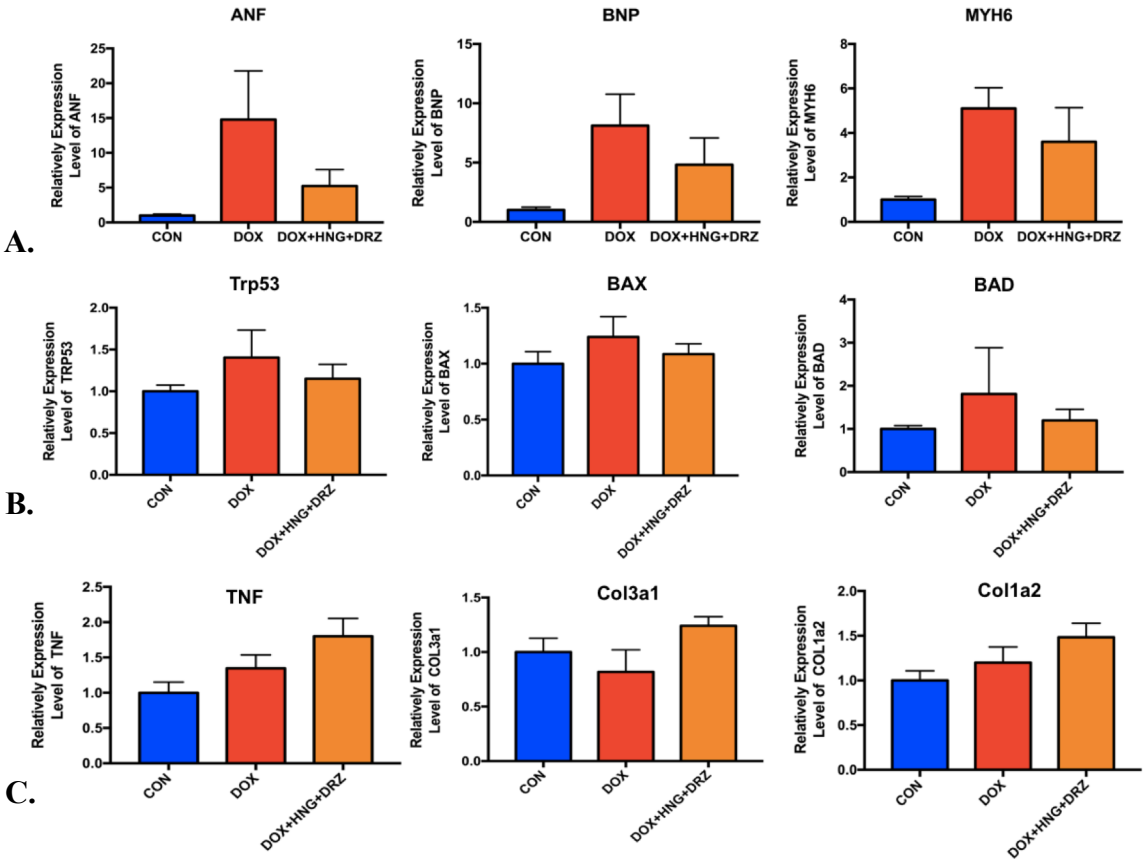

Figure 3: Gene expression analysis. A. Expression of molecular markers which signify cardiomyopathy; from left to right: ANF, BNP, MYH16. B. Expression of molecular markers of apoptosis; from left to right: Trp53, BAX, BAD. C. From left to right expression of TNF a molecular marker that can initiate either cell death or survival, Col3a1, Col1a2, molecular markers for fibrosis expression. Changes not statistically significant.

Figure 4

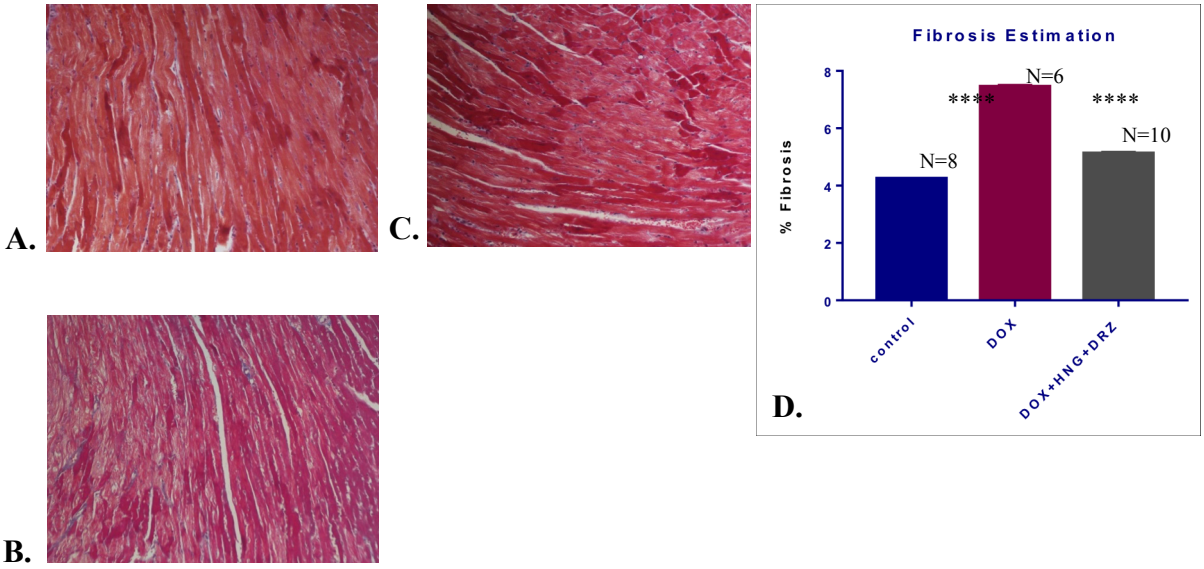

Figure 4. Fibrosis estimation via histology. Representative images of MT staining of heart tissue from A. control mice, B. DOX treated mice and C. DOX+HNG+DRZ treated mice. D. Estimation of fibrosis amount using microscopy. Error bars included, **** indicates P-value <0.0001, obtained via Tukey's multiple comparison test.

Figure 5

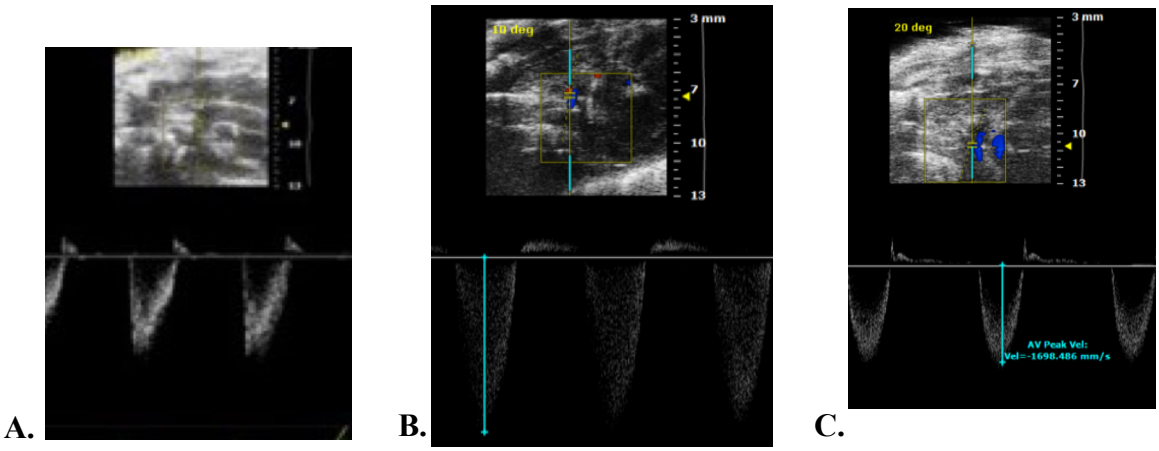

Figure 5. Representative images of Aortic Arch with Aortic Valve Peak Velocity Doppler Imaging for A. Control, V≈890mm/s (A.), banding, V≈3200mm/s (B.) and de-banding V≈1650mm/s (C.) mice.

Figure 6

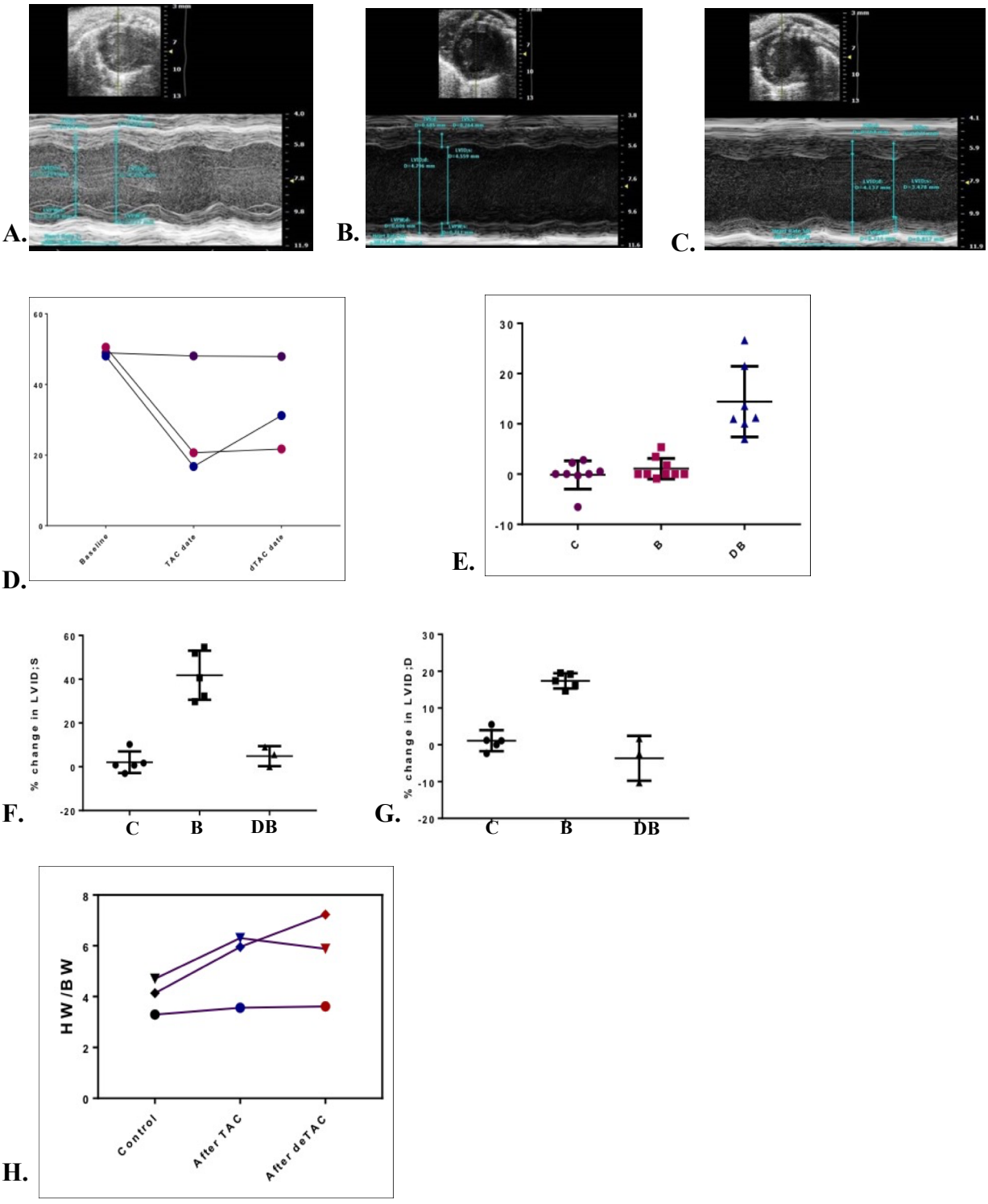
A.
B.
C.
D.
Baseline
TAC date
dTAC date
E.
C
B
DB
F.
% change in LVID;S
C
B
DB
G.
% change in LVID;D
C
B
DB
H.
H W /B W
Control
After TAC
After deTAC

Figure 6. Analysis of heart function via echocardiography. Representative images of M-Mode View of the LV short axis from which ejection fraction was calculated for control (A.), banding (B.) and de-banding (C.) mice. D. The average EF at the start of the experimental procedure (Baseline), after the TAC and after dTAC procedure; $P<0.0001$. E. EF recovery after dTAC procedure; Dunn's multiple comparisons test $P=0.0014$. F. Change in LVID systole; Dunn's multiple comparisons test $p= 0.0137$. G. Change in LVID diastole; Dunn's multiple comparisons test $p= 0.0002$. H. The average total heart weight (normalized by body weight) at the start of the experimental procedure (Baseline), after the TAC and after dTAC procedure Mann Whitney test $p=.048$. Purple color denotes control group, magenta denotes TAC/B group and navy denotes dTAC/DB group for all of the graphs.

Figure 7

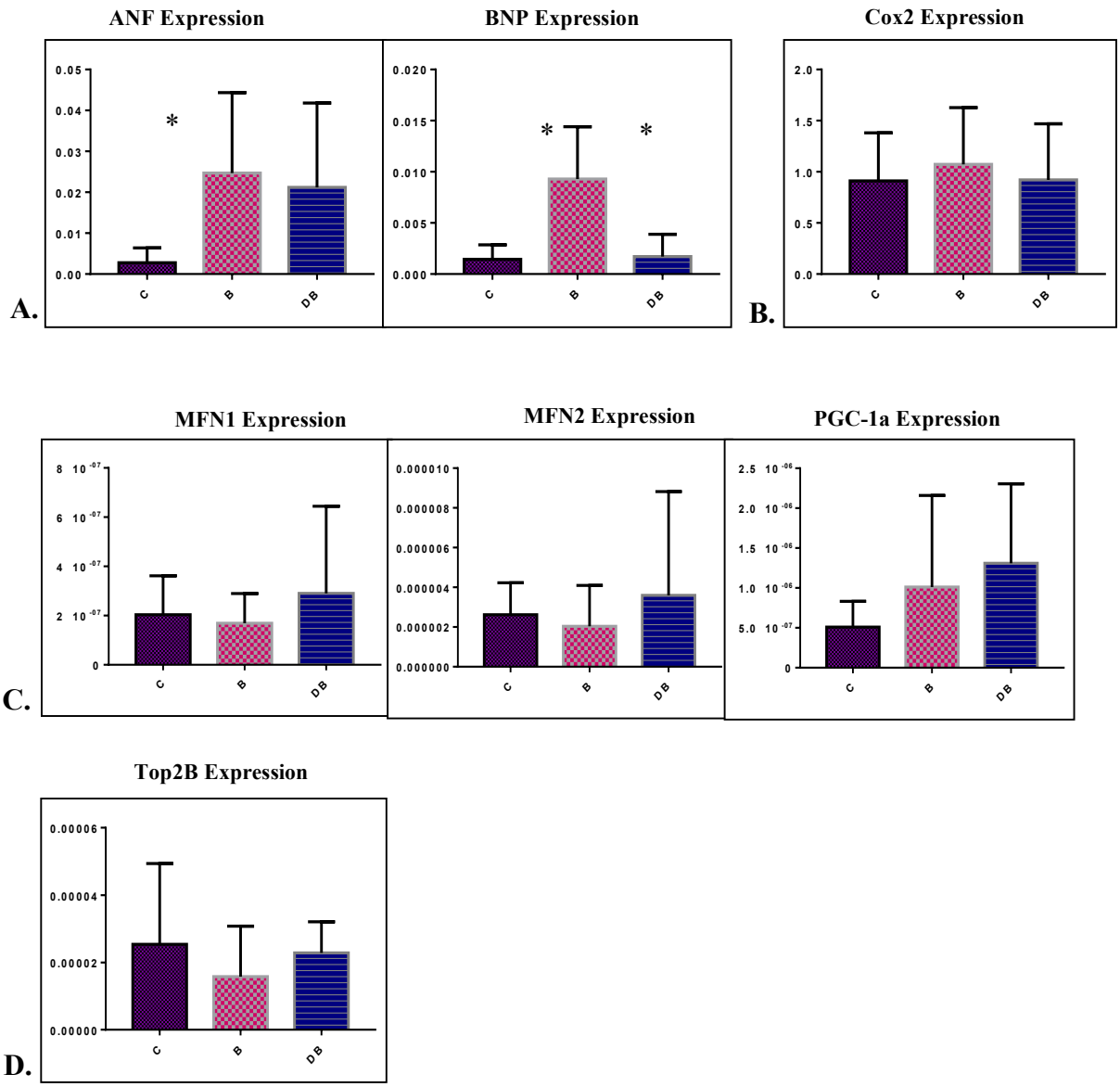

Figure 7: Gene expression analysis of Reverse remodeling group. A. Expression of molecular markers which signify cardiomyopathy; from left to right: ANF, BNP. B. Expression of Cox2 which has been linked with mitophagy. C. From left to right expression of Mfn1, Mfn2 markers

involved in mitochondrial fusion and PGC-1a—molecular marker for mitochondrial biogenesis. D. Expression of Top2B which has been shown to interfere with PGC-1a levels in certain conditions. Only change in ANF statistically significant; C vs B, $p=0.025$, BNP C vs B $p=0.041$, B vs DB $p=0.028$; Dunn's multiple comparisons test used.

Figure 8

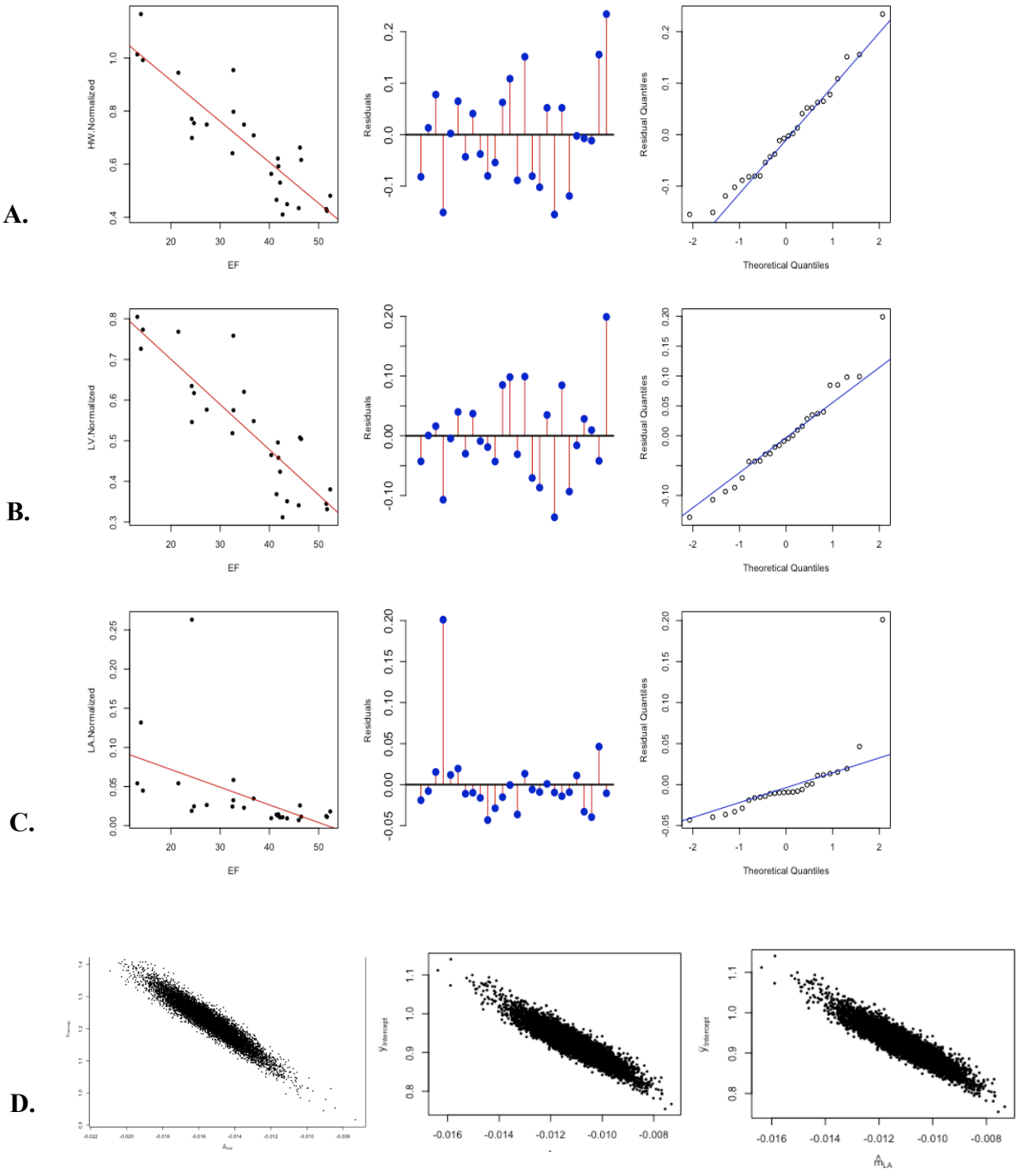
A.
HW.Normalized
EF
Residuals
Residual Quantiles
Theoretical Quantiles
B.
LV.Normalized
EF
Residuals
Residual Quantiles
Theoretical Quantiles
C.
LA.Normalized
EF
Residuals
Residual Quantiles
Theoretical Quantiles
D.
$y_{intercept}$
$\hat{m}_{LA}$

Figure 8: Interaction between EF and heart weight. From left to right, interaction plot, Rediduals and QQplot of the residuals for total heart weight (A), LV (B), and LA (C). D. Correlation Plot for total heart weight, LV and LA from left to right.

Figure 9

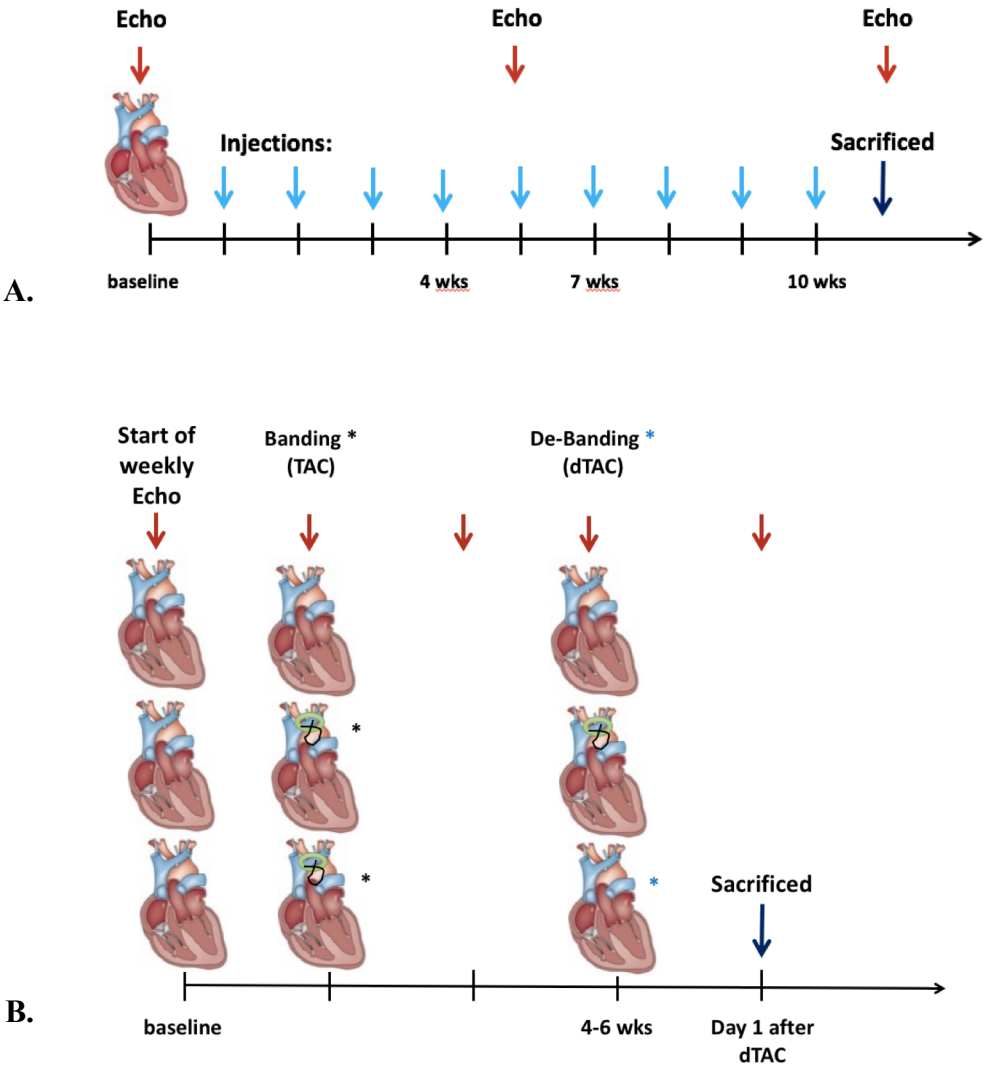

Figure 9: Experimental Design. A. Experimental design for the first part of the thesis work; establishing cardio-protective features of combined treatment of DRZ+HNG. B. Experimental design for the second part of the thesis work; establishing functional recovery in TAC-induced heart failure model.

Table 1

Correlation	Spearman.Correlation.Coefficients	Spearman.CI	Spearman.P.Values
EF and Total Heart Weight	-0.8577778	(-0.942,-0.688)	0e+00
EF and LV Weight	-0.8611966	(-0.939,-0.696)	0e+00
EF and LA Weight	-0.7367521	(-0.858,-0.502)	1e-04

Table 1: Interaction between EF and heart weight Spearman Correlation Analysis. P-values calculated using Null Hypothesis Significance Testing procedure in R software.