Published in final edited form as:

Toxicol Lett. 2019 October 15; 315: 87–95. doi:10.1016/j.toxlet.2019.08.010.

N-Acetylcysteine prevents the decreases in cardiac collagen I/III ratio and systolic function in neonatal mice with prenatal alcohol exposure

Van K. Ninh^a, Elia C. El Hajj^a, Martin J. Ronis^b, Jason D. Gardner^{a,*}

^aLSU Health Sciences Center, Department of Physiology, 1901 Perdido Street, New Orleans, LA, 70112, United States

^bLSU Health Sciences Center, Department of Pharmacology, 1901 Perdido Street, New Orleans, LA, 70112, United States

Abstract

Prenatal alcohol exposure (PAE) is often associated with congenital heart defects, most commonly septal, valvular, and great vessel defects. However, there have been no known studies on whether PAE affects the resulting fibroblast population after development, and whether this has any consequences in the postnatal period. Our previous study focused on the effects of PAE on the postnatal fibroblast population, which translated into changes in cardiac extracellular matrix (ECM) composition and cardiac function in the neonatal heart. Moreover, our lab has previously demonstrated that alcohol-induced fibrosis is mediated by oxidative stress mechanisms in adult rat hearts following chronic alcohol exposure. Thus, we hypothesize that PAE alters cardiac ECM composition that persists into the postnatal period, leading to cardiac dysfunction, and these effects are prevented by antioxidant treatment. To investigate these effects, pregnant mice were intraperitoneally injected with 2.9 g EtOH/kg body weight on gestation days 6.75 and 7.25. Controls were injected with vehicle saline. Randomly selected dams in both groups were then treated with 100 mg/kg body weight of the antioxidant *N*-acetylcysteine (NAC) immediately after EtOH or vehicle administration. Left ventricular (LV) chamber dimension and function were assessed in sedated animals on neonatal day 5 using echocardiography. Ejection fraction decreased in the PAE group. NAC treatment prevented this depression of systolic function in PAE neonates. Hearts were analyzed for expression of fibroblast activation markers. Alpha smooth muscle actin (α -SMA) increased in PAE neonatal hearts, and this increase was prevented by NAC treatment. In PAE pups, collagen I decreased, but collagen III expression increased compared to saline animals; the overall collagen I/III ratio significantly decreased. When PAE mice were treated with NAC, collagen I/III ratio did not change. Overall, our data demonstrate that prenatal alcohol exposure produces changes in collagen subtype in neonatal cardiac ECM and a decline in systolic function, and these adverse effects were prevented by NAC treatment.

*Corresponding author at: LSUHSC Physiology, 1901 Perdido St., MEB 7205, New Orleans, LA, 70112, United States., jgardn@lsuhsc.edu (J.D. Gardner).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Keywords

Fetal alcohol spectrum disorder; Neonate; Neonatal heart; Antioxidant; Heart; Extracellular matrix; Hypertrophy

1. Introduction

The Office of the United States Surgeon General currently recommends that no amount of alcohol should be consumed by women during pregnancy because of the risk of birth defects (Seiler, 2016). The range of behavioral and congenital birth defects caused by alcohol consumption during pregnancy is collectively known as Fetal Alcohol Spectrum Disorder (FASD), with Fetal Alcohol Syndrome being the worst category on the spectrum (Jones and Smith, 1973). There is extensive evidence in the literature that alcohol exposure during gastrulation is harmful to the developing fetus; this period during development reflects an early time point in which a woman may not be aware that she is pregnant (Linask and Han, 2016; Serrano et al., 2010; Boschen et al., 2018; Cavieres and Smith, 2000). Though there is a large body of literature on alcohol as a teratogen, most of these studies focus on neurodevelopmental defects. Comparatively, there is much less known on the effects of alcohol on the developing heart, and even less on how this may affect the newborn (Henderson et al., 1995; Daft et al., 1986; Kockaya and Akay, 2006; Blakley and Scott, 1984). Alcohol exposure during pregnancy causes severe congenital heart defects in fetal alcohol syndrome. However, standard screening of newborns on the fetal alcohol spectrum may not detect more understated cardiac changes, which may increase the risk for cardiovascular diseases (Parkington et al., 2010).

As such, there is growing interest in the adverse effects of FASD beyond the immediate cardiac congenital defects and how this may impact the cardiovascular health of exposed offspring. In our own studies using a preclinical model of prenatal alcohol exposure (PAE) in mice, we observed reductions in the heart weight to body weight ratio, changes in extracellular matrix (ECM) protein expression of collagens I and III, and cardiac dysfunction in exposed neonates (Ninh et al., 2019). The ECM provides structural scaffolding for the cardiomyocytes and thus is critical for normal heart formation and function after birth (Deb and Ubil, 2014). The composition and stiffness of the ECM directly regulates ventricular relaxation, force development, and mechanotransduction during contraction (Collier et al., 2012). Changes in the collagen profile of the ECM affects the physical properties of the heart, and the decrease in the collagen I/III ratio in the PAE pups may play a key role in the reduced cardiac function we observed (Collier et al., 2012).

However, the mechanism by which alcohol exposure alters the development of the myocardium and cardiac ECM is unknown. Unlike many other teratogens and drugs, alcohol does not have a direct receptor or target, and molecules readily cross through the plasma membrane of cells (Zakhari, 2006). The metabolism of alcohol into reactive aldehydes causes damage to biomolecules such as proteins, lipids, and nucleic acids (Zakhari, 2006). As such, oxidative injury has been widely speculated to play a major role in the problems seen in prenatal alcohol exposure (Brocardo et al., 2011). Additionally, in utero alcohol

exposure has been shown to cause oxidative damage to lipids and decrease the antioxidant glutathione in rat fetus (Henderson et al., 1995; Fernandez-Checa et al., 1991). Corroborating the role of oxidative damage in FASD defects, there are numerous studies that show the beneficial effects of antioxidant treatment against fetal alcohol exposure (Mitchell et al., 1999; Cano et al., 2001; Chen et al., 2004; Ojeda et al., 2018). As such, we hypothesize that prenatal alcohol exposure creates an environment of oxidative stress, leading to a more compliant ECM, and cardiac dysfunction in neonates, and treatment with an antioxidant can prevent these effects.

2. Materials and methods

2.1. Prenatal alcohol exposure (PAE) model and N-acetylcysteine antioxidant treatment

All experimental studies were performed on C57Bl/6 mice purchased from Envigo (Indianapolis, IN) and their offspring. Studies and animal handling adhered to the principles of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals 8th edition, and approved by LSU Health Sciences Center's Institutional Animal Care and Use Committee. Approximately eight week old male and female mice were mated overnight. The detection of a mucosal vaginal plug the next morning was designated as gestation day 0.5, and the breeding pairs were separated. Pregnant mice were randomly separated into 4 experimental treatment groups. All groups received treatment through two intraperitoneal injections on gestation days 6.75 and 7.25 (approximately 11:00 AM and 5:00 PM, respectively). The prenatal alcohol exposure (PAE) group were administered two intraperitoneal injections of 2.9 g ethanol/kg maternal body weight of a 30% (volume/volume) ethanol solution in sterile saline. Control mice designated as the Saline group were administered an equal volume of vehicle saline based upon body weight per injection. The *N*-acetylcysteine (NAC) treatment group was administered 100 mg/kg maternal body weight dissolved in vehicle saline per injection (Sigma Aldrich A7250–100 g). The last treatment group designated as NAC PAE received 2.9 g ethanol/kg maternal body weight of 30% (volume/volume) ethanol and 100 mg/kg maternal body weight *N*-acetylcysteine dissolved in vehicle saline per administration. After the treatments, the gestational period continued uninterrupted. Upon birth of neonates in each treatment group, pups were aged to 5 days old. Cardiac function was analyzed using echocardiography. The neonates were weighed and measured from the crown of their skull to their rump in order to assess body length. Heart weight to body weight ratio was calculated based on these measurements. Once the morphological assessments were obtained, the neonates were sacrificed and whole hearts were then collected for tissue analysis. Approximately 130 pups and 60 dams were involved in this study. For each experiment, n = 4–9 per treatment group.

2.2. Maternal blood alcohol content

Blood alcohol content (BAC) were assessed in pregnant mice in PAE and NAC PAE treatment groups. Six dams in each group were sacrificed 10 min after the second intraperitoneal (IP) injection of respective treatments in order to assess representative peak blood alcohol concentration for the animals in our study. Trunk blood was collected, centrifuged, and the plasma was analyzed using Analox GM7 analyzer (London, UK).

2.3. Echocardiography

5 day old neonates in each experimental group were sedated (2% isoflurane) and echocardiography was performed with MX700 transducer to assess left ventricular (LV) chamber dimension and function (VEVO 3100, VisualSonics; Toronto, CA). B-mode two-dimensional images and M-mode tracings were obtained from a short axis-view of the left ventricle. Using these images, LV mass, LV chamber diameter, and posterior and anterior wall thickness were measured and assessed at both systole and diastole. These parameters were used to calculate ejection fraction, fractional shortening, and LV volumes using the VevoLab software. No significant differences in heart rate between groups were observed (average rate of 410 ± 25 BPM).

2.4. Measurement of lipid peroxidation

Approximately 10–15 mg of cardiac tissue per sample were briefly homogenized using RIPA buffer (#899900; Pierce Antibody Products; ThermoFisher Scientific; Waltham, MA) with HALT protease inhibitor (#78440; ThermoFisher Scientific). Samples were then assessed for alcohol-induced lipid peroxidation by measuring malondialdehyde concentration per gram of tissue. Malondialdehyde concentration was assessed through controlled reactions with thiobarbituric acid reactive substances (TBARS) assay (#10009055; Cayman Chemical; Ann Arbor, MI).

2.5. Western blot analysis

Western blot analyses were performed by randomly selecting one male neonate from each litter, and the assay was performed as previously described (Ninh et al., 2018). The unit of analysis was one litter, or more specifically, one male from each litter. Male neonates were collected into a single cage, and one male was blindly selected. This random selection was performed in order to avoid biases in selecting and analyzing neonates based on perceived weight, appearance, or illness. In future studies, analysis of female neonates are warranted. Sample sizes for Western Blot analyses ranged from $n = 5-9$ for each group. Briefly, 10–15 mg of ventricular tissue for each sample was homogenized in RIPA buffer (#899900; Pierce Antibody Products ThermoFisher Scientific) with HALT protease and phosphatase inhibitor cocktail (#78440; ThermoFisher Scientific). Bradford Assay was used to assess protein concentration of homogenized tissue for each sample. 50 μ g of protein from each sample were mixed with Laemmli Buffer (#161–0737; Bio-Rad; Hercules, CA) in 1:1 ratio and were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF). PVDF membranes were incubated overnight at 4 °C with the following dilutions of primary antibody against NADPH oxidase 4 (1:1000, Abcam; ab133303; Cambridge, UK), Nrf2 (1:1000, Sigma #SAB4501984; Sigma-Aldrich; St. Louis, MO), Nitrosylated tyrosine (1:500, Abcam ab42789), collagen I (1:1000, Abcam ab34710), collagen III (1:5000, Abcam ab7778), α smooth muscle actin (1:1000, Abcam ab5694). Histone H3 (1:5000, Abcam ab1791) or GAPDH (1:1000, Abcam ab9485) was used as a loading control. The membranes were incubated with goat anti-rabbit secondary antibody (1:1000, Abcam #97051) for 1 h at room temperature. Visualization of protein bands were performed by exposure to Western ECL substrate kit (Clarity; Biorad) and images collected with ImageQuant LAS 4000 imager (GE Healthcare Life Sciences; Malborough, MA).

Densitometry was performed using CareStream software, and all bands were normalized to either histone H3 or GAPDH protein expression.

2.6. Total cardiac collagen content

Total collagen content was quantitatively estimated by measuring hydroxyproline content of whole ventricular samples. Hydroxyproline was quantified using an established protocol (Stegemann, 1958; El Hajj et al., 2014). 30–40 mg of wet LV tissue were dried at 65 °C overnight and hydrolyzed in 6 N HCl overnight. The solution was then filtered, evaporated and resuspended in 1 ml of citrate buffer, pH 6.0. The resulting product was then reacted in a 16-well plate with isopropanol, antioxidant solution (Chloramine T) and Ehrlich Reagent (p-dimethylamino benzaldehyde) and measured at an absorbance of 540 nM. Data were expressed as ug of hydroxyproline per mg of dry tissue.

2.7. Statistics

Data processing and analysis were performed using Graphpad 7.0 (Prism; La Jolla, CA). Main effect and interactions between ethanol treatment and N-acetylcysteine antioxidant treatment were determined with two-way ANOVA. If significant interactions were observed, Tukey's post-hoc analysis was performed. A p-value of < 0.05 was considered significant. All values are shown as mean $\pm$ SEM.

3. Results

3.1. NAC treatment prevents decrease in neonatal body weight and length after PAE

Neonatal body weight and crown to rump length were measured prior to sacrifice on day 5 after birth; left ventricular mass was obtained via echocardiography and normalized to measured body weights. Representative maternal blood alcohol concentration was obtained from PAE and NAC PAE dams after the second alcohol exposure (Fig. 1). Body weight decreased significantly in PAE neonates compared to Saline counterpart. PAE animals treated with NAC had significantly higher body weights compared to PAE alone and NAC treatment alone (Fig. 1A). Crown to rump length decreased in PAE neonates compared to Saline, and NAC treatment prevented the decrease in body length in PAE animals (Fig. 1B). There is a main effect of PAE in LV mass/body weight ratio, but there is no significant interaction (Fig. 1C). The changes seen between PAE neonates and PAE neonates treated with NAC were not due to a difference in maternal blood alcohol concentration, as blood alcohol levels were not different between groups (Fig. 1D).

3.2. NAC treatment decreases oxidative stress markers in ventricles of PAE neonates

Oxidative stress results from an imbalance in reactive oxidant species and antioxidant capacity to scavenge free radicals. The NADPH oxidase 4 (NOX4) enzyme is a major producer of reactive oxidant species in cardiac tissue; whereas Nrf2 transcription factor regulates expression of antioxidant enzymes. Whole ventricular tissues from neonates of each experimental group were analyzed for markers of oxidative stress such as NOX4, Nrf2, nitrotyrosine, and malondialdehyde (Fig. 2). Protein expression of NOX4 significantly increased in PAE hearts compared to Saline; NAC treatment prevented this increase in PAE and NAC control animals compared to PAE alone (Fig. 2A). Ventricular tissue was then

analyzed for protein expression of Nrf2, and PAE decreased protein expression whereas NAC treatment prevented this decrease in PAE neonates (Fig. 2B). Nitrosylated tyrosine residues were used as a marker of oxidative damage to proteins. There was a main effect of NAC to reduce nitrotyrosine, but there was no significant interaction (Fig. 2C). Likewise, malondialdehyde was used as a marker of oxidative damage to lipids, and NAC treatment in PAE animals decreased oxidative stress markers compared to neonates with PAE alone (Fig. 2D).

3.3. NAC treatment prevents a decrease in the collagen I/III ratio in PAE neonatal hearts

To determine whether oxidative stress produced changes in the cardiac ECM, profibrotic markers were analyzed in whole heart tissue (Fig. 3). Alpha smooth muscle actin is a indicating myofibroblast transition, and this protein significantly increased in PAE animals compared to Saline controls. There was no significant change in expression of α -smooth muscle actin in NAC and NAC PAE animals compared to Saline, but NAC did decrease α -SMA expression compared to PAE animals (Fig. 3A). Collagen 1 confers more tensile strength and stiffness to the cardiac extracellular matrix, whereas collagen III confers a more compliant phenotype. There were no significant differences in collagen I and collagen III protein expression between any groups (Fig. 3B, 3C). However, when collagen I/III ratio was analyzed, PAE decreased the ratio significantly compared to Saline controls. NAC treatment in PAE animals prevented this decrease in the collagen I/III ratio compared to PAE alone (Fig. 3D). Despite these changes in the ratio of collagen subtypes, there were no changes in total collagen content between any of the groups (Fig. 3E).

3.4. NAC treatment prevents a decrease in systolic function in PAE neonates

Systolic function was assessed in 5 day old neonates using echo-cardiography in order to determine whether the changes in the ECM produced cardiac functional changes (Fig. 4). There were no differences between any of the groups when left ventricular diastolic volume (LV Vol;d) was measured (Fig. 4A). However, PAE significantly increased left ventricular systolic volume (LV Vol;s) compared to Saline. NAC treatment prevented the elevation in LV Vol;s compared to PAE animals (Fig. 4B). PAE caused a reduction in ejection fraction as compared to Saline. NAC control animals were not different than Saline controls. For the NAC PAE animals, the depression of systolic function was prevented (Fig. 4C). Similar results were found when fractional shortening was analyzed; however, NAC PAE animals had increased fractional shortening compared to NAC controls (Fig. 4D).

3.5. NAC prevents eccentric index and wall stress in PAE neonates

Left ventricular interior diameter and posterior wall thickness were measured at diastole and at systole by echocardiography. These parameters were used to calculate eccentric index during diastole and systole by the equations $LVID;d/(2*LVPW;d)$ and $LVID;s/(2*LVPW;s)$, respectively (Fig. 5). During diastole, LVID;d did not change between any of the groups (Fig. 5A). However, both NAC control and PAE produced a significant decrease in the thickness of the left ventricular posterior wall compared to Saline animals. NAC treatment prevented this decrease in PAE treated animals versus NAC control and PAE alone (Fig. 5B). Eccentric index at diastole increased in PAE animals compared to Saline controls. NAC alone decreased the eccentric index from PAE alone, and NAC treatment prevented the

increase in PAE animals (Fig. 5C). According to La Place's Law, a decrease in wall thickness at a given interior diameter will lead to increased ventricular wall stress. The results from our eccentric index calculation suggest that PAE caused an elevation of wall stress due to LV wall thinning, which was prevented by NAC. Likewise, the same parameters were measured during systole. NAC treatment and NAC treatment alone and to PAE animals significantly reduced LVID;s compared to PAE alone (Fig. 5D). PAE caused a decrease in the thickness of the left ventricular posterior wall compared to Saline. However, NAC treatment given to PAE animals prevented the decrease in wall thickness compared to PAE alone (Fig. 5E). Again, the eccentric index was increased in PAE animals compared to Saline, NAC alone, and with NAC treatment, preventing the increase in eccentric index, and hence, wall stress, compared to the PAE alone (Fig. 5F).

4. Discussion

It was previously estimated that fetal alcohol spectrum disorders (FASD) affected 10 per 1000 children in the United States, or about 1% of children. However, an investigation published in the Journal of the American Medical Association used weighted prevalence measures in 4 distinct US communities and determined that FASD has a weighted prevalence of 31.1–98.5 per every 1000 children (May et al., 2018). In one estimate, Burd et al. reviewed dozens of clinical case studies and suggested that FASD and congenital heart defects (CHD) had comorbidity rates of 29.6% (Burd et al., 2007). Even so, healthcare professionals only give a clinical diagnosis of FAS based on official CDC guidelines, whereas other conditions in the spectrum are not clinically recognized by the American Academy of Pediatrics (Bertrand et al., 2005; Hoyme et al., 2005). As such, 80% of children born with FASD may remain undiagnosed and have no obvious physical symptoms.

Indeed, the potential risk in underdiagnoses of FASD may be represented in several published meta-analyses of clinical studies. These recent meta-analyses found no association between PAE and congenital heart defects (Yang et al., 2015; Zhu et al., 2015; Sun et al., 2015). It is plausible that the studies used in the analyses may not provide an accurate representation of the cardiac effects of PAE since the authors only analyzed the prevalence of the most common CHDs in FAS patients, such as AV septal and valve defects. More subtle or undefined cardiac effects may have been overlooked or not used in the inclusion criteria. Published results using animal models of PAE produce much more robust cardiac effects; however, CHDs are not always produced in these models either. Less obvious cardiovascular phenotypes may not be noticed clinically, and children born with FASD may be at risk for adverse cardiac events in adulthood (Parkington et al., 2010; Saenz et al., 1999; Hoyme et al., 2016).

There is growing concern regarding the effects of FASD on the overall cardiovascular health of exposed children that is supported by several controlled rodent studies (Parkington et al., 2010; Wold et al., 2001; Ren et al., 2002; Syslak et al., 1994). For example, in utero alcohol exposure reduced the myofibril volume of individual cardiomyocytes in rat pups, and this effect persisted into the postnatal period (Syslak et al., 1994). Further, PAE produced decreases in peak tension development in cardiac muscle of rat offspring aged to 10–12 weeks, suggesting that alcohol-induced cardiac dysfunction persists into adulthood (Wold et

al., 2001). Outside of the myocardium, PAE increases vascular stiffness and could have profound implications on blood pressure and cardiovascular health in both human subjects and animal models (Parkington et al., 2010; Morley et al., 2010). These alcohol-induced cardiovascular abnormalities may result from changes in the cardiac extracellular matrix, which play a crucial role in proper cardiovascular development (Lockhart et al., 2011). To the best of our knowledge, our lab was the first to show that prenatal alcohol exposure altered the composition of the cardiac extracellular matrix (ECM) to a more compliant phenotype when analyzed on postnatal day 5 (Ninh et al., 2019).

However, the molecular events by which PAE causes alterations in the cardiac ECM are currently unknown. Our current study focuses on investigating a mechanism by which prenatal alcohol exposure affects the neonatal cardiac extracellular matrix and whether the effects may be preventable. The teratogenic effects of alcohol are widespread and affect many, if not all, organ systems. This suggests the possibility of a central causative path contributing to the adverse effects, such as oxidative stress. The metabolism of alcohol by the enzyme alcohol dehydrogenase generates acetaldehyde, a highly toxic and reactive molecule (Zakhari, 2006). In addition to forming reactive aldehydes, ethanol and acetaldehyde have been shown to upregulate the enzyme NADPH oxidase (NOX), although the exact mechanism is unclear. NOX enzymes are a key source of producing reactive oxidant species, including in the developing fetus (Hill et al., 2014). Indeed, Dong et al demonstrated that PAE produces oxidative damage to lipids and DNA in a NOX-dependent manner (Dong et al., 2010)

As such, this current study focuses on whether PAE-induced oxidative stress contributes to changes in the ECM and cardiac dysfunction we previously saw in neonates. The glutathione analogue N-acetylcysteine (NAC) was chosen as the antioxidant treatment in order to test the hypothesis. NAC behaves as both a scavenger of reactive oxidant species and a precursor for glutathione synthesis, and its use in pregnant women has been approved by the Food and Drug Administration (Lauterburg et al., 1983; Mokhtari et al., 2017; Sansone and Sansone, 2011). In the current study, we demonstrated that PAE increased NADPH oxidase 4 (NOX4) protein expression in the ventricular tissue of 5-day-old neonates compared to control saline-administered neonates. Further, PAE decreased the protein expression of Nrf2 transcription factor. Upon activation by excess reactive oxidant species and inflammatory mediators, Nrf2 translocates to the nucleus and binds to the antioxidant response element. This promotor targets transcription of antioxidant enzymes such as superoxide dismutase, heme oxygenase, and glutamate cysteine ligase (Tonelli et al., 2018; Dong et al., 2008). A decrease in the Nrf2 transcription factor would suggest a decrease in virtually all cellular antioxidant enzymes. These results suggest that PAE increases reactive oxidant species through upregulation of NOX4 while concomitantly decreasing Nrf2-mediated antioxidant pathways. Thus, these changes can be associated with an environment of oxidative stress in the ventricles of PAE-exposed neonates. This potential alteration in the redox state of ventricles was assessed via markers of oxidative damage to lipids and tyrosine residues on proteins. As predicted, NAC treatment decreased these markers of oxidative damage in PAE pups.

The increase in NOX4 protein expression is of particular interest to this study, since it is the predominant isoform expressed in cardiac fibroblasts (Cucoranu et al., 2005; Rocic and

Lucchesi, 2005). Since it is a constitutively active enzyme, its protein expression can be correlated with enzymatic activity (Martyn et al., 2006). It is well-established that NADPH oxidase isoform 4 (NOX4) promotes the phenotypic transition of quiescent fibroblasts into activated myofibroblasts, which are the key regulators of collagen synthesis and hence the cardiac ECM (Cucoranu et al., 2005; Jiang et al., 2014; Ghatak et al., 2017). Myofibroblasts upregulate the synthesis of collagens, growth factors, cytokines, and other ECM proteins. The increased expression of NOX4 supports the idea that the oxidative stress generated by the enzyme promotes fibroblast activation in the LV of PAE pups. Indeed, the upregulation of α -smooth muscle actin (α -SMA) in PAE neonates mark myofibroblast transition and coincides with altered collagen expression. The cardiac ECM primarily consists of fibrillar collagens I and III. Collagen I confers a stiffer and more rigid property to the myocardium, whereas collagen III contributes to a more compliant and pliable phenotype (Collier et al., 2012; Hutchinson et al., 2011; Lapiere et al., 1977). We observed decreases in the collagen I/III ratio in PAE pups, and this is associated with a more compliant ventricle. Although increases in compliance usually result in increased LV diastolic volume, there were no differences observed between any of the groups. Even so, the decrease in the collagen I/III ratio is consistent with the thinner LV posterior wall we observed in the echocardiography measurements in PAE, and likewise, PAE animals treated with NAC prevented the decreases in collagen I/III ratio and LV wall thinning. NAC treatment in PAE animals restored α -SMA expression to saline-control levels and was significantly reduced compared to PAE alone animals.

Despite these changes in fibroblast activation and classically profibrotic markers, there was a lack of difference in total collagen content between each experimental group as measured by ventricular hydroxyproline concentration. Hydroxyproline residues are mainly unique to collagen proteins and therefore represents the total collagen content, which includes both soluble and insoluble fibers (Goldsmith et al., 2014; van der Rest and Garrone, 1991). Only the accumulation of insoluble fibers in the ECM contributes to fibrosis. Adult fibroblast expression of α -SMA signifies activation and transition to myofibroblasts and is followed by increases in collagen secretion and other profibrotic markers (Santiago et al., 2010). This typically results in increased collagen accumulation and fibrosis. However, the PAE neonatal hearts have an increased expression of α -SMA without concomitant increases in collagen content. This may demonstrate that α -SMA expression does not necessarily reflect a profibrotic phenotype in neonatal fibroblasts. Instead, it provides evidence that PAE and oxidative stress may alter fibroblast function. Regardless, decreases in collagen I/III without a change in collagen content is further supportive of a compliant, less stiff ventricle in the PAE neonates (Collier et al., 2012; Nagao et al., 2018; Weber et al., 1988).

The increased oxidative stress and alterations to the cardiac ECM ultimately produced a depression in LV systolic function in PAE pups. The ECM provides structure to the myocardium as well as scaffolding for the myocytes to contract against (Weber et al., 1994; Bowers et al., 2010). A more compliant and less stiff ECM hinders force development by the myocytes and disrupts mechanotransduction (Hutchinson et al., 2011; Baicu et al., 2003). Indeed, the compliant phenotype in PAE ventricles produced a trending increase in LV end systolic volume. Further supporting systolic dysfunction in PAE animals are the decreases in both ejection fraction and fractional shortening. NAC treatment of PAE animals restored all

systolic parameters to normal control levels. However, we acknowledge that reductions in peak tension development and peak cell shortening have been observed in ventricular papillary muscles of adult rats that had been exposed to alcohol in utero, and this may also contribute to the decreases in systolic function (Wold et al., 2001; Ren et al., 2002). A limitation in our studies is the lack of myocyte-related measurements, such as contractility.

Morphological measurements of neonates provided additional evidence of adverse cardiac function. PAE pups had both lower body weight and body length compared to their saline-administered controls. Failure to thrive and low body weight are key features of FASD (Hoyme et al., 2016). NAC treatment prevented these decreases in growth retardation even in the presence of alcohol. However, PAE had a main effect in reducing neonatal heart weight to body weight ratio. Despite this main effect in PAE treated animals, there was significant statistical interaction in NAC treatment preventing an increase in eccentric index and wall thinning in PAE animals. As such, NAC treatment in PAE animals may have lower heart weight, but they maintain proper wall thickness and cardiac function compared to their PAE alone counterparts. Lower heart weights in PAE pups could indicate decreases in ventricular wall thickness and smaller cardiomyocyte volumes.

When LV posterior wall thickness was measured in echocardiography images, PAE pups did indeed have thinner LV walls compared to control. Interestingly, NAC treatment prevented the reduction in wall thickness in PAE pups compared to PAE alone. The decrease in wall thickness was then compared to the LV interior diameter in order to assess whether the thinner walls would be detrimental to whole heart mechanics. There were no changes in interior diameter between any of the experimental groups; as such, even at the same interior diameter, LV wall thickness was decreased in the PAE animals alone. This abnormal wall thickness-to-chamber diameter ratio is also known as the eccentric index, and it is often associated with cardiac wall stress according to LaPlace's Law (James et al., 1995). We observed that eccentric index increased in PAE pups, and the associated increase in cardiac wall stress was prevented by NAC treatment even with alcohol administration. Although cardiac atrophy measurements were beyond the scope of this study, it is evident that measurements of cardiomyocyte quantity, maturation, and myofibril assembly are needed to fully understand the mechanism responsible for the decrease in LV mass.

There is an increasing amount of evidence that PAE results in more complex and adverse outcomes than the typical congenital heart defects associated with FASD. These less characterized effects have been shown to persist after birth, and some studies show that they are still present in adulthood. Our current study provides novel evidence that prenatal alcohol exposure alters the cardiac extracellular matrix through oxidative stress, which ultimately results in depressed systolic function in neonatal mice. Treatment with the glutathione analogue, N-acetylcysteine, prevented most of the adverse cardiac outcomes in 5 day old neonates exposed to in utero alcohol early in fetal development during the gastrulation phase. This study provides insight into the mechanisms involved in adverse cardiovascular health of offspring affected by prenatal alcohol exposure and fetal alcohol spectrum disorders. Future studies are warranted to further examine the cellular mechanisms responsible and whether these changes in the neonatal heart contribute to susceptibility to cardiovascular diseases in adulthood.

Funding

Saving Tiny Hearts Society (JDG); T32AA007577-19(Patricia E. Molina); 1F31HL134263(ECE).

References

- Baicu CF, Stroud JD, Livesay VA, Hapke E, Holder J, Spinale FG, Zile MR, 2003 Changes in extracellular collagen matrix alter myocardial systolic performance. *Am. J. Physiol. Heart Circ. Physiol* 284 (1), H122–132. 10.1152/ajpheart.00233.2002. [PubMed: 12485818]
- Bertrand J, Floyd LL, Weber MK, Fetal Alcohol Syndrome Prevention Team DoBD, Developmental Disabilities NCoBD, Developmental Disabilities CfDC, Prevention, 2005 Guidelines for identifying and referring persons with fetal alcohol syndrome. *MMWR Recomm. Rep* 54 (RR-11), 1–14.
- Blakley PM, Scott WJ Jr, 1984 Determination of the proximate teratogen of the mouse fetal alcohol syndrome. 2. Pharmacokinetics of the placental transfer of ethanol and acetaldehyde. *Toxicol. Appl. Pharmacol* 72 (2), 364–371. [PubMed: 6695380]
- Boschen KE, Gong H, Murdaugh LB, Parnell SE, 2018 Knockdown of Mns1 increases susceptibility to craniofacial defects following gastrulation-stage alcohol exposure in mice. *Alcohol. Clin. Exp. Res* 42 (11), 2136–2143. 10.1111/acer.13876. [PubMed: 30129265]
- Bowers SL, Banerjee I, Baudino TA, 2010 The extracellular matrix: at the center of it all. *J. Mol. Cell. Cardiol* 48 (3), 474–482. 10.1016/j.yjmcc.2009.08.024. [PubMed: 19729019]
- Brocardo PS, Gil-Mohapel J, Christie BR, 2011 The role of oxidative stress in fetal alcohol spectrum disorders. *Brain Res. Rev* 67 (1–2), 209–225. 10.1016/j.brainresrev.2011.02.001. [PubMed: 21315761]
- Burd L, Deal E, Rios R, Adickes E, Wynne J, Klug MG, 2007 Congenital heart defects and fetal alcohol spectrum disorders. *Congenit. Heart Dis* 2 (4), 250–255. 10.1111/j.1747-0803.2007.00105.x. [PubMed: 18377476]
- Cano MJ, Ayala A, Murillo ML, Carreras O, 2001 Protective effect of folic acid against oxidative stress produced in 21-day postpartum rats by maternal-ethanol chronic consumption during pregnancy and lactation period. *Free Radic. Res* 34 (1), 1–8. [PubMed: 11234991]
- Cavieres MF, Smith SM, 2000 Genetic and developmental modulation of cardiac deficits in prenatal alcohol exposure. *Alcohol. Clin. Exp. Res* 24 (1), 102–109. [PubMed: 10656199]
- Chen SY, Dehart DB, Sulik KK, 2004 Protection from ethanol-induced limb malformations by the superoxide dismutase/catalase mimetic, EUK-134. *FASEB J.* 18 (11), 1234–1236. 10.1096/fj.03-0850fje. [PubMed: 15208273]
- Collier P, Watson CJ, van Es MH, Phelan D, McGorrian C, Tolan M, Ledwidge MT, McDonald KM, Baugh JA, 2012 Getting to the heart of cardiac remodeling; how collagen subtypes may contribute to phenotype. *J. Mol. Cell. Cardiol* 52 (1), 148–153. 10.1016/j.yjmcc.2011.10.002. [PubMed: 22008391]
- Cucoranu I, Clempus R, Dikalova A, Phelan PJ, Ariyan S, Dikalov S, Sorescu D, 2005 NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circ. Res* 97 (9), 900–907. 10.1161/01.RES.0000187457.24338.3D. [PubMed: 16179589]
- Daft PA, Johnston MC, Sulik KK, 1986 Abnormal heart and great vessel development following acute ethanol exposure in mice. *Teratology* 33 (1), 93–104. 10.1002/tera.1420330112. [PubMed: 3738814]
- Deb A, Ubil E, 2014 Cardiac fibroblast in development and wound healing. *J. Mol. Cell. Cardiol* 70, 47–55. 10.1016/j.yjmcc.2014.02.017. [PubMed: 24625635]
- Dong J, Sulik KK, Chen SY, 2008 Nrf2-mediated transcriptional induction of antioxidant response in mouse embryos exposed to ethanol in vivo: implications for the prevention of fetal alcohol spectrum disorders. *Antioxid. Redox Signal* 10 (12), 2023–2033. 10.1089/ars.2007.2019. [PubMed: 18759561]
- Dong J, Sulik KK, Chen SY, 2010 The role of NOX enzymes in ethanol-induced oxidative stress and apoptosis in mouse embryos. *Toxicol. Lett* 193 (1), 94–100. 10.1016/j.toxlet.2009.12.012. [PubMed: 20026259]

- El Hajj EC, El Hajj MC, Voloshenyuk TG, Mouton AJ, Khoutorova E, Molina PE, Gilpin NW, Gardner JD, 2014 Alcohol modulation of cardiac matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs favors collagen accumulation. *Alcohol. Clin. Exp. Res* 38 (2), 448–456. 10.1111/acer.12239. [PubMed: 24033327]
- Fernandez-Checa JC, Garcia-Ruiz C, Ookhtens M, Kaplowitz N, 1991 Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol-fed rats. Tracer kinetic studies in vitro and in vivo and susceptibility to oxidant stress. *J. Clin. Invest* 87 (2), 397–405. 10.1172/JCI115010. [PubMed: 1991826]
- Ghatak S, Hascall VC, Markwald RR, Feghali-Bostwick C, Artlett CM, Gooz M, Bogatkevich GS, Atanelishvili I, Silver RM, Wood J, Thannickal VJ, Misra S, 2017 Transforming growth factor beta1 (TGFbeta1)-induced CD44V6-NOX4 signaling in pathogenesis of idiopathic pulmonary fibrosis. *J. Biol. Chem* 292 (25), 10490–10519. 10.1074/jbc.M116.752469. [PubMed: 28389561]
- Goldsmith EC, Bradshaw AD, Zile MR, Spinale FG, 2014 Myocardial fibroblast-matrix interactions and potential therapeutic targets. *J. Mol. Cell. Cardiol* 70, 92–99. 10.1016/j.yjmcc.2014.01.008. [PubMed: 24472826]
- Henderson GI, Devi BG, Perez A, Schenker S, 1995 In utero ethanol exposure elicits oxidative stress in the rat fetus. *Alcohol. Clin. Exp. Res* 19 (3), 714–720. [PubMed: 7573798]
- Hill AJ, Drever N, Yin H, Tamayo E, Saade G, Bytautiene E, 2014 The role of NADPH oxidase in a mouse model of fetal alcohol syndrome. *Am. J. Obstet. Gynecol* 210 (5), e461–465. 10.1016/j.ajog.2013.12.019.466.
- Hoyme HE, May PA, Kalberg WO, Kodituwakku P, Gossage JP, Trujillo PM, Buckley DG, Miller JH, Aragon AS, Khaole N, Viljoen DL, Jones KL, Robinson LK, 2005 A practical clinical approach to diagnosis of fetal alcohol spectrum disorders: clarification of the 1996 institute of medicine criteria. *Pediatrics* 115 (1), 39–47. 10.1542/peds.2004-0259. [PubMed: 15629980]
- Hoyme HE, Kalberg WO, Elliott AJ, Blankenship J, Buckley D, Marais AS, Manning MA, Robinson LK, Adam MP, Abdul-Rahman O, Jewett T, Coles CD, Chambers C, Jones KL, Adnams CM, Shah PE, Riley EP, Charness ME, Warren KR, May PA, 2016 Updated clinical guidelines for diagnosing fetal alcohol spectrum disorders. *Pediatrics* 138 (2). 10.1542/peds.2015-4256.
- Hutchinson KR, Guggilam A, Cismowski MJ, Galantowicz ML, West TA, Stewart JA Jr, Zhang X, Lord KC, Lucchesi PA, 2011 Temporal pattern of left ventricular structural and functional remodeling following reversal of volume overload heart failure. *J. Appl. Physiol* (1985) 111 (6), 1778–1788. 10.1152/japplphysiol.00691.2011. [PubMed: 21885799]
- James MA, MacConnell TJ, Jones JV, 1995 Is ventricular wall stress rather than left ventricular hypertrophy an important contributory factor to sudden cardiac death? *Clin. Cardiol* 18 (2), 61–65. [PubMed: 7720291]
- Jiang F, Liu GS, Dusting GJ, Chan EC, 2014 NADPH oxidase-dependent redox signaling in TGF-beta-mediated fibrotic responses. *Redox Biol.* 2, 267–272. 10.1016/j.redox.2014.01.012. [PubMed: 24494202]
- Jones KL, Smith DW, 1973 Recognition of the fetal alcohol syndrome in early infancy. *Lancet* 302 (7836), 999–1001. [PubMed: 4127281]
- Kockaya EA, Akay MT, 2006 Histological changes in the liver of fetuses of alcohol-treated pregnant rats. *Cell Biochem. Funct* 24 (3), 223–227. 10.1002/cbf.1215. [PubMed: 16634090]
- Lapiere CM, Nusgens B, Pierard GE, 1977 Interaction between collagen type I and type III in conditioning bundles organization. *Connect. Tissue Res* 5 (1), 21–29. [PubMed: 141359]
- Lauterburg BH, Corcoran GB, Mitchell JR, 1983 Mechanism of action of N-acetylcysteine in the protection against the hepatotoxicity of acetaminophen in rats in vivo. *J. Clin. Invest* 71 (4), 980–991. [PubMed: 6833497]
- Linask KK, Han M, 2016 Acute alcohol exposure during mouse gastrulation alters lipid metabolism in placental and heart development: Folate prevention. *Birth Defects Res. A Clin. Mol. Teratol* 106 (9), 749–760. 10.1002/bdra.23526. [PubMed: 27296863]
- Lockhart M, Wirrig E, Phelps A, Wessels A, 2011 Extracellular matrix and heart development. *Birth Defects Res. A Clin. Mol. Teratol* 91 (6), 535–550. 10.1002/bdra.20810. [PubMed: 21618406]

- Martyn KD, Frederick LM, von Loehneysen K, Dinauer MC, Knaus UG, 2006 Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. *Cell. Signal* 18 (1), 69–82. 10.1016/j.cellsig.2005.03.023. [PubMed: 15927447]
- May PA, Chambers CD, Kalberg WO, Zellner J, Feldman H, Buckley D, Kopald D, Hasken JM, Xu R, Honerkamp-Smith G, Taras H, Manning MA, Robinson LK, Adam MP, Abdul-Rahman O, Vaux K, Jewett T, Elliott AJ, Kable JA, Akshoomoff N, Falk D, Arroyo JA, Hereld D, Riley EP, Charness ME, Coles CD, Warren KR, Jones KL, Hoyme HE, 2018 Prevalence of fetal alcohol Spectrum disorders in 4 US communities. *JAMA* 319 (5), 474–482. 10.1001/jama.2017.21896. [PubMed: 29411031]
- Mitchell JJ, Paiva M, Heaton MB, 1999 Vitamin E and beta-carotene protect against ethanol combined with ischemia in an embryonic rat hippocampal culture model of fetal alcohol syndrome. *Neurosci. Lett* 263 (2–3), 189–192. [PubMed: 10213167]
- Mokhtari V, Afsharian P, Shahhoseini M, Kalantar SM, Moini A, 2017 A review on various uses of N-Acetyl cysteine. *Cell J.* 19 (1), 11–17. [PubMed: 28367412]
- Morley R, Dwyer T, Hynes KL, Cochrane J, Ponsonby AL, Parkinson HC, Carlin JB, 2010 Maternal alcohol intake and offspring pulse wave velocity. *Neonatology* 97 (3), 204–211. 10.1159/000252973. [PubMed: 19864927]
- Nagao K, Inada T, Tamura A, Kajitani K, Shimamura K, Yukawa H, Aida K, Sowa N, Nishiga M, Horie T, Makita T, Ono K, Tanaka M, 2018 Circulating markers of collagen types I, III, and IV in patients with dilated cardiomyopathy: relationships with myocardial collagen expression. *ESC Heart Fail.* 5 (6), 1044–1051. 10.1002/ehf2.12360. [PubMed: 30273997]
- Ninh VK, El Hajj EC, Mouton AJ, El Hajj MC, Gilpin NW, Gardner JD, 2018 Chronic ethanol administration prevents compensatory cardiac hypertrophy in pressure overload. *Alcohol. Clin. Exp. Res* 10.1111/acer.13799.
- Ninh VK, El Hajj EC, Mouton AJ, Gardner JD, 2019 Prenatal alcohol exposure causes adverse cardiac extracellular matrix changes and dysfunction in neonatal mice. *Cardiovasc. Toxicol* 10.1007/s12012-018-09503-8.
- Ojeda L, Nogales F, Murillo L, Carreras O, 2018 The role of folic acid and selenium against oxidative damage from ethanol in early life programming: a review. *Biochem. Cell Biol* 96 (2), 178–188. 10.1139/bcb-2017-0069. [PubMed: 29040815]
- Parkington HC, Coleman HA, Wintour EM, Tare M, 2010 Prenatal alcohol exposure: implications for cardiovascular function in the fetus and beyond. *Clin. Exp. Pharmacol. Physiol* 37 (2), e91–98. 10.1111/j.1440-1681.2009.05342.x. [PubMed: 19930419]
- Ren J, Wold LE, Natavio M, Ren BH, Hannigan JH, Brown RA, 2002 Influence of prenatal alcohol exposure on myocardial contractile function in adult rat hearts: role of intracellular calcium and apoptosis. *Alcohol Alcohol.* 37 (1), 30–37. [PubMed: 11825854]
- Rocic P, Lucchesi PA, 2005 NAD(P)H oxidases and TGF-beta-induced cardiac fibroblast differentiation: Nox-4 gets Smad. *Circ. Res* 97 (9), 850–852. 10.1161/01.RES.0000190403.87462.bf. [PubMed: 16254216]
- Saenz RB, Beebe DK, Triplett LC, 1999 Caring for infants with congenital heart disease and their families. *Am. Fam. Phys* 59 (7), 1857–1868.
- Sansone RA, Sansone LA, 2011 Getting a knack for NAC: N-acetyl-cysteine. *Innov.Clin. Neurosci* 8 (1), 10–14.
- Santiago JJ, Dangerfield AL, Rattan SG, Bathe KL, Cunningham RH, Raizman JE, Bedosky KM, Freed DH, Kardami E, Dixon IM, 2010 Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. *Dev. Dyn* 239 (6), 1573–1584. 10.1002/dvdy.22280. [PubMed: 20503355]
- Seiler NK, 2016 Alcohol and pregnancy: CDC's health advice and the legal rights of pregnant women. *Publ. Health Rep* 131 (4), 623–627. 10.1177/0033354916662222.
- Serrano M, Han M, Brinez P, Linask KK, 2010 Fetal alcohol syndrome: cardiac birth defects in mice and prevention with folate. *Am. J. Obstet. Gynecol* 203 (1), 75 10.1016/j.ajog.2010.03.017. e77–75 e15.

- Stegemann H, 1958 Microdetermination of hydroxyproline with chloramine-T and pdimethylaminobenzaldehyde. *Hoppe Seylers Z. Physiol. Chem* 311 (1–3), 41–45. [PubMed: 13548908]
- Sun J, Chen X, Chen H, Ma Z, Zhou J, 2015 Maternal alcohol consumption before and during pregnancy and the risks of congenital heart defects in offspring: a systematic review and meta-analysis. *Congenit. Heart Dis* 10 (5), E216–224. 10.1111/chd.12271. [PubMed: 26032942]
- Syslak PH, Nathaniel EJ, Novak C, Burton L, 1994 Fetal alcohol effects on the postnatal development of the rat myocardium: an ultrastructural and morphometric analysis. *Exp. Mol. Pathol* 60 (3), 158–172. 10.1006/exmp.1994.1015. [PubMed: 7957776]
- Tonelli C, Chio IIC, Tuveson DA, 2018 Transcriptional regulation by Nrf2. *Antioxid. Redox Signal* 29 (17), 1727–1745. 10.1089/ars.2017.7342. [PubMed: 28899199]
- van der Rest M, Garrone R, 1991 Collagen family of proteins. *FASEB J.* 5 (13), 2814–2823. [PubMed: 1916105]
- Weber KT, Pick R, Janicki JS, Gadodia G, Lakier JB, 1988 Inadequate collagen tethers in dilated cardiopathy. *Am. Heart J* 116 (6 Pt 1), 1641–1646. [PubMed: 3195449]
- Weber KT, Sun Y, Tyagi SC, Cleutjens JP, 1994 Collagen network of the myocardium: function, structural remodeling and regulatory mechanisms. *J. Mol. Cell. Cardiol* 26 (3), 279–292. 10.1006/jmcc.1994.1036. [PubMed: 8028011]
- Wold LE, Norby FL, Hintz KK, Colligan PB, Epstein PN, Ren J, 2001 Prenatal ethanol exposure alters ventricular myocyte contractile function in the offspring of rats: influence of maternal Mg2+ supplementation. *Cardiovasc. Toxicol* 1 (3), 215–224. [PubMed: 12213974]
- Yang J, Qiu H, Qu P, Zhang R, Zeng L, Yan H, 2015 Prenatal alcohol exposure and congenital heart defects: a meta-analysis. *PLoS One* 10 (6), e0130681 10.1371/journal.pone.0130681. [PubMed: 26110619]
- Zakhari S, 2006 Overview: how is alcohol metabolized by the body? *Alcohol Res.Health* 29 (4), 245–254. [PubMed: 17718403]
- Zhu Y, Romitti PA, Caspers Conway KM, Shen DH, Sun L, Browne ML, Botto LD, Lin AE, Druschel CM, National Birth Defects Prevention S, 2015 Maternal periconceptional alcohol consumption and congenital heart defects. *Birth Defects Res. A Clin. Mol. Teratol* 103 (7), 617–629. 10.1002/bdra.23352. [PubMed: 26118863]

Fig. 1. Neonatal morphological measurements. A. PAE significantly decreased neonatal body weight compared to Saline. NAC PAE neonates had significantly higher body weight compared to PAE and NAC controls. B. Crown to rump length was decreased in PAE animals vs Saline, and both NAC and NAC PAE had significantly longer body lengths compared to PAE alone. C. PAE and NAC PAE had significantly lower LV mass when body weight was taken into consideration compared to Saline controls. D. There were no differences in maternal blood alcohol content between PAE and NAC PAE dams. (* $p < 0.05$ versus saline control; # $p < 0.05$ versus PAE; & $p < 0.05$ versus NAC; LV = left ventricle).

Fig. 2.

Oxidative stress markers in neonatal ventricular tissue. A. PAE had significantly higher protein expression of NADPH oxidase 4 (NOX4) compared to Saline animals. NAC PAE animals had lower NOX4 expression compared to PAE. C. PAE reduced Nrf2 protein expression compared to Saline, and NAC PAE had significantly higher Nrf2 expression compared to PAE alone. C. NAC had a main effect of reducing nitrosylated tyrosine residues. D. NAC PAE neonates had lower concentrations of malondialdehyde compared to PAE ventricles. (* $p < 0.05$ versus saline control; # $p < 0.05$ versus PAE; +Main effect of NAC $p < 0.05$).

Fig. 3.

Protein expression of profibrotic markers in left ventricular tissue. A. PAE animals had significantly higher α -SMA than Saline controls, and NAC treatment prevented this increase. B. There were no significant changes in collagen I expression between any of the experimental groups. C. There were no significant differences in collagen III expression between any groups. D. PAE significantly decreased collagen I/III ratio compared to Saline. NAC PAE prevented this decrease in the collagen I/III ratio compared to PAE alone. E. There were no differences in total collagen content between any experimental groups. (* $p < 0.05$ versus saline control; # $p < 0.05$ versus PAE; α -SMA = α smooth muscle actin).

Fig. 4.

Systolic function of 5 day old neonates. A. There were no significant differences in LV end diastolic volume between any experimental groups. B. PAE had significantly higher LV end systolic volume versus Saline. NAC control and NAC PAE had significantly lower LV vol;s compared to PAE alone. C. Ejection fraction was significantly lower in PAE neonates vs. Saline. NAC control and NAC PAE had higher ejection fraction compared to PAE animals. D. PAE animals had decreased fractional shortening compared to Saline and NAC controls. NAC treatment prevented this decrease in PAE animals. NAC PAE had higher fractional shortening compared to NAC controls. (* $p < 0.05$ versus saline control; # $p < 0.05$ versus PAE; & $p < 0.05$ versus NAC; LV Vol;d = left ventricular end diastolic volume; LV Vol;s = left ventricular end systolic volume).

Fig. 5.

Cardiac wall stress was calculated as an association between the LV interior diameter and posterior wall thickness. A. There were no significant changes in LVID;d between any experimental groups. B. LVPW;d was decreased in PAE and in NAC controls versus Saline animals. NAC PAE had significantly higher LVPW;d versus PAE and NAC control groups. C. Eccentric Index was used as a measure of LV wall stress. PAE increased eccentric index versus Saline ventricles. NAC and NAC PAE had significantly lower eccentric index compared to PAE. D. NAC and NAC PAE had significantly lower LVID;s compared to PAE. E. PAE had thinner LV posterior wall compared to Saline. Both NAC and NAC PAE neonates had higher LVPW;s compared to PAE. F. Eccentric index was higher in PAE animals compared to Saline. NAC and NAC PAE animals had significantly lower wall stress at systole compared to PAE. (* $p < 0.05$ versus saline control; # $p < 0.05$ versus PAE; & $p < 0.05$ versus NAC; LVID;d = left ventricular interior diameter at diastole; LVPW;d = left ventricular posterior wall at diastole; LVID;s = left ventricular interior diameter at systole; LVPW;s = left ventricular posterior wall at systole).