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Cardiomyopathy reverses with recovery of liver injury, cholestasis and cholanemia in mouse model of biliary fibrosis

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Abstract

Background—Triggers and exacerbants of cirrhotic cardiomyopathy (CC) are poorly understood, limiting treatment options in patients with chronic liver diseases. Liver transplantation alone reverses some features of CC, but the physiology behind this effect has never been studied.

Aims—We aimed to determine whether reversal of liver injury and fibrosis in mouse affects cardiac parameters. The second aim was to determine whether cardiomyopathy can be induced by specifically increasing systemic bile acid (BA) levels.

Methods—6–8 week old male C57BL6J mice were fed either chow (n=5) or 3, 5-diethoxycarbonyl-1, 4-dihydroxychollidine (DDC) (n=10) for 3 weeks. At the end of 3 weeks, half the mice in the DDC fed group were randomized to chow (the reversed [REV] group). Serial ECHOs and electrocardiographic analysis was conducted weekly for 6 weeks followed by liver tissue and serum studies. Hearts were analyzed for key components of function and cell signaling. Cardiac physiologic and molecular parameters were similarly analyzed in Abcb11$^{-/-}$ mice (n=5/grp) fed 0.5% cholic acid supplemented diet for 1 week.

Results—Mice in the REV group showed normalization of biochemical markers of liver injury with resolution of electrocardiographic and ECHO aberrations. Catecholamine resistance seen in DDC group resolved in the REV group. Cardiac recovery was accompanied by normalization of cardiac troponin-T2 as well as resolution of cardiac stress response at RNA level. Cardiovascular physiologic and molecular parameters correlated with degree of cholanemia. Cardiomyopathy was reproduced in cholanemic BA fed Abcb11$^{-/-}$ mice.

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Conclusions—Cardiomyopathy resolves with resolution of liver injury, is associated with cholanemia, and can be induced by BA feeding.

Keywords

bile acid-myocardial interaction; cardiac adaptation; hepatic - cardiopathy; liver injury; cholanemia

Introduction

Patients with end stage cirrhotic liver disease are known to exhibit unique abnormalities in cardiovascular physiology, known collectively as “cirrhotic cardiomyopathy” (CC) (1, 2). The key features of this cardiomyopathy, are a) hyperdynamic left ventricular (LV) contractility at baseline, b) left ventricular and septal hypertrophy, c) diastolic dysfunction, d) electrophysiological abnormalities and e) systolic incompetence during exercise, catecholamine or hemodynamic stress (1, 3). Though the exact prevalence of CC is unknown, recent literature (2, 4) supports that upwards of 50% adults and children with advanced cirrhosis have CC. Presence of CC has been shown to peri-operative outcomes in adults and children(5–8).

Despite its clinical significance and impact on health care in cirrhosis, this cardiomyopathy has been vastly understudied, especially the triggers that initiate and maintain the cardiac dysfunction are relatively unknown, and as a consequence, no feasible treatment option exists to support these patients through successful transplantation and recovery. Liver transplantation, alone has so far been shown to reverse some of the features of cardiac dysfunction (9, 10), but the degree to which CC is reversible is unknown.

In this multifactorial and complex, but potentially reversible disease process, role for bile acid excess (cholanemia) – a pathognomonic feature of liver disease has been suggested (11–17), but not pursued in physiologic and molecular detail. The degree to which these circulating bile acids contribute to CC is unknown.

Therefore the goal of this study was to answer two key questions. The first is whether liver injury and fibrosis in mouse is reversible and if reversibility affects cardiac parameters. The second is whether key features of cardiomyopathy can be induced by specifically increasing systemic bile acid levels. We hypothesized that cardiomyopathy in the established mouse model of DDC fed biliary fibrosis (18) resolves with recovery of liver injury, correlate with the degree of cholanemia and is reproduced by bile acid feeding. Specifically we examined biochemical and histologic markers of liver injury after withdrawal of DDC diet and evaluated its effect on cardiac function and cell signaling pathways. We also induced pathologic cholanemia by feeding cholic acid (bile acid) to mice with genetic deletion of bile salt export pump ($Abcb11^{-/-}$) to evaluate cardiac function and cell signaling pathways.

MATERIALS AND METHODS

Animals and diet

Separate experiments were performed to answer each of the two key questions. To test reversibility of biliary fibrosis induced cardiomyopathy, biliary fibrosis was induced in juvenile male 6–8 week old C57BL6J mice (Jackson Labs; BarHarbor, ME.) by feeding 0.1% 3,5-diethoxycarbonyl-1,4-dihydroxychollidine (DDC) [Sigma-Aldrich. St. Louis, MO.] supplemented chow for a period of 3 weeks. Diet prepared at Harlan-Teklad Inc. San Diego, California (TD.07868), by mixing 1 gram of DDC powder with 1 kilogram of isocaloric chow, irradiated and stored per company instructions. It is known that this supplementation results in biliary fibrosis, cholestasis and cholanemia. (18). After 3 weeks, DDC-fed mice were further randomized to either chow (reversal group [REV]) or were continued on DDC diet for further 3 weeks (6 week DDC fed group). Age matched male C57BL6J mice fed isocaloric chow (Harlan-Teklad Inc. San Diego, CA.) were used as controls for all experiments. Thus total duration of these experiments was 6 weeks and involved 3 groups: Group 1 - chow fed for 6 weeks (Chow group); Group 2 - DDC fed for 6 weeks (DDC group); and Group 3 - DDC fed for 3 weeks and then reversed to chow for 3 weeks (REV group). To evaluate if bile acid feeding induces functional and molecular changes in the heart, male 6–8 week old, bile acid transport in sister of P-glycoprotein (Abcb11) knockout (Abcb11$^{-/-}$) mice (bred in our animal facility from heterozygous mating pair (B6.129S6-Abcb11 tm1Wng/J) obtained form Jackson Labs; BarHarbor, ME and their littermate controls were used. These mice were either fed 0.5% cholic acid (CA) supplemented diet (Harlan-Teklad Inc. San Diego, CA.) or isocaloric chow (Harlan-Teklad Inc. San Diego, CA.) for a period of 1 week. For all experiments, feeds and bedding were weighed and changed twice a week to provide a rough estimate of cumulative food intake per mouse. Mice were fed ad libitum and had free access to water. Food was withdrawn 4 hours before subjecting mice to any experiments and all experiments were conducted consistently during the day time to maintain circadian rhythm. All experiments were done in accordance with IACUC approved protocols at Baylor College of Medicine.

Cardiac Parameters

Continuous electrocardiograms were recorded noninvasively in unsedated mice using EC Genie (Mouse Systems Inc, Quincy MA). Two-dimensional echocardiography (2DE) was performed in the Mouse Phenotype Core (BCM) on sedated mice (Vevo 770 Digital RF, VisualSonic Inc. Toronto, CN) (18).

Catecholamine challenge and stress echocardiography

Sedated mice were challenged with a single intra-peritoneal dose of 0.02mg/kg of isoprenaline (Sigma-Aldrich; St. Louis, MO). Pre and post injection cardiac parameters were evaluated by echocardiography and cardiac response to isoprenaline stress was compared between chow and DDC fed mice.

Serum analyses

Sera were collected from the inferior vena cava (IVC) and analyzed for Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), total and conjugated bilirubin levels (Cobas Integra 400+; Roche) at the Center of Comparative Medicine (BCM). Serum bile acid levels were evaluated by colorimetric methods (BioQuant Inc, SanDiego, CA. & Roche) as before (18).

Liver Histology

Liver was stained with routine H/E and Mason-trichrome for presence of collagen. All histological studies were performed by the Texas Gulf Coast Digestive Disease Center. Histological presence of collagen was evaluated quantitatively for color intensity using the program Image J (NIH). Briefly Mason-trichrome stained hearts (n=3 /group) were photographed on an Olympus BH-2 light microscope (Olympus Corp, NY USA), using a 20× objective lens. QCapture Pro V 6.0 (QImaging Inc, Surrey, British Columbia, Canada) imaging software was used to acquire the images. Four 20× images sections were taken randomly per slide with the same exposure settings and images were analyzed for color intensity using the program Image J (NIH) and reported as percent area stained blue.

Transmission Electron-Microscopy (EM): Ultrastructural analysis of left ventricles of hearts of the 3 week DDC fed mice was conducted using TEM (Hitachi H-7500) at Texas Medical Center Digestive Disease Core.

Serum Analysis of cardiac troponin-T2 (cTNNT2) by ELISA: Serum was analyzed for mouse cardiac TNNT2 levels using Enzyme-linked Immunosorbent Assay Kit (USCN Life Science Inc; Houston, TX), per company instructions.

Quantitative real time PCR (qRTPCR)

RNA quantitation was conducted following standard protocol, using probes and primers as previously published (18). Briefly, total RNA was isolated from snap frozen homogenized heart samples and cDNA was synthesized and analyzed by qRTPCR according to standard techniques (19) using SYBR® Green™ (Applied Biosytems, Foster city, CA.). Relative RNA expression was calculated by delta Ct method. Gene expression of target genes were normalized to an internal standard (GAPDH) and the difference expressed as amount of gene expressed per internal standard.

Immunoblotting

Proteins from whole homogenized hearts were extracted using standard procedures (19). Protein concentrations were measured using the Pierce BCA kit (Thermo Scientific, Rockford, IL). After gel electrophoresis and immunoblotting, the gels were analyzed for expression of various proteins with specific antibodies as described before (18). Equal protein loading was confirmed by α-tubulin (Sigma-Aldrich. St. Louis, MO). Results were analyzed by densitometry (Kodak software) and reported as fold change compared to chow fed hearts.

Statistical Analysis

Data are presented as Means ± SD (unless specified). Data was analyzed using non-parametric Mann-Whitney test (unless specified) All statistical calculations were done using the PRISM 3.0 software program (Graph-Pad Prism, San Diego, CA, USA). $P < 0.05$ was selected as the level of significance.

RESULTS

Liver injury, cholestasis and cholanemia resolve with reversal to chow diet

Animals fed DDC diet showed significant increases in serum ALT, bilirubin (total and conjugated) and bile acid levels, consistent with studies done by us (18) and others (20), at the end of 1, 3 and 6 weeks of feeding as compared to CHOW controls (Fig. 1A). Serum ALT and bile acid levels were the highest (~30 fold) at 3 weeks as compared to 1 week (~7 fold) and 6 week (~15 fold) of feeding when compared to CHOW group. REV mice exhibited a trend to normalization of serum ALT, bilirubin and bile acid levels once a normal chow diet was restored. By 6 weeks, mean ALT, bilirubin and bile acid levels were modestly elevated (2.5 fold, 2 fold, and 3 fold respectively) in the REV compared to CHOW groups, but the differences were not significant. Histologically, mice in the DDC group showed severe biliary hyperplasia and fibrosis compared to the CHOW group (Fig. 1A and 1B), whereas REV mice demonstrated attenuated biliary fibrosis when compared to the 6 week DDC fed group (Fig. 1B).

Electrocardiographic, functional and structural aberrations resolve with reversal of liver injury

Electrocardiography and ECHO analysis was conducted serially over the period of 6 weeks. As shown in Fig. 2, DDC-fed mice, demonstrated bradycardia, prolonged corrected QT (QTc) interval, hyperdynamic contractility of the left ventricle, [increased shortening fractions (%FS) and ejection fractions (%EF)], in line with our previous observations (18). The alterations in heart rate, QT interval, LV contractility, posterior wall thickness normalized in the REV group to chow fed levels. No differences in any of these parameters were evident at one week of DDC feeding (Fig. 2).

Catecholamine insensitivity resolves with resolution of liver injury

When mice were challenged with 0.02mg/kg of single dose of intraperitoneal injection of isoprenaline there was an attenuated increase in mean heart rates (180±20 vs. 250±40 bpm), mean ejection fractions (20±8% vs. 50±5%) as well as mean shortening fraction (35±5% vs. 70±5%) in the DDC fed mice when compared to CHOW. This reflected in a significant decrease in the cardiac index in the DDC fed mice compared to chow fed mice on isoprenaline challenge. (Fig. 3A). Catecholamine resistance resolved in REV group (Fig. 3B), as evidenced by normalization of cardiac response to isoprenaline challenge (%EF, %FS and CI) to basal chow levels.

Reversal of fetal gene expression in the heart with resolution of liver injury

The common response of the heart to various pathophysiologic conditions including hemodynamic, metabolic and hypoxic stress is the return to the “fetal gene program”. This response is adaptive, and includes preference of glucose over fatty acids as substrates for energy, as well as isoform switches of sarcomeric proteins (myosin heavy chains).(21, 22) Important changes in RNA expressions of these key stress mediated genes occur through the course of liver injury and resolution. Genes regulating fatty acid oxidation such as uncoupling protein-3 (UCP-3), fatty acid binding protein in the heart (h-FABP) and mitochondrial carnitine palmitoyltransferase 2 (m-CPT2), collectively were ~50–60% downregulated as expected at 3 weeks(18) and stayed downregulated at 6 weeks of feeding. In the reversal group, the expressions of UCP3 and h-FABP normalized to chow fed levels. (Fig. 4A). Glucose transporter-1 (GLUT-1), which regulates basal glucose uptake, which was upregulated (~1.8 fold) in the 3 week DDC fed mice showed normalization after diet reversal. Pyruvate dehydrogenase kinase isoform 4 (PDK4), regulates cardiac glucose oxidation.. Suppression of PDK4 indicates increased glucose oxidation (utilization) and is one of the adaptive mechanisms of a failing/stressed heart (23). DDC fed mice show a 60% suppression of cardiac PDK4 at 3 and 6 weeks interval. PDK4 gene expression normalizes after reversal of diet. Beta-myosin heavy chain (β-MyH7), a key sarcomeric protein and a marker of cardiac stress and hypertrophy is robustly upregulated (~20–40 fold) in the DDC fed mouse hearts. Upregulation of (β-MyH7), was noted as early as 1 week of DDC feeding, preceded any change in the cardiac physiologic parameters, and remained modestly (3 fold) elevated even after liver injury is reversed (Fig. 4A).

High Sensitive cardiac troponin T2 (cTNNT2; HS-TNT), is a well established clinically relevant circulating serum biomarker of myocardial stress. An increase in serum cTNNT2 is predictive of cardiac failure, myocardial fibrosis and cardiovascular mortality (24, 25). Serum levels of cTNNT2 were significantly higher (~ 4 fold) in the 3 and 6 week DDC fed mice. cTNNT2 levels normalized to chow levels in the Reversal group (Fig. 4B). As cTNNT2 levels were maximal in the 3 week fed mice, we analyzed cardiac muscle isolated from the left ventricles of those mice by Electron Microscopy to evaluate for ultrastructural changes in the myocytes. EM did not detect major myocyte injury, myocyte loss or destruction (Fig. 4C), suggesting that the increase in the highly sensitive cTNNT2 is indicative of a reversible micro-injury to the heart muscle (25, 26).

High serum bile acid levels are associated with key measures of cardiac dysfunction

We evaluated whether the degree of cholanemia affects cardiovascular parameters at a physiologic and molecular level. When serum levels of bile acids in 6 week DDC fed (n=5), chow fed (n=5) and those reversed (n=5) were plotted against structural and functional parameters as obtained from ECHO and ECG, we found that HR, and raw un-indexed cardiac mass had a negative relationship with bile acid levels, while %EF (hyperdynamic LV) showed a positive association (Fig. 5A). There was a positive relationship between degree of cholanemia and GLUT-1 RNA, while PDK4 showed a negative association (Fig. 5B). Concomitantly, genes regulating fatty acid oxidation, UCP-3 and h-FABP showed a negative association with bile acid levels (Fig. 5B). β-MYH7 and heme oxygenase-1 (HO-1), both of which are inducible stress-response genes, positively correlated with serum

bile acid levels (Fig. 5B). Pearson Correlation analysis was used and p value less than 0.05 was considered significant. Though a relatively small sample size may overestimate the significance, physiologic and molecular parameters of dysfunction, appeared to be associated with the degree of cholanemia in this model.

Bile acid feeding induces cardiomyopathy in Abcb11$^{-/-}$ mice

To further establish the role for bile acids as signaling molecules targeting the heart in liver injury, we evaluated if features of cardiomyopathy could be reproduced in a mouse by feeding bile acid in the diet. Male Abcb11$^{-/-}$ mice, (correlates of the human cholestatic liver disease PFIC2) (27, 28) were fed either bile acid (0.5% cholic acid [CA]) supplemented diet (Harlan Teklad) or isocaloric chow. Within 1 week of bile acid supplementation, Abcb11$^{-/-}$ mice demonstrated liver injury, cholestasis and cholanemia (Supplemental Fig. 1). Male Abcb11$^{-/-}$ get moribund with longer duration of CA feeding and hence feeding was restricted to 1 week duration. Bile acid levels were modestly elevated (~ 10 fold) in the wild type CA fed mice compared to chow fed WT or Abcb11$^{-/-}$ mice, and robustly (~30–40 fold) elevated in CA fed Abcb11$^{-/-}$ mice (Fig. 6B). ECHO and ECG evaluations reveal bradycardia, and hyperdynamic LV (higher %EF and %FS), similar to that seen in the DDC fed mice (Fig. 6A). At the protein level, there was evidence of activation (2 fold increase in phosphorylation) of AKT and inhibition (1.5 fold increase in phosphorylation) of GSK3β (Fig. 6B). At the RNA level, there was molecular evidence of increased glucose uptake, as evidenced by a modest (2 fold) but significant increase in GLUT-1 and increased glucose oxidation, as evidenced by ~75% decrease in PDK4 gene expression. Concomitantly, there was suppression of UCP-3, m-CPT-2 and h-FABP at the RNA level, suggesting decreased fatty acid oxidation. This re-expression of fetal metabolic genes (Fig. 6B) suggests a stressed heart in cholanemic Abcb11$^{-/-}$ mice. There was a 40 fold upregulation in βMyHC gene expression and a 50% downregulation of αMYHC, indicating pathologic structural remodeling at RNA level (Fig. 6B). There was no evidence of cardiac hypertrophy on ECHO or myocardial fibrosis, lipid accumulation or myocardial fibrosis on histology (data not shown).

Discussion

In this study, we show, for the first time that cardiac dysfunction, and molecular aberrations, as well as the myocardial stress response resolve after resolution of liver injury and cessation of the cholestatic insult. This study also proposes a novel association between elevated bile acids, and the pathophysiologic and molecular markers of cardiac function. The role of high serum levels of circulating bile acids as inducers of myocardial dysfunction, is further emphasized in this study, by reproducing key physiologic and molecular features of cardiomyopathy in Abcb11$^{-/-}$ mice made cholanemic by bile acid feeding.

Chronic feeding of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) is a well established mouse model of biliary fibrosis, long been used to study Mallory–Denk body formation, which are hepatocellular inclusion bodies characteristically associated with alcoholic and non-alcoholic steatohepatitis, metabolic liver diseases (eg, Wilson's disease and other forms of copper toxicities) and chronic cholestatic liver diseases (29–33). In addition, Fickert et al

have previously shown that DDC feeding in mice results in cholangitis with pronounced ductular reaction, onion-skin-type like periductal fibrosis and finally liver fibrosis of the biliary type. Though not a model of micro or macronodular cirrhosis, the cholangiopathy and biliary fibrosis induced by DDC feeding shares several specific pathognomic features of some human cholangiopathies associated with biliary type of liver fibrosis, in particular primary and secondary sclerosing cholangitis (20). In this study, mice fed DDC supplemented diet for 1 week have increased serum ALT and bile acid levels (Fig. 1), without significant cholestasis or biliary fibrosis (histologic data not shown). By 3 weeks, as shown by us and others (18, 20), there is a substantial evidence of further liver injury as evidenced by further elevations of ALT, increases in bile acid levels, significant cholestasis and histological evidence of peri-portal fibrosis and biliary hyperplasia. Though the degree of cholestasis and biliary fibrosis remains comparable with the 3 week DDC fed group, ALT and bile acid levels show a modest but significant decrease in the 6 week DDC fed group. This effect may be indicative of some active compensatory mechanisms in the hepatocytes with persistent DDC induced insults. Serum ALT, bile acids and bilirubin normalized within 3 weeks to near control levels, when mice were switched from DDC to chow feeding (the Reversal group). Histologically biliary fibrosis was less pronounced in the Reversal group (Fig. 1B), in line with observations made by Fickert et al (32).

Using this model, we have previously described structural, functional, electrocardiographic and molecular consequences of biliary fibrosis on the heart (18). Though no animal model can perfectly reproduce human clinical disease, many cardiac findings in DDC fed mouse model of biliary fibrosis can be extrapolated to human clinical disease. Electrocardiographic abnormalities, more specifically prolongation of QT interval are seen both, in adults (34, 35) as well as children with end stage cirrhotic liver disease (5). Patients have abnormal ECHOs, characterized by hypertrophy of the left ventricle and septal wall (6, 10). The LV ejection fractions (%EF) are higher at baseline (6, 10), but fail to show an appropriate response to catecholamine stress (10), which perhaps is one of the most pathognomonic feature of cirrhotic cardiomyopathy(1, 4, 36, 37). Hearts of the 3 week DDC fed mice also show prolongation of QT interval, LV and septal thickening, increased LV ejection and shortening fractions (18), along with attenuated response to isoprenaline (Fig. 3A) replicating clinical observations. Cirrhotic patients have a low systemic vascular resistance as a result of circulating vasodilatory mediators resulting from liver dysfunction and portal hypertension. This is often manifested as high heart rate and hyperdynamic circulation. The DDC fed mice however have low heart rate. This major discrepancy from clinical cirrhosis could be explained by the severity of cholestasis and cholanemia in these mice (Fig. 1), as it has long been known that patients with obstructive jaundice have bradycardia – a phenomenon described as “icterus bradycardia”, first described by Rohrig et al in 1863 (38, 39). Prolongation of QT interval, left ventricular geometry as well as catacholamine insensitivity resolves with after liver transplantation, thus making cirrhotic cardiomyopathy one of the “reversible cardiomyopathies” (5, 10, 34, 40). In this mouse model, similar to clinical observations, we show that electrocardiographic and echocardiographic aberrations (Fig. 2), as well as catecholamine response (Fig. 3b) recover after resolution of liver injury (Fig. 1).

Studies done by Ma et al in 1999 (41), show that isolated ventricular papillary muscles from rats with biliary fibrosis induced by Common Bile Duct Ligation (CBDL) demonstrate an

attenuated contractile response to isoprenaline, in line with our observation in vivo. At a molecular level, Ma et al show that cAMP production in response to isoprenaline as well as forskolin is attenuated in the CBDL papillary muscles. Response to isoprenaline (which acts at the beta-adrenergic receptor level) remains attenuated despite choledochojejunostomy - an intervention which normalized serum bile acid levels. However, response to forskolin (acting at the adenyl cyclase level downstream from the beta receptors) returns to normal in the choledochojejunostomy animals, suggesting that bile acids regulate adenyl cyclase activity downstream of beta-receptors (41). The observation that choledochojejunostomy normalizes forskolin effect on the isolated papillary muscles, suggests that the myocardium restores the adenyl cyclase activity, if not beta-adrenergic sensitivity in vitro after normalization of bile acid levels. Some of the observed differences between the Ma study and our study, could be due to differences in species, model and *in vivo* nature of our study.

Our observations are also in line with in-vitro studies conducted by Gorelik et al in 2002 (14), where isolated rat cardiomyocytes show a reversible attenuation in rate and amplitude of contractility when exposed to increasing concentrations of taurocholate.

Cardiac dysfunction in cirrhosis is complex and multifactorial. Portal hypertension, cholestasis, cholanemia, metabolic derangement, increased circulating cytokines, occult endotoxemia and malnutrition are features of end stage liver disease. Each of these contribute to myocardial dysfunction and thus is an ongoing challenge to try to dissect out the individual and combined roles in the pathophysiology of cirrhotic cardiomyopathy. In the DDC fed mouse model of biliary fibrosis, splenic pulp pressures (surrogate for portal pressures) were similar (data not shown), suggesting that cardiomyopathy in this model was independent of portal pressures. Though the correlations between bile acid levels and key physiologic and molecular parameters will have to be interpreted with caution, because the relatively small sample size, it is nevertheless interesting that cardiac physiologic and molecular parameters correlated with the degree of cholanemia.

To further examine the contribution of elevated bile acids to cardiac dysfunction, cholanemia was induced in Abcb11$^{-/-}$ mice, by feeding 0.5% cholic acid supplemented diet for 1 week. This clinically relevant model exhibits biliary fibrosis, cholestasis and cholanemia within 1 week of feeding (Fig. 6A) (27, 28). 2DE, electrocardiography, protein and gene analysis of the hearts of these mice reproduce essential features of the cardiomyopathy seen in DDC fed mice. These mice show bradycardia, increased LV ejection and shortening fractions, phosphorylation (activation) of AKT and phosphorylation (inhibition) of GSK3β, as well as fetal gene expressions, all of which are hallmarks of reverse cardiac remodeling.

Bile acids have long been known to be toxic to the heart. Studies conducted by Joubert in 1978, show that cholic acid has a dose-dependent negative chronotropic effect on isolated atria (15) as well as whole hearts (16) in rats. Studies further show that cholic acid antagonizes effect of isoprenaline on the heart of Wistar rats (15). Despite the fact that the dose of cholic acid used in those experiments was supraphysiologic, these experiments were the first to suggest a direct bile acid-myocardial interaction. Series of in vitro experiments done by Gorelick et al (14, 42–44) have consistently shown that taurocholic acid affects

cardiomyocyte contractility and calcium transients. Removal of taurocholic acid restores the contractility of the cardiomyocytes (14) emphasizing reversibility. Zavek et al show cholic acid, but not ursodeoxycholic acid produced a decrease in basal cardiac contractility and responsiveness to beta-adrenoceptor activation suggesting that hydrophobic properties of bile acid determine its effect on cardiac physiology (17). Recently, Rainer and colleagues have shown that hydrophobic bile acids at high concentrations (often seen in cholestatic diseases) also induce arrhythmias in adult human hearts (45). At a molecular level, we have shown activation of AKT and inhibition of GSK3β in isolated neonatal mouse cardiomyocytes when challenged with tauro-chenodexycholic acid and lithocholic acid (18). These in vivo, ex vivo and in vitro studies support the concept of pathologic bile acid-myocardial interaction as drivers of pathology.

As mentioned before, cardiac dysfunction in end stage liver disease is multifactorial. The fact that it resolves with resolution of liver injury (both in humans as well as in experimental models), proves that altered milieu induced by liver injury causes cardiomyopathy. Though we still do not know what initiates and maintains the cardiac dysfunction, we can only speculate on its resolution. Here we speculate a role for bile acids and cholanemia as critical contributors of cardiomyopathy. Our speculation is strengthened by multiple in vitro and ex vivo studies (12, 14–17, 39, 42, 43, 45–49) which nicely compliment our current in vivo observation. We propose (Fig. 7) that circulating mediators, such as high levels of bile acids – a known consequence of liver injury, induces myocardial stress, leading to functional, structural and metabolic adaptations. These adaptations, when overwhelmed, with secondary stressors such as pharmacologic stress (catecholamines), infectious stress (sepsis), physiologic stress (exercise), hypovolemic stress (hemorrhage) and surgical stress (TIPS, transplantations etc), lead to decompensations and cardiac failure. On resolution of liver injury, after normalization of bile acids, vascular tone, nutritional status, inflammatory milieu, the heart remodels back to normal, as seen in patients post-transplant (9). Elucidating the molecular mechanisms and pathways of initiation, maintenance and resolution of cirrhotic cardiomyopathy, with specific focus on bile acid pathobiology is the obvious next step of these initial studies. Specifically the role of membrane receptors like the muscarinic receptors, TGR5, and nuclear receptors such as FXR need further in depth study. How bile acids affect myocardial contractility, its effects on calcium transients, and effect on proteins regulating calcium flux into and out of the sarcoplasmic reticulum needs to be studied in physiologic and molecular detail.

In summary, functional, structural, electrocardiographic and molecular aberrations as well as catecholamine insensitivity seen in DDC fed mouse model of biliary fibrosis resolve with resolution of liver injury. Pathophysiologic and molecular alterations that define this cardiomyopathy appear to correlate with serum levels of circulating bile acids. Bile acid feeding reproduces essential features of cirrhotic cardiomyopathy. These studies provide support for an association between liver injury and cardiac dysfunction and demonstrate that the heart responds to signals from the diseased liver, suggesting a critical role for a liver-heart interaction. This study also proposes the concept of pathologic bile acid-myocardial interaction, as a key mediator of cardiac dysfunction, which should be rigorously evaluated. Modulating this interaction could offer a viable therapy for optimizing hemodynamic status and reversing cirrhotic cardiomyopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2DEcho	Two-dimensional echocardiography
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
AKT	v-akt murine thymoma viral oncogene/Protein kinase B
βAR	β-adrenergic receptor
BA	bile acids
β MyHC	β myosin heavy chain
Abcb11	bile acid transport in sister of P-glycoprotein
cardiac TNNT2	cardiac troponin T2
DDC	0.1% 3, 5-diethoxycarbonyl-1, 4-dihydroxychollidine
%EF	ejection fractions
%FS	shortening fractions
FAO	fatty acid oxidation
GLUT	Glucose transporter proteins
GSK3β	Glycogen Synthase Kinase-3β
h-FABP/FABP-3	heart-type fatty acid binding protein
mCPT-2	mitochondrial-carnitine palmitoyl transferase-2
UCP-3	uncoupling protein-3

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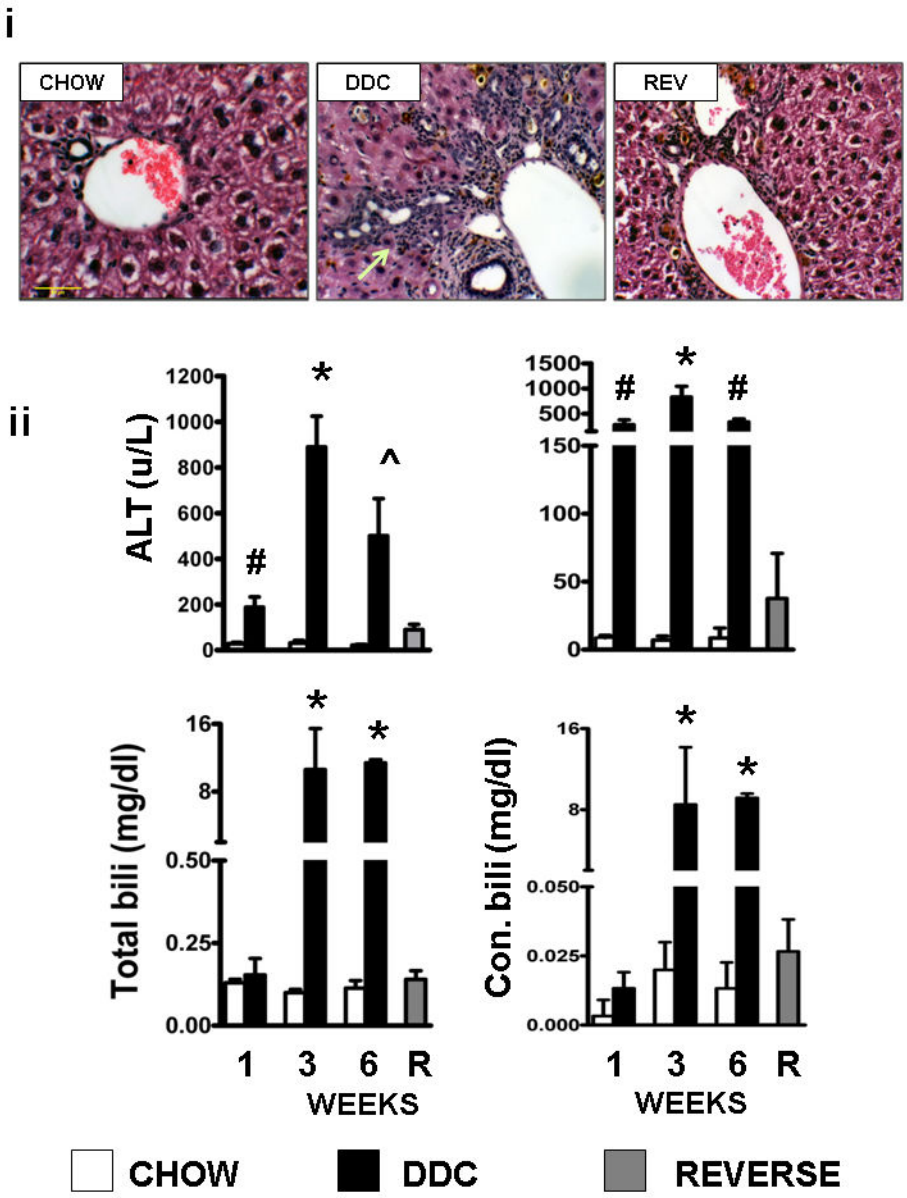


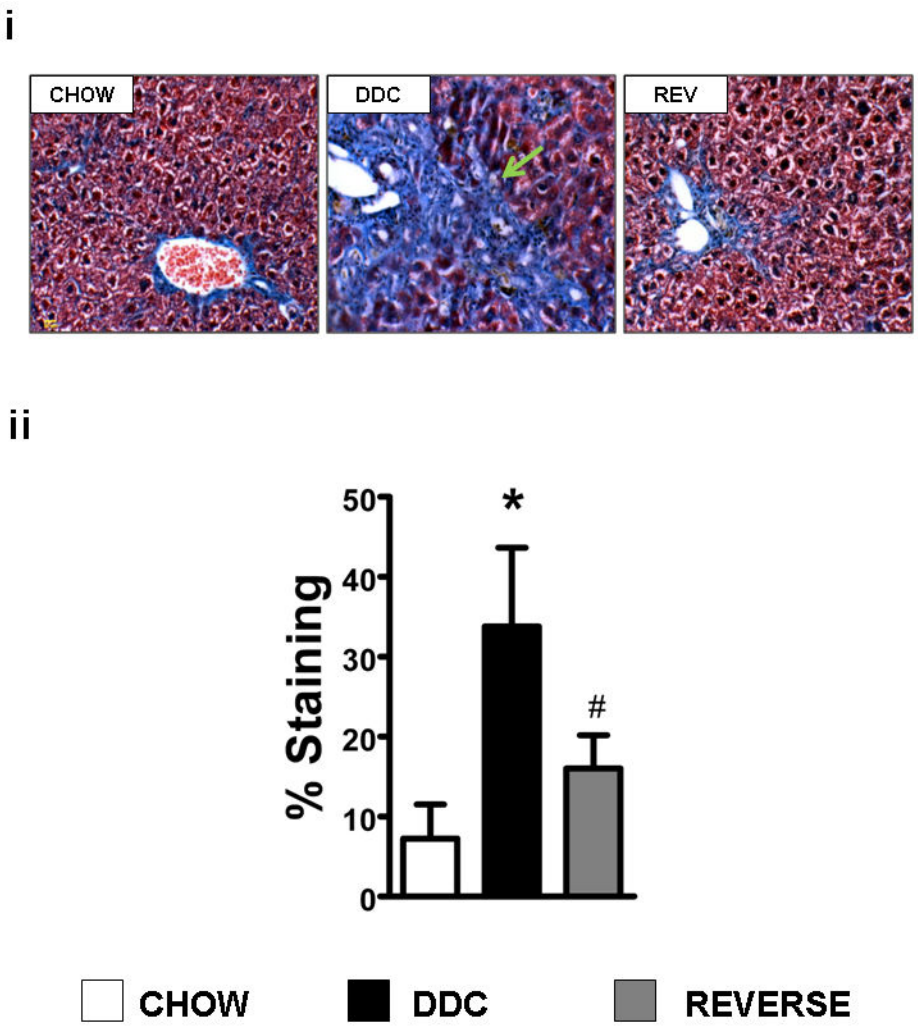


Figure 1.

Fig. 1A: Liver injury resolves with reversal of DDC diet: **(i)** Hematoxyline-Eosin stained representative liver sections of diet-reversed mice (REV) shows recovery but not complete resolution of biliary hyperplasia when compared to livers of mice fed either chow or DDC for 6 weeks. Note severe biliary hyperplasia in 6 week DDC fed mice (arrow). Mag: 40×; scale bar=50μ. **(ii)** denotes bar graphs of ALT, total and conjugated bilirubin and serum bile acid levels in the mice fed 1 week, 3 weeks and 6 weeks of chow, DDC diet and the reversal group. Note normalization of liver injury, degree of cholestasis and cholanemia in the diet-reversed group (R) (n=5 per group; Results: Mean±SD; Stats: ANOVA with Neuman–Keuhls post –hoc).

Fig. 1B: Partial resolution of biliary fibrosis with reversal of DDC diet: **(i)** Mason-Trichrome stained representative liver sections of diet-reversed mice (REV) shows recovery but not complete resolution of biliary fibrosis when compared to livers of mice fed DDC for 6 weeks. Note severe biliary hyperplasia and fibrosis in 6 week DDC fed mice (arrow). Magnification used 20×; scale bar=50μ. **(ii)** Bar graphs showing quantitative analysis of fibrosis using Image J (NIH). Results reported as percent area stained blue by collagen. (n=3

mice/group; 4 randomly selected images in 20× magnification; Results: Mean±SD; Stats: ANOVA with Neuman–Keuhls; * $p<0.001$; # $p<0.05$)

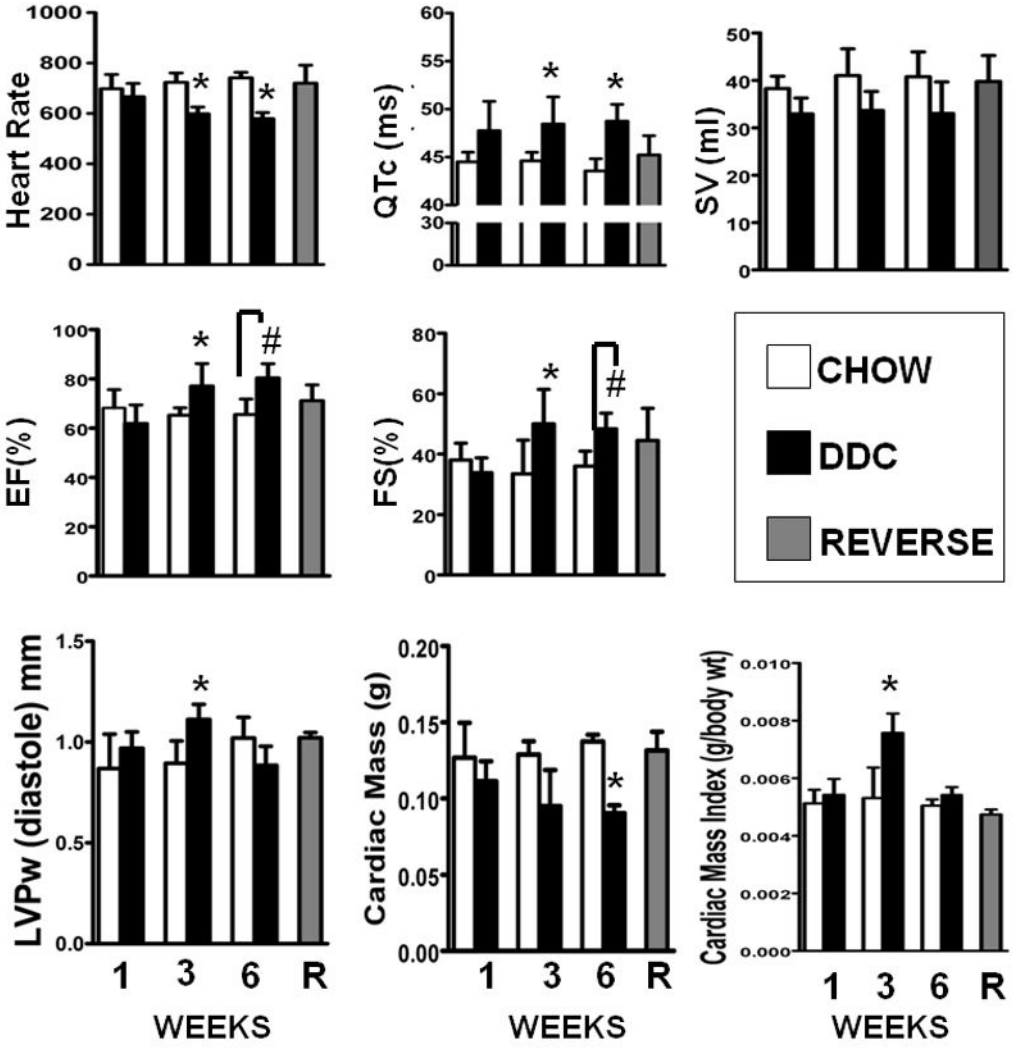


Fig. 2. Normalization of key ECHO parameters on resolution of liver injury

Bar graphs denote key cardiac physiologic parameters, heart rate (HR), corrected QT interval (QTc) as analyzed by rhythm strips, shortening fractions (%FS), ejection fractions (%EF), Left ventricular posterior wall thickness at end diastole (LVPw), cardiac mass, cardiac mass index and stroke volume as assessed by sedated mouse 2DEchoes. Note normalization of the physiologic indices in reversed (REV) group. (* $p<0.05$; Results: Mean ±SD; n=6).

Fig.3A(i)

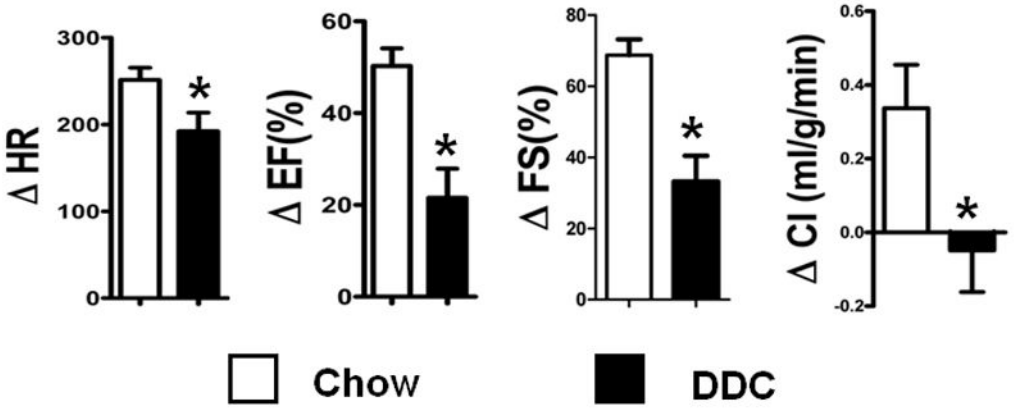
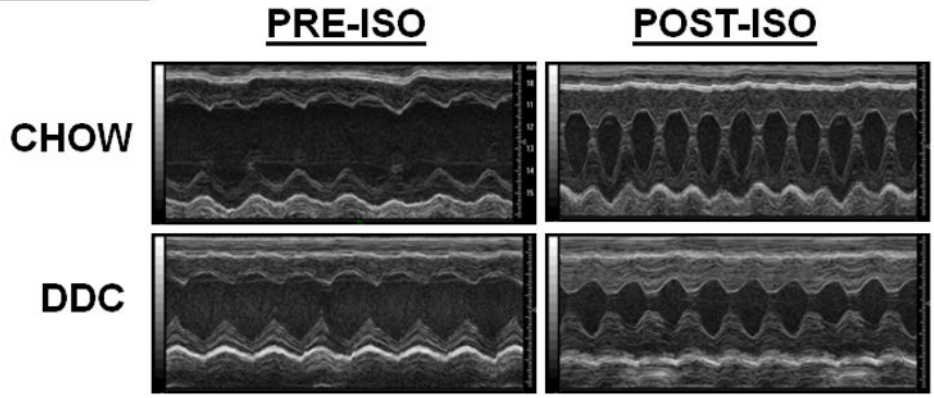


Fig.3A(ii)



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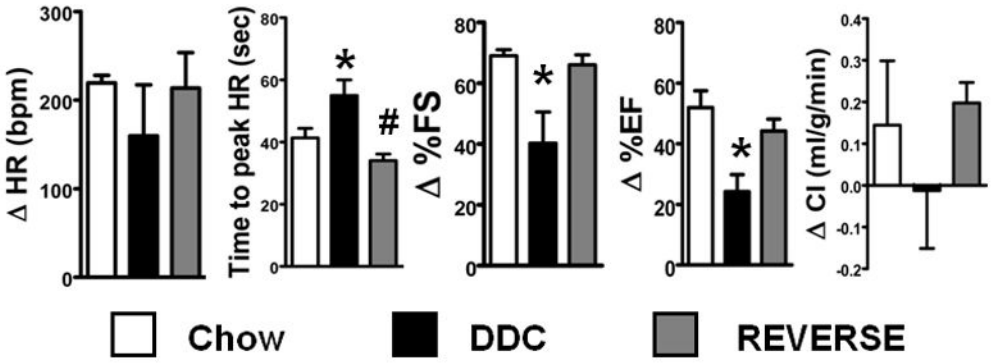


Fig.3B(ii)

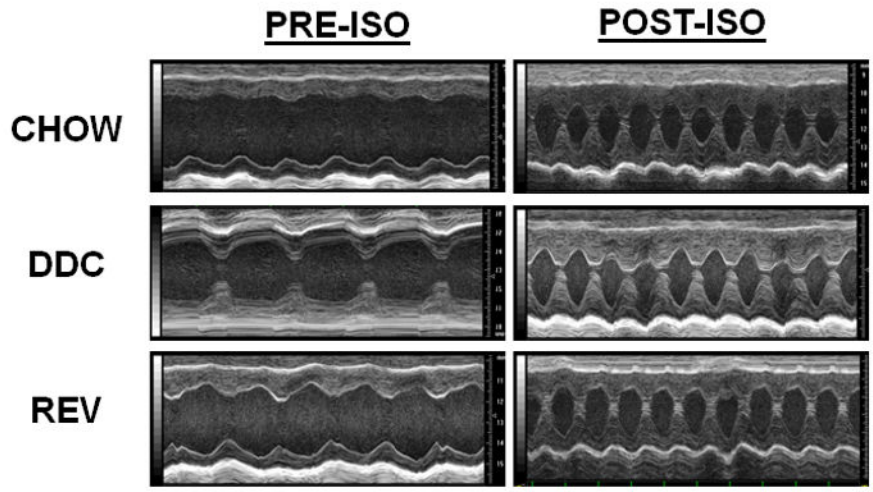
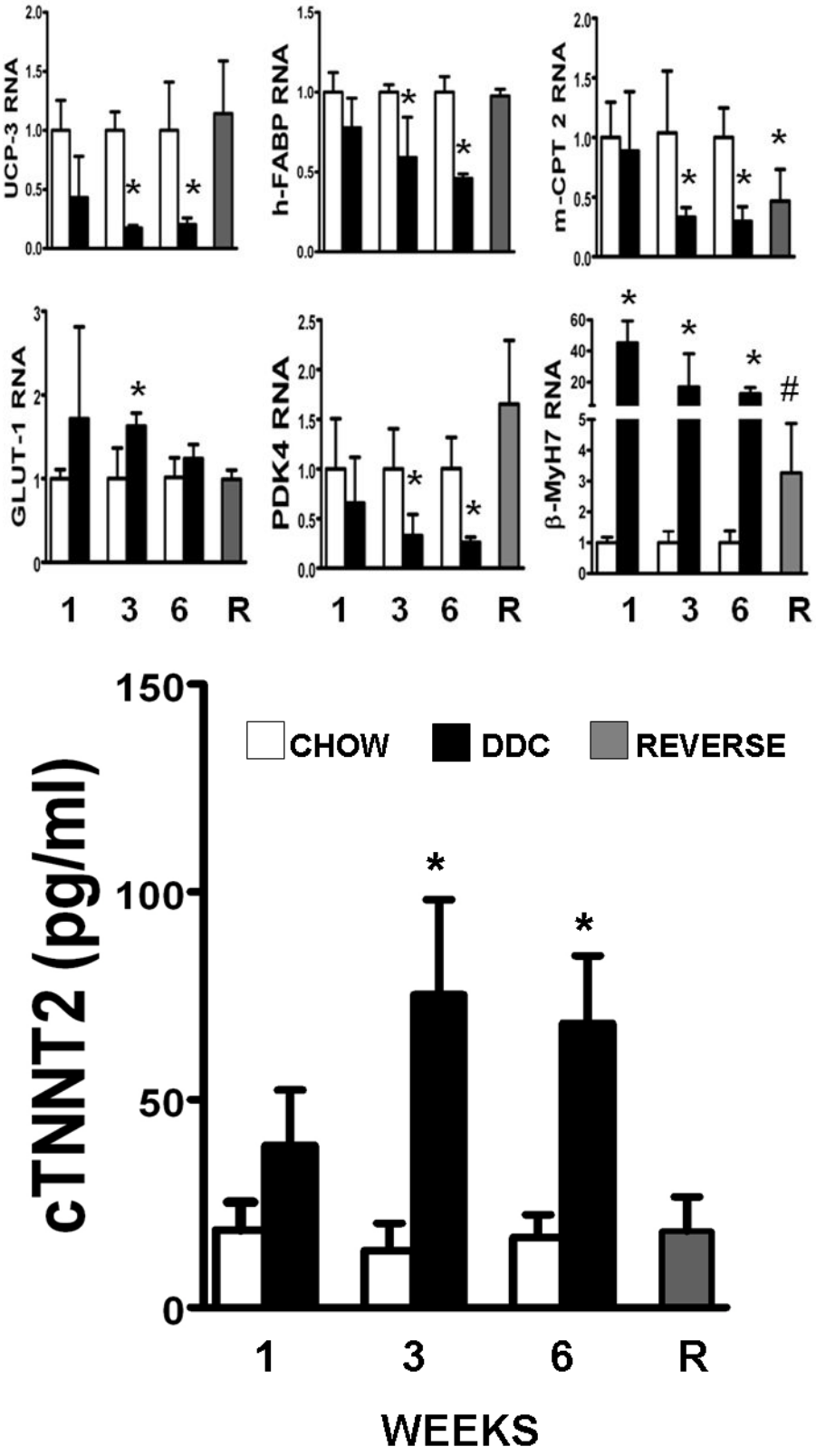


Figure 3.

Fig. 3 (A): Catecholamine insensitivity in mice with biliary fibrosis. Mice with biliary fibrosis induced by 3 weeks of DDC feeding show inappropriate cardiac response to isoprenaline challenge when compared to normal chow fed counterparts. **(i**) Note attenuated changes (Δ) in key ECHO parameters of heart rate (HR), Ejection fraction (EF), shortening fraction (FS) and cardiac output indexed to weight (CI). **(ii)** shows representative ECHO pictures in M-mode showing attenuated contractility in DDC fed mice post isoprenaline challenge. (n=5 per group; Results: Mean±SD;* $p<0.05$).

Fig. 3 (B): Catecholamine insensitivity resolves with resolution of liver injury: **(i)** shows bar graphs of heart rate, ejection and shortening fraction and cardiac index in mice in the reversal group as compared to chow and 6 week DDC fed group. Note normalization of response to isoprenaline after reversal of liver injury. **(ii)** shows representative M-mode ECHOs showing improved isoprenalien response in reversal group.(n=5 per group; Results: Mean±SD;* $p<0.05$; # $p<0.05$ compared to chow).



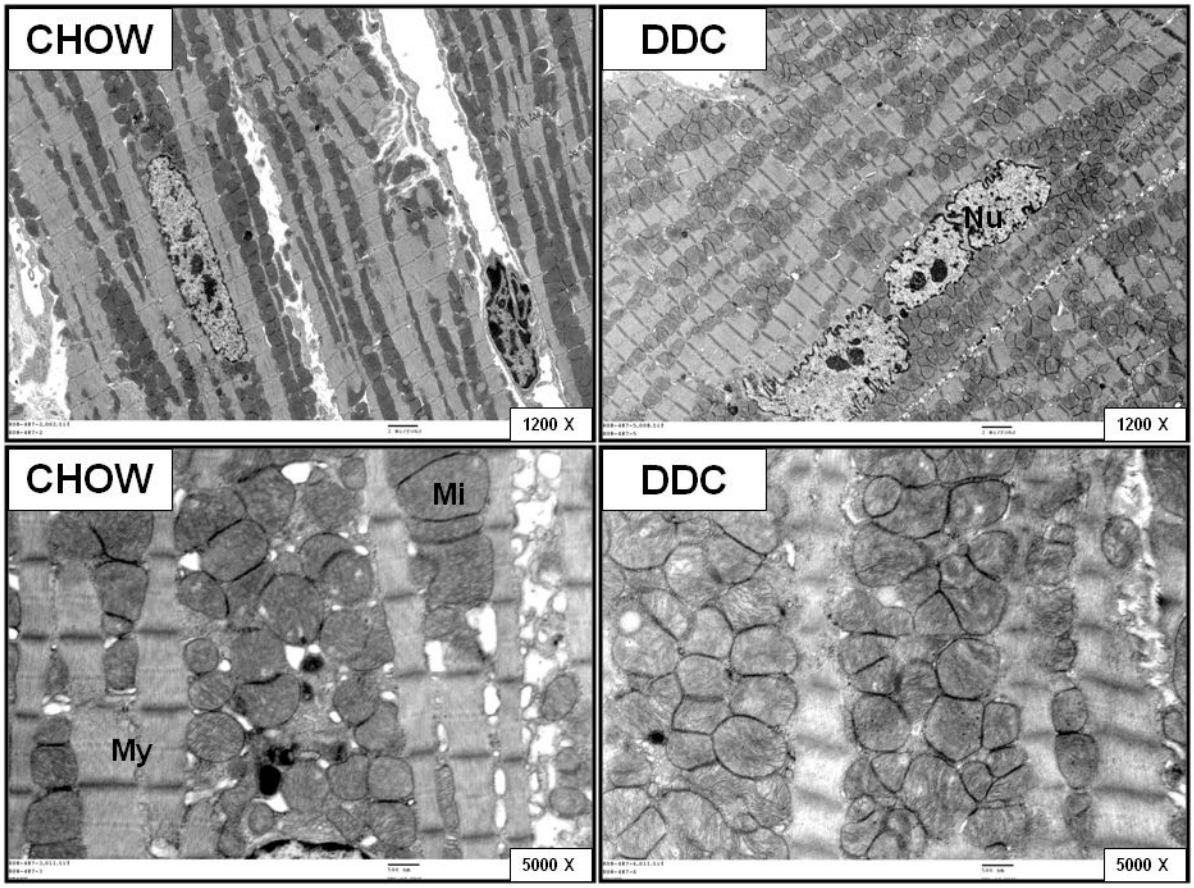
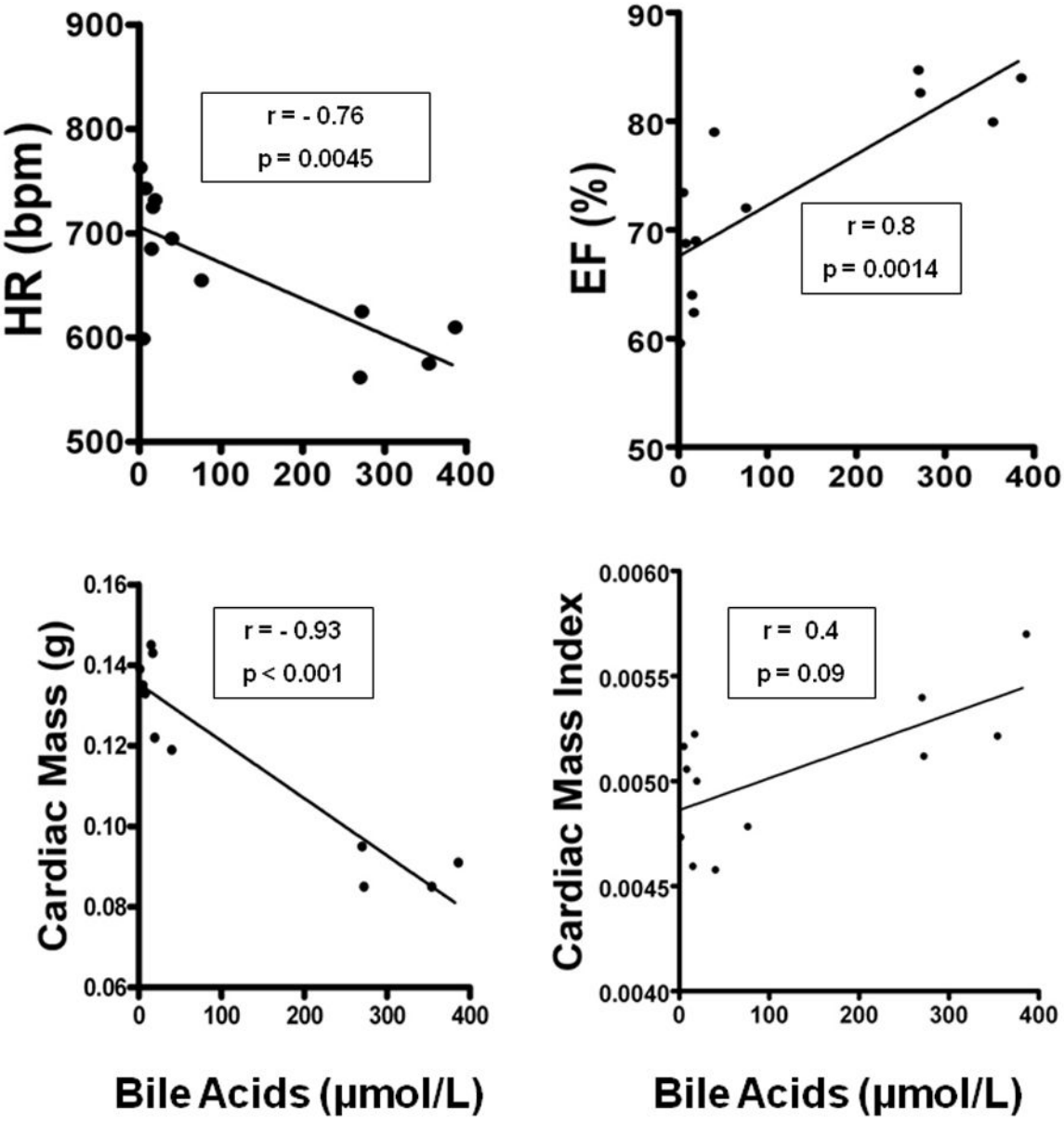


Figure 4.

Fig. 4A: Heart re-models to adult metabolic gene program after reversal of liver injury: Bar graph shows QPCR results of key “fetal” genes expressed by heart in stress. Note RNA levels UCP3, h-FABP (key genes regulating fatty acid oxidation); GLUT-1 (glucose uptake) and PDK4 (glucose oxidation) return to chow fed (non-stressed) levels. βMYH7 RNA expression falls significantly after diet reversal compared to the DDC fed mice. This suggests that fetal profile of the stressed DDC fed hearts reverses to non-stressed adult profile after liver injury is reversed. RNA levels are standardized to GAPDH and denoted as fold change compared to chow fed hearts within each group.(*$p<0.05$ vs. all groups; #$p<0.05$ vs. all groups; Results: mean±SD; n=5 in each group).

Fig. 4B: Bar graph showing serum circulating levels of cTNNT2, as analyzed by ELISA. Note 4 fold increase in cTNNT2 in 3 and 6 week DDC fed animals as compared to chow. cTNNT2 levels normalized to chow levels in the Reversal (R) group. n=4–5/group; Results: Mean±SD; Stats: ANOVA with Neuman-Keuhls post–hoc; *$p<0.05$)

Fig. 4C: Representative Transmission Electron Microscopic pictures (EM) of muscles isolated form the left ventricles of 3 weeks of chow or DDC feeding (n=3 in each group). There was no evidence of myocyte destruction/loss/apoptosis in the DDC fed mice. Here myocytes (My), nucleus (Nu) and mitochondria (Mi) do not show any damage or injury.



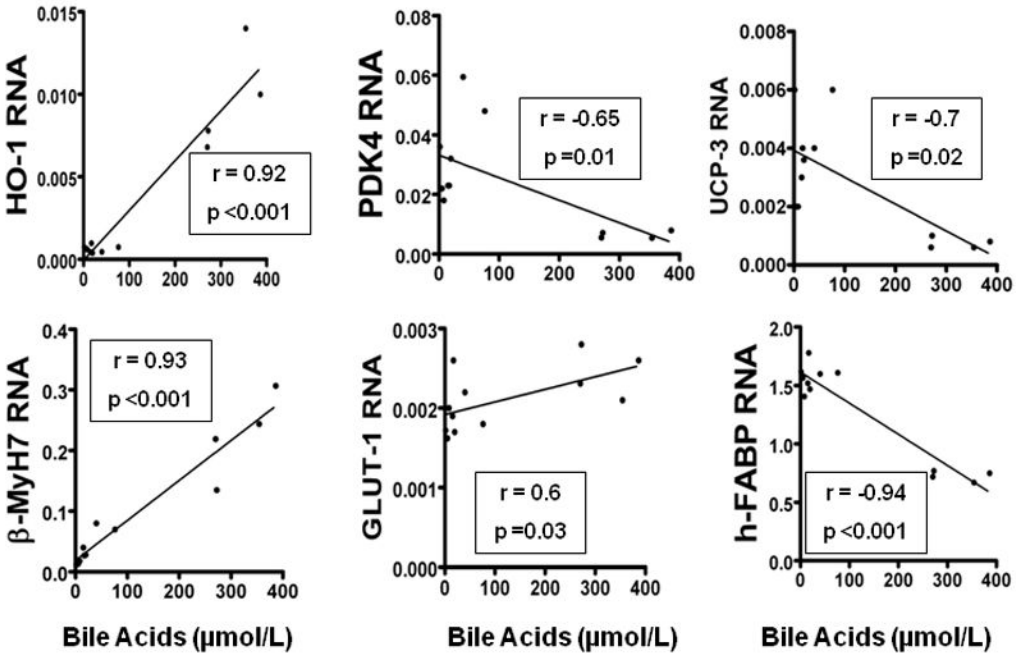
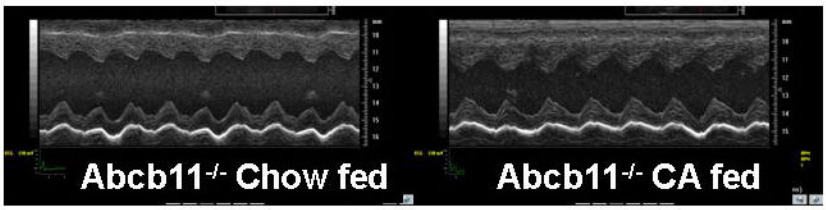
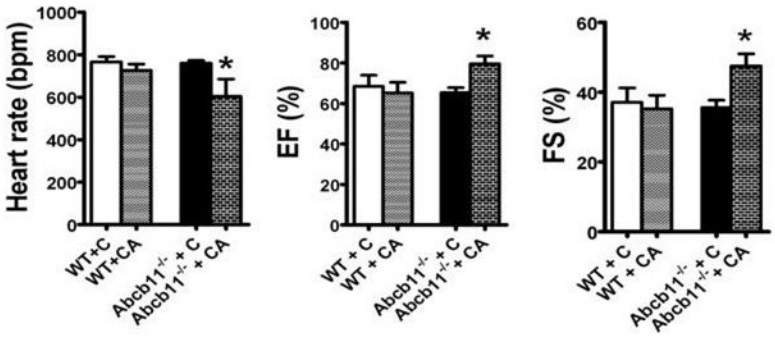


Figure 5.

Fig. 5A: Circulating serum bile acid levels are associated with cardiac physiologic alterations: Regression analysis of key cardiac physiologic parameters (heart rate (HR), ejection fraction (EF), un-indexed and indexed cardiac mass and serum bile acid levels at the end of 6 weeks in chow/DDC/Reversal group, show a negative association between HR, and cardiac mass (unindexed) and a positive association between bile acid levels and LV ejection fraction. (Stats: Pearson correlation, n=15).

Fig. 5B: Circulating serum bile acid levels are associated with gene alterations in the heart: Regression analysis of key cardiac RNA levels suggest a positive association between bile acids levels and HO-1, GLUT-1 and βMYH7 RNA and a negative association between UCP-3, h-FABP and PDK4 and bile acid levels at the end of 6 weeks in groups fed either chow, DDC or reversal of diet. (Stats: Pearson correlation, n=15).





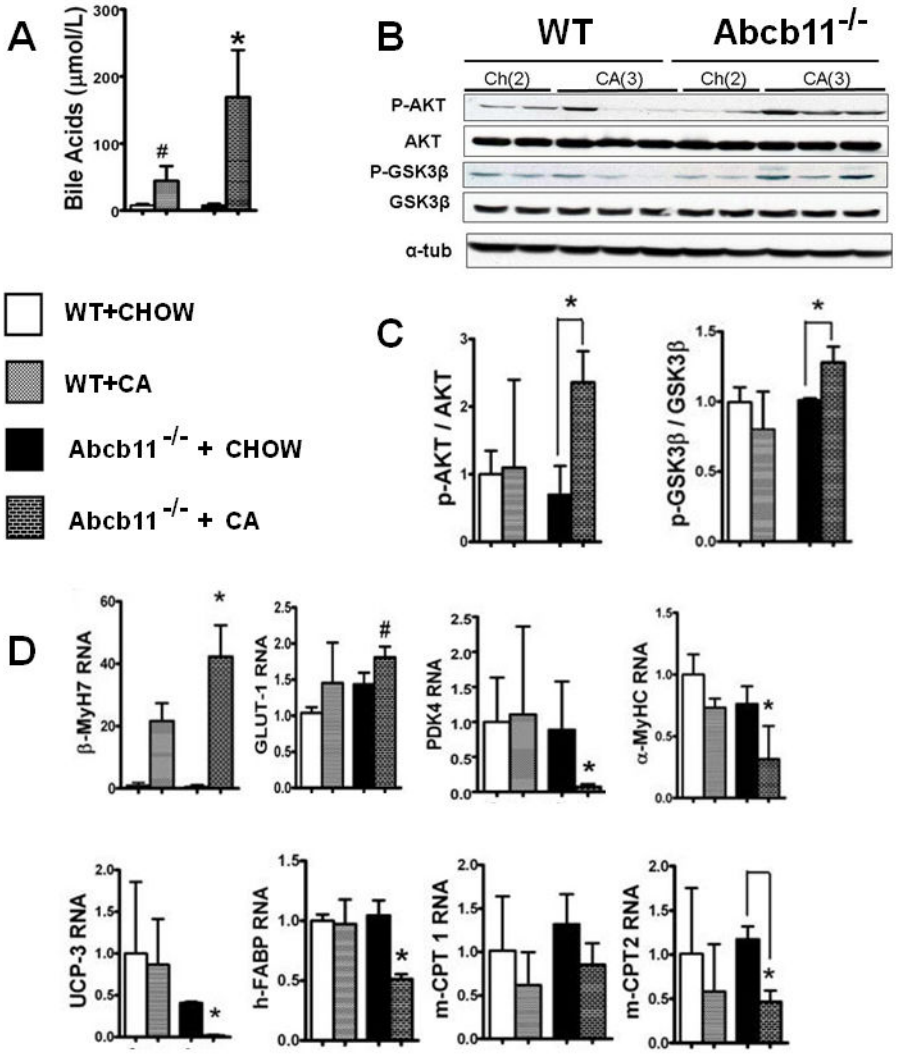


Figure 6.

Fig. 6A: Cholanemic CA fed Abcb11−/− mice show altered cardiac physiology. Top panel shows representative M-mode 2DEs depicting increased LV contractility in CA fed cholanemic Abcb11$^{-/-}$ mice. Bar graph below shows CA fed cholanemic Abcb11$^{-/-}$ mice demonstrate lower heart rate, increased ejection (EF) and shortening fraction (FS). (*p<0.05; n=4/grp; ANOVA, Neuman–Keuhls, Results:Mean±SD).

Fig. 6 (B): Bile acid fed cholanemic Abcb11−/− mice show altered myocardial signaling. **(A)** Denotes serum bile acid levels in WT and Abcb11−/− mice. **(B)** shows representative western blots for AKT, *Ser*473-phospho-AKT, GSK3β, *Ser*9-phospho-GSK3β, with α-tubulin. **(C)** shows analysis of the respective bands normalized to α-tubulin depicting fold change in phosphorylation of AKT and GSK3β. **(D)** QRTPCR results for β-MyH7, α-MyHC, GLUT-1, PDK4, UCP-3, h-FABP, m-CPT-1 and 2. Values are normalized to GAPDH and denote fold change compared to chow fed WT mice. Results: Mean±SD, ANOVA, n=4/group; *p<0.05; # p<0.05 vs. chow fed).

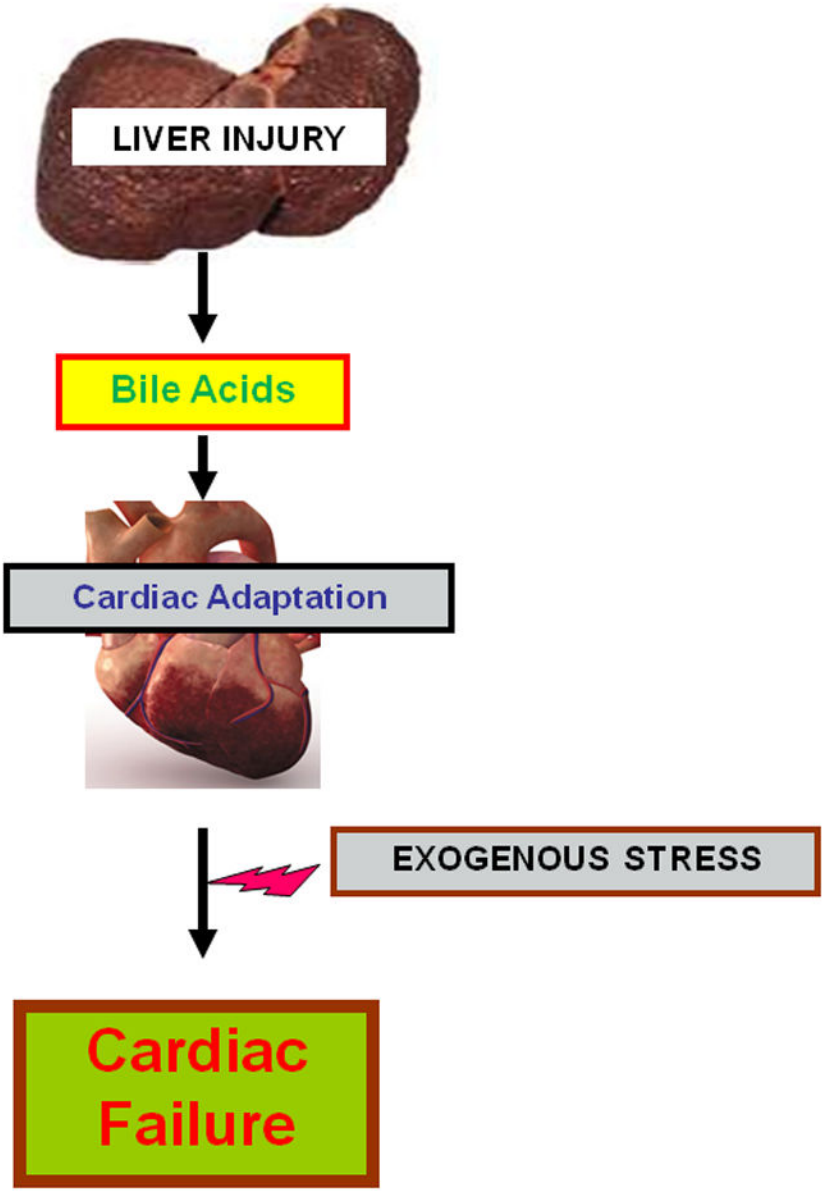


Fig. 7. Postulated mechanism for cirrhotic cardiomyopathy

Heart adapts to bile acid induced stress. The adaptive response is overwhelmed when combined with exogenous stressors such as isoprenaline (catecholamine) resulting in cardiac failure.