DISRUPTED ADIPONECTIN-CONNEXIN43 SIGNALING UNDERLIES EXACERBATED MYOCARDIAL DYSFUNCTION IN DIABETIC FEMALE RATS

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ABSTRACT

The 2015 American Heart Association scientific statement has advocated for sex-specific research to understand the paradoxically higher sensitivity of women to type 2 diabetes mellitus (T2DM)-associated cardiovascular morbidity and mortality. The primary goal of this study was to address this need by elucidating the mechanisms by which these females lose inherent cardioprotection and become more susceptible to detrimental cardiovascular-related outcomes than males. The central hypothesis of this study was "*estrogen (E_2) exacerbation of diabetes-evoked disruption of cardiac adiponectin (APN)-connexin 43 (Cx43) signaling underlies heightened myocardial dysfunction in diabetic females*". The study provides novel data on the modulation of the cardiac E_2-APN-Cx43 axis and its critical role in maintaining a delicate redox balance in cardiomyocytes, how this cardioprotective signaling becomes detrimental in T2DM, and identified a molecular target for developing potential pharmacological interventions for diabetic females. Our findings demonstrate that E_2 exacerbates female autonomic and cardiac dysfunction in a rodent model of T2DM, increasing left

ventricular (LV) mass and Tau as well as decreasing LVDP, $dP/dt_{max,}$ dP/dt_{min}, contractility index (CI) and fractional shortening (FS). Additionally, the restoration of the APN-Cx43 signaling axis via a small molecule APN agonist virtually reverses all of these cardiac dysfunction parameters as well as mitigates detrimental molecular alterations and a proinflammatory milieu in all female rats by restoring cardiac levels of Cx43, decreasing heme oxygenase 1 (HO-1), tumor necrosis factor (TNF) α and circulating levels of asymmetric dimethylarginine (ADMA). The research project replicates the unexplained paradoxically higher mortality risk in women and provides novel mechanisms for this cardiovascular health problem. Collectively, these findings support the hypothesis and proposed underlying mechanism and further demonstrations that the restoration of Cx43 emerges as a promising novel target in diabetic cardiovascular disease for the development of future therapeutics for women.

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by

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To my Husband, Brett, and children: Chloe, Declan, Asher and Grace.

Thank you for supporting my dreams.

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LIST OF SYMBOLS/ABBREVIATIONS

AdipoR1: adiponectin receptor 1

AdipoR2: adiponectin receptor 2

ADMA: asymmetric dimethylarginine

AKT: protein kinase B

APN: adiponectin

Cav-3: caveolin-3

CI: contractility index

CVD: cardiovascular disease

Cx43: connexin43

DAF-FM Diacetate: Difluorofluorescein diacetate

DCFH-DA: 2',7'-dichlorofluorescein diacetate

DM: diabetes mellitus/diabetic (used interchangeably with T2DM)

DMSO: Dimethyl sulfoxide

E_2: estrogen/estradiol

Echo: echocardiography

EDP: end diastolic pressure

EF: ejection fraction

ERα: estrogen receptor alpha

ERβ: estrogen receptor beta

ERK: extracellular regulating kinases

ERT: estrogen replacement therapy

FS: fractional shortening

GPER: g-protein coupled estrogen receptor/GPR30

GTT: glucose tolerance test

HO-1: heme oxygenase 1

HR: heart rate

HF: high frequency

IP: intraperitoneal

IV: intravenous

LF: low frequency

LV: left ventricular

LVDP: left ventricular developed pressure

MAP: mean arterial pressure

MAPK: mitogen activated protein kinase

NO: nitric oxide

OVX: ovariectomy

ROS: reactive oxygen species

SO: sham operated

STZ: streptozotocin

T2DM: type 2 diabetes mellitus/diabetic (used interchangeably with DM)

TNFα: tumor necrosis factor-alpha

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Scientific Premise

Recent scientific statements from both the National Institutes of Health (NIH) and American Heart Association (AHA) have highlighted the need for conducting preclinical female sex specific research. Specifically, there is a need to understand the mechanisms underlying a paradoxical increase in the cardiovascular morbidity and mortality in women with type 2 diabetes mellitus (T2DM) compared with age-matched diabetic men (Regensteiner et al., 2015). T2DM is increasing at a rapid rate in younger individuals, and its treatment constitutes a significant financial burden (Regensteiner et al., 2015). Physiologically, premenopausal women lose their inherent cardioprotection, and are more impacted by the diabetes-associated cardiovascular anomalies when compared to age-matched T2DM men (Juutilainen et al., 2004). A recent report showing no negative cardiovascular consequences of estrogen (E_2) replacement therapy (ERT) in women (Manson et al., 2017) will likely increase future ERT. Thus, this preclinical research focused on elucidating the mechanisms by which ovarian hormones, particularly estrogen (E_2), render females more susceptible to diabetes mellitus (DM)/hyperglycemia-related cardiovascular anomalies than males. The findings will also yield new insights into the unstudied mechanisms of E_2-mediated upregulation of adiponectin (APN)-connexin 43 (Cx43) function in females under physiological conditions and how its disruption in pathological states, such as T2DM, contributes to this unexplained paradox. The restoration of this critical signaling axis emerges as a novel and effective therapeutic target in diabetic females.

1.2 Adiponectin (APN)

APN, an adipokine with anti-inflammatory and antidiabetic properties (Guo et al., 2007; Durand et al., 2012; Fisman and Tenenbaum, 2014), is reduced in obesity, T2DM, and coronary artery disease (Kadowaki et al.; Takemura et al., 2007; Zhu et al., 2008; Tian et al., 2009; van Stijn et al., 2015) and binds to two transmembrane adiponectin receptors, AdipoR1 and AdipoR2. Sexual dimorphism showing higher APN levels and APN protection of left ventricular (LV) function from oxidative stress in eNOS deficient mice (Durand et al., 2012) support a greater cardioprotective role for APN in female mice. This premise is further supported by the ability of APN supplementation, in *aged* female rats and porcine models, to reduce infarct size (Tomicek et al., 2015) and oxidative stress (Tao et al., 2007). However, it remains unstudied if this sexually dimorphic cardioprotection, conferred by APN or its downstream effector Cx43, is compromised in *young* DM females. Realizing that cellular oxidative stress transforms E_2 into a proinflammatory molecule (White et al., 2005), and that DM causes oxidative stress (Rochette et al., 2014) and reduces APN, studies are needed to determine if these consequences are more prominent in DM females in an E_2-dependent manner.

1.3 Connexin43 (Cx43)

While Cx43 constitutes a promising therapeutic target for cardiovascular disease (CVD) (Schulz et al., 2015), its role in DM associated cardiovascular anomalies in females has not been studied. Interestingly, cardiac Cx43 expression and distribution are higher in females and its deficiency is implicated in sex-related differences in cardiac mortalities (Stauffer et al., 2011; Michela et al., 2015). Physiologically, Cx43

formation of gap junctions at the outer cardiac sarcolemma ends mediates favorable effects on ion flow, energy metabolism, ROS handling and cardiac rhythm (Ruiz-Meana et al., 2008). By marked contrast, Cx43 disorganization (lateralization), downregulation or reduced phosphorylation (pCx43) creates aberrant hemichannels (Michela et al., 2015), and leads to cardiac dysfunction (Ai and Pogwizd, 2005) in models of myocardial infarction (Ruiz-Meana et al., 2008; Schulz et al., 2015). Therefore, the reliance of cardiac function on higher cardiac Cx43 expression in females (Stauffer et al., 2011; Michela et al., 2015), likely increases their susceptibility to DM-evoked Cx43 disorganization and subsequent myocardial oxidative stress and dysfunction. The apparent critical role of Cx43 supports the proposed studies to test this hypothesis.

1.4 Role of E_2 in APN-Cx43 signaling in the heart

Alterations in estrogen receptor (ER) expression (upregulation of ERα) in gestational diabetes (Kleiblova et al., 2010) and human heart failure (Mahmoodzadeh et al., 2006) may contribute to sex differences in LV hypertrophy and heart failure (Regitz-Zagrosek et al., 2010). It is well established that in conditions of oxidative stress, estrogen can transform from an anti-inflammatory into a proinflammatory molecule (White et al., 2005). Additionally, ERα KO mice exhibit disrupted APN signaling (Mauro et al., 2014) and E_2 up- and down-regulates Cx43 and pCx43 expression (Moinfar et al., 2016) which contribute to cardioprotection (Murphy and Steenbergen, 2014; Murphy et al., 2017). Further, E_2 regulates nitric oxide synthase (NOS) and numerous calcium transporters (Keusch et al., 2008; Murphy et al., 2017) and Cx43 regulates Ca^{2+} ion channel flow in the myocardium (Xiao et al., 2016). There are no functional or

molecular studies on this signaling pathway in DM females. The proposed studies on the E_2-APN-Cx43 axis are critical to understanding the molecular mechanisms underlying exacerbated cardiac dysfunction in DM females, and whether the restoration of the function of this axis serves as a novel therapeutic modality for alleviating myocardial dysfunction in DM females.

1.5 An orally active small molecule APN receptor agonist: AdipoRon

High molecular weight (HMW) adiponectin mitigates detrimental cardiac remodeling in disease states (Takemura et al., 2007; Tian et al., 2009) and exogenous HMW APN or globular APN efficaciously represses deteriorating cardiac function (Tao et al., 2007; Zhu et al., 2008; Fisman and Tenenbaum, 2014). Yet, the use of APN as a pharmacological therapeutic tool in T2DM is limited due to its short half-life, large size and lack of GI absorption (Kadowaki et al.; Zhang et al., 2018). However, AdipoRon, an orally available small molecule agonist at AdipoR1 and AdipoR2 (Okada-Iwabu et al., 2013) constitutes a clinically feasible alternative. AdipoRon alleviates post-ischemic cardiac remodeling and apoptosis in APN KO male mice (Zhang et al., 2015), decreases cardiac hypertrophy (Zhang et al., 2018) and mitigates overall DM symptoms (Okada-Iwabu et al., 2013; Choi et al., 2018). Additionally, AdipoRon ameliorated diabetic nephropathy and oxidative stress in a male mouse model (Choi et al., 2018). However, a potential cardioprotective effect of AdipoRon has not been evaluated in female DM models.

1.6 Diabetes Mellitus and Oxidative Stress

Diabetes Mellitus (DM) is a cluster of metabolic disorders where blood glucose levels are elevated over a prolonged period and physiological glycemic control is inadequate. It is caused by absolute or relative insulin deficiency, sometimes associated with insulin resistance. Type 1 diabetes is an autoimmune disease where the immune system attacks and destroys pancreatic beta cells. Type 2 diabetes (T2DM) is a syndrome with multiple etiologies rather than a single specific disease. However, it is known that as the hyperglycemia of diabetes becomes chronic, the sugar that normally serves as a substrate fuel and signal takes on the darker role of toxin in the structure and function of organs (Robertson, 2004) and importantly, in the heart (Rutter et al., 2003). This “toxic sugar” creates an overproduction of reactive oxygen species (ROS) in the organs and diabetes is well established as a condition of elevated oxidative stress (Ceriello and Motz, 2004; Rochette et al., 2014).

The pathology of DM is a key risk factor for the development of heart anomalies and dysfunction and the focus of this research project. There are key differences between the sexes, in large part because of hormonal regulation (Pradhan, 2014), that are not fully understood. The prevalence of DM, a leading risk factor for CVD, is increasing at a rapid rate (Regensteiner et al., 2015; Bjornstad et al., 2016) and CVD is the leading cause of morbidity and mortality worldwide. Despite the outlined heightened risks for female cardiovascular complications and negative outcomes in the diabetic population, clinicians are less likely to aggressively treat women with appropriate therapeutics as opposed to men (Victor et al., 2012). Additionally, current pharmacotherapies for CVD in the diabetic population have been suggested to be less

effective in women than in men (Regensteiner et al., 2015) and mechanisms underlying the differential response are undefined.

1.7 Innovation

These studies are the first to deal with the mechanisms of the unexplained hypersensitivity to myocardial dysfunction in diabetic females. The multilevel approach and a well-planned research strategy (longitudinal and cross-sectional design) generated first-hand data on the role of the female sex/E_2-dependent APN-Cx43 signaling in regulating cardiac redox status and function in healthy and diabetic females. These findings identified a novel molecular target for developing a precision therapeutic to alleviate the exacerbated DM-induced myocardial dysfunction in E_2-replete females.

1.8 Authentications and Aims of the Study

The proposed experimental design, validated methods, appropriate sample size and statistical analyses led to unbiased robust results. Most notable, selecting established conditions for T2DM induction (Srinivasan and Ramarao, 2007; Sasidharan et al., 2013) ensures reproducibility of data within and between labs. Scientific rigor was ensured by: (i) the use of soy/phytoestrogens-free diets to avoid their confounding effects on generated data; (ii) consistent with current recommendations (Griendling et al., 2016), at least 2 different methods were used for biochemical measurements of oxidative stress markers; (iii) the use of authenticated antibodies and validating their specificity in our model system.

Specific Aim 1: Test the hypothesis that estrogen exacerbates T2DM-evoked cardiac and autonomic dysfunction.

Rationale: There is unmet need to understand why E_2 paradoxically increases diabetes-induced cardiac dysfunction. Cardiac remodeling in female mice is more exacerbated by decreased APN levels than male mice—suggesting a greater cardioprotective role for APN in females (Durand et al., 2012). Whether the reduction in cardiac APN in diabetic male rats (El-Sayed et al., 2016) is more evident in diabetic female rats needs to be investigated. This is important because there is ongoing debate on the role of APN in cardiac pathology in diabetes (Kadowaki et al.) and APN interaction with estrogen regulation of nitric oxide synthases (Zhu et al., 2008; Durand et al., 2012) and Cx43 (Stauffer et al., 2011). Therefore, the present studies elucidated the role of the APN-E_2-Cx43 axis in healthy and diabetic female (in the absence or presence of E_2) and male rats. These integrative studies addressed unresolved questions about this clinically important problem impacting women's cardiovascular health. These experiments will address the questions:

1. Are hemodynamic and cardiovascular dysfunction worse in diabetic estrogen-replete females?
2. What are the effects on mean arterial pressure, left ventricular developed pressure and contractility?
3. How are cardiac parameters (dP/dt_{max}, dP/dt_{min}, Tau, ejection fraction, fractional shortening and autonomic control) affected?

4. Does estrogen evoke heightened diabetic glycemic dysregulation and cardiac hypertrophy? Studies will examine glucose tolerance testing and LV mass/hypertrophy.

Specific Aim 2: Test the hypothesis that disruption of the APN-Cx43 axis underlies the sex/estrogen-dependent exacerbation of diabetic myocardial dysfunction.

Rationale: The dependence of females on higher Cx43 expression to maintain cardiac function and redox regulation (Stauffer et al., 2011) may become detrimental in the presence of DM. This untested premise is supported if the DM-evoked Cav3 inhibition and Cx43 disorganization in male rat hearts (Ruiz-Meana et al., 2008; Michela et al., 2015; Schulz et al., 2015) is exacerbated in females. Further, it is not known if the ERα mediation of the Cav3 dependent disorganization of Cx43 in isolated cardiac myocytes (Chung et al., 2009) occurs in DM females. Preliminary data show ERα upregulation in DM female rats and previous lab findings implicated ERα in the sex/E_2-dependent myocardial oxidative stress and dysfunction caused by alcohol (Yao and Abdel-Rahman, 2017). These studies focused on the roles of APN, Cx43 and ovarian hormones, particularly estrogen, because these molecules are linked, and contribute to, cardioprotection under normal physiological conditions (Murphy and Steenbergen, 2007; Keusch et al., 2008; Murphy and Steenbergen, 2014; Schulz et al., 2015). While reported studies in male diabetic rats (Guo et al., 2007; Okada-Iwabu et al., 2013; Schernthaner and Stangl, 2013; Fisman and Tenenbaum, 2014; Hossain et al., 2015; Koentges et al., 2015; Regensteiner et al., 2015) and our preliminary studies partly

support our scientific premise, there are currently no detailed studies on this signaling cascade in diabetic female rats, particularly in the presence of estrogen. These studies will address the questions:

1. Do diabetic females exhibit greater E_2 dependent disorganization/deactivation of cardiomyocyte Cx43 and its signaling cascade?
    I. ERα, ERβ and GPER
    II. APN, AdipoR1 and AdipoR2
    III. Cx43
2. Does suppression of downstream cardioprotective molecules of APN signaling underlie exacerbated female diabetic myocardial dysfunction?
    I. pAKT/AKT
    II. pERK/ERK

Specific Aim 3: Does restoration of APN-Cx43 signaling (as a novel molecular target) reverse heightened female T2DM-evoked cardiac dysfunction?

Rationale: The research plan will identify novel molecular targets and add supporting evidence to the diabetes evoked disruption of the E_2-APN-Cx43 signaling axis as an underlying mechanism for female hypersensitivity to cardiovascular dysfunction. The long-term goal focuses on developing precision-based diagnostics and therapeutics for mitigating cardiac anomalies in diabetic females. While females produce more super oxide, they handle it more efficiently than males, a vital mechanism for their cardio protection (Murphy and Steenbergen, 2007; Keusch et al., 2008; Murphy and Steenbergen, 2014). These studies focused on restoring the function of the two

interrelated molecules APN and Cx43 because they are implicated in this sex-dependent phenomenon. Notably, APN dampens myocardial oxidative stress (Bugger and Abel, 2010; Koentges et al., 2015), at least partly, via triggering Cx43 signaling (Murphy and Steenbergen, 2007; Ruiz-Meana et al., 2008; Schulz et al., 2015) and E_2 increases APN and Cx43 levels (Stauffer et al., 2011; Wang et al., 2015). However, the possibility that restoring the APN and CX43 function reverses DM-evoked cardiac dysfunction in females has not been investigated. The research project applied a pharmacological strategy to achieve this goal: administration of the APN agonist, AdipoRon (Okada-Iwabu et al., 2013), which also activates Cx43 signaling. These studies will address the questions:

1. Will “AdipoRon” (a small molecule AdipoR1 and AdipoR2 agonist) rescue cardiac function in E_2-replete diabetic females? Does it ameliorate reduction of cardioprotective Cx43, ERK or Akt?
2. Does restoration of APN-Cx43 signaling (via AdipoRon) reverse oxidative stress and other biochemical alterations in E_2 replete females?
3. How are circulating levels of the cardiovascular risk marker, asymmetric Dimethylarginine (ADMA), affected by estrogen and AdipoRon dosing?

CHAPTER TWO: MATERIALS AND METHODS

2.1 Animals

Young (8 weeks of age) female and male Wistar rats (170-200g, Charles River Laboratories, Raleigh, NC) were kept in the University animal care center and housed in pairs in standard plastic cages. Rats were allowed free access to water and chow (Prolab Rodent Chow; Granville Milling, Creedmoor, NC) until the beginning of the special phytoestrogen-free diet. After initial intake measurements, rodents received ad libitum control (AIN-93G Growth Purified diet; 57W5 TestDiet) or high fat (DIO Rodent Purified diet with 45% energy from fat; 58V8 TestDiet) diet (Granville Milling, Creedmoor, NC) as reported (Srinivasan and Ramarao, 2007; Brown and Panchal, 2011; Sasidharan et al., 2013); dietary specifics are included in Table 1. Rats were maintained on a 12-12 hour light-dark cycle and the temperature was maintained at 23 ± 1 ºC, humidity at 50 ± 10%. All surgical procedures were conducted under sterile conditions and rats received preoperative analgesia (buprenorphine 0.03 mg/kg every 8 hours, S.Q. or meloxicam 1mg/kg, 0.2ml/day, P.O) 30 min prior to surgery.

A whole body chamber ventilated with a 1-2% isoflurane, balanced oxygen gas mixture was used as anesthesia for the OVX, echocardiography and E_2 containing silastic tube implantation, while I.P. ketamine/xylazine (90/10 mg/kg, respectively) anesthesia was utilized for the terminal femoral and left ventricular catheterization surgery. The disappearance of corneal reflex, response to pain sensation (toe pinch) and loss of muscle tension were utilized as signs of appropriate anesthesia. Post-operative analgesia consisted of a minimum of three days of buprenorphine (0.03 mg/kg/S.Q. every 8 hours) or meloxicam

(1mg/kg, 0.2ml/day, P.O). All procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institute of Health and the National Research Council Committee Update of the Guide for the Care and Use of Laboratory Animals, 2015.

2.2 Ovariectomy

Ovariectomy (OVX) was conducted as reported in our previous studies (El-Mas and Abdel-Rahman, 2000) in the University Department of Comparative Medicine surgical suites under sterile conditions. Rats were placed in a prone position and flank shaved with electric clippers. Surgical incision area was prepped with three consecutive cleanses of a povidone-iodine scrub, followed with a 70% ethanol rinse. An approximately 1-2 cm incision was made in the skin and symmetrical 1 cm incisions on either side of the underlying muscle from the second to fifth lumbar vertebrae. The peri-ovarian fat was pulled out through the muscle incision, ovaries located, tied off with sterile suture, and removed. The muscle was closed using absorbable suture (Roboz Surgical Instrumental Co., Gaithersburg, MD), skin closed with surgical clips (Mikron Precision Inc., Gardena, CA) and removed 10-14 days later. Rats were allowed a two week “wash out” period post OVX to ensure depletion of ovarian hormones and most endogenous E_2 in accordance with an established protocol in our laboratory (El-Mas and Abdel-Rahman, 2000). Estrogen replacement was achieved using subcutaneous implantation of silastic tubing (10 mm length, 1.57 mm inner diameter × 3.18 mm outer diameter, Silastic®, Dow Corning) filled with approximately 25 mg of 17β-estradiol-3-benzoate [1,3,5(10)-estratriene-3,17β-diol-3-benzoate]. Silastic tubing was sealed with

medical adhesive type A (Silastic®, Dow Corning), gas sterilized, and implanted subcutaneously at the back of the neck in rats. Serum estradiol levels were measured at the conclusion of the study.

2.3 Induction of diabetes mellitus (DM)

We adopted the high fat diet plus two low doses streptozotocin (STZ) (Brown and Panchal, 2011; Sasidharan et al., 2013) to induce T2DM. Four weeks after the initiation of the special diet regimen, rats were injected with freshly prepared STZ (35 mg/kg; I.P.) in 0.1M citrate buffer (pH 4.0) or the buffer (control). One week after the first injection, a second STZ injection was given under the same conditions. Three days after the second STZ injection, non-fasting blood glucose levels were measured by a Blood Glucose Monitoring System (Freestyle-Precision Neo, Abbott, Alameda, CA) with tail vein blood. Onset of diabetes was identified by polydipsia, polyuria and BGL ≥ 250 mg/dL. Rats that exhibited these characteristics were considered diabetic, and buffer-treated rats were used as non-diabetic controls as reported (Srinivasan and Ramarao, 2007).

2.4 Body and heart weights

Body weights were determined, in grams, at baseline (prior to initiation of special diet) and then once weekly until the termination of the study. On the final day, prior to vascular catheterization, body weights were recorded, and following euthanasia, the hearts were excised, weighed and stored at -80ºC for subsequent biochemical studies (see below).

2.5 Echocardiography

Echocardiography was performed at baseline and biweekly by two experienced researchers, one of which was blinded to the experimental groups. Both researchers

performed echo and analyzed recorded images, with images randomly verified against their match. Rats were lightly anesthetized with isoflurane using a whole body chamber ventilated with a 1-2% isoflurane, balanced oxygen gas mixture. After anesthesia induction, animals were transferred to an imaging platform equipped with a heating pad to maintain body temperature and anesthesia maintained via nose-cone delivery of the same gas mixture, typically for <10 min. Hair was removed from the chest wall using a chemical depilator and the skin cleansed with warm water. Non-toxic acoustic gel was placed on the chest wall and non-invasive ultrasound images of the heart were collected and stored for analysis according to a standard protocol (Visual Sonics Vevo 2100 (Phase I) and 3100 (Phase II) Imaging System, FujiFilm and VevoLab Software v.2.1.0, Toronto, Ontario, Canada). Rats were removed from the nose cone, returned to their cage, and monitored until awakened spontaneously. M-mode and B-mode images of LV end-diastolic diameter, interventricular septum and posterior LV wall thicknesses at end diastole were measured and averaged over five beat cycles along with ejection fraction, fractional shortening, contractility index and Tau (the mean time constant for the isovolumic-pressure decline).

2.6 Intravascular and left ventricular catheterization

As described in previous studies (Yao and Abdel-Rahman, 2017), a catheter consisting of 5cm PE-10 tubing bound to 15cm PE-50 tubing was placed in the abdominal aorta via left femoral artery and a PE-50 tubing, filled with heparinized saline, was inserted through the carotid artery into the LV for the measurement of arterial blood pressure and LV function, respectively. Hemodynamic recordings were collected when arterial and left ventricular catheters were connected to Gould-Statham (Oxnard, CA) pressure transducers and flushed with heparinized saline (100 IU/ml). BP and left ventricular indices were

simultaneously recorded by ML870 (PowerLab 8/30; Colorado Springs, CO) and analyzed by LabChart (v.7) pro software (AD Instruments, Colorado Springs, CO).

2.7 Blood and tissue collection

At the conclusion of hemodynamic measurements, prior to sacrifice, blood was collected in heparinized tubes and centrifuged at 2000g for 10 min. Serum was collected and stored at -80ºC until biochemical analysis was performed. Rats were euthanized after blood collection according to authorized AUP. Hearts were excised, weighed and flash frozen in 2-methylbutane (Sigma-Aldrich, St. Louis, MO) on dry ice. Tissue was stored at -80 ºC until processed for biochemical studies.

2.8 Western blot analysis

All western blots were conducted following the established protocols in our lab (El-Sayed et al., 2016; Yao and Abdel-Rahman, 2017). LV tissue was homogenized on ice in lysis buffer (20mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM Sodium pyrophosphate, 1mM beta-glycerolphosphate, 1mM activated sodium orthovanadate and 1µg/ml leupeptin with a protease inhibitor cocktail (Roche, Indianapolis, IN), sonicated and centrifuged (12,000 x g for 20 min). Supernatant protein was extracted and samples were quantified for protein using Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA). Protein extracts (50 µg/lane) were separated in a 4-12% gel electrophoresis (Novex Tris-Glycine gel; Life Technologies, Carlsbad, CA) at 150 V (Bio-Rad Laboratories, Hercules, CA). After semidry transfer to nitrocellulose membrane for 30 minutes at 25 V, 1 A (Bio-Rad Laboratories, Hercules, CA), membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 2 hours. Post initial blocking,

membranes were incubated in primary antibody overnight at 4ºC on a rocker. Primary antibodies used were as follows: Rabbit polyclonal anti-APN, anti-AdipoR1, anti-TNFα, anti-HO-1 and anti-ERα (1:200 dilution, Abcam, Cambridge MA), Rabbit polyclonal anti-AKT and anti-pERK (1:200 dilution, Cell Signaling Technology, Danvers MA), Goat polyclonal anti-Cx43 and anti-GPER (1:200 dilution, Abcam, Cambridge MA) and Mouse monoclonal anti-GAPDH (1:1000 dilution, Abcam, Cambridge, MA) and Mouse anti-pAKT and anti-ERK (1:200 Cell Signaling Technology, Danvers MA). Afterward, secondary membranes were washed and incubated with secondary antibody prepared by IRDye680-conjugated goat anti-mouse and IRDye800-conjugated goat anti-rabbit, or IRDye680 conjugated donkey anti-mouse and IRDye800-conjugated donkey anti-goat (depending upon respective primary antibody utilized at a dilution of 1:5000) for sixty minutes in the dark at room temperature on rocker. GAPDH was used as a loading control. Molecular weights of proteins for western blots are shown, and representative blots for a particular protein in healthy and diabetic groups were run on the same gel; whenever random order of the blots necessitated splicing gel lanes, this was identified with a gap in the image. Arbitrary units reported are the target protein normalized to respective GAPDH on the same gel, or the phosphorylated (pERK and pAKT) protein to its respective total protein. Bands were detected by Odyssey Infrared Imager and quantified by integrated intensities with Odyssey application software version 3 (LI-COR Biosciences).

2.9 Colocalization immunofluoresent microscopy

To corroborate western blot findings, spatial distribution of Cx43 was investigated by dual labeling immunofluorescence in heart sections as previously described (Steagall et al., 2017). Briefly, hearts were equlibrated to -20ºC and sectioned with a cryostat (HM 505E;

Microm International GmbH, Waldorf, Germany). Three heart tissue cyrostat sections (20 µm thick) were post-fixed in 4% paraformaldehyde on Polysine coated microscope slides (Thermo Scientific LLC, Portsmouth, NH) and blocked for 2 hours with 10% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) in Tris-buffered saline containing 0.1% Tween-20 (TBS) and 10% Triton (X-100). After incubation overnight at 4ºC with primary antibody (1:100 dilution v/v), sections were washed four times with TBS and incubated with the appropriate secondary antibody (1:200 dilution v/v) for 60 minutes and washed four times with TBS. Coverslips were applied with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Primary antibodies used were goat anti-Cx43 (Abcam, Cambridge, MA) and mouse anti-Cav3 (Santa Cruz Biotech, CA). Secondary antibodies were Cyanine 3 (Cy3)-conjugated donkey anti-goat IgG (H + L) (Jackson ImmunoResearch) and Fluorescein (FITC)-conjugated donkey anti-mouse IgG (H + L) (Jackson ImmunoResearch). Control sections were incubated with only secondary antibodies to determine non-specific staining. Three representative images across the heart sections, chosen according to similar spatial orientation, were acquired by laser scanning at two wavelengths, 488 nm (Argon/2) and 543 nm (HeNe1), using the Zeiss LSM 700 confocal microscope. Image analysis software ZEN 2012 (Carl Zeiss, Jena, Germany) kept parameters constant throughout the acquisition process.

2.10 Measurements of estradiol (E_2)

For confirmation of the E_2 depletion in ovariectomized (OVX) and its restoration to physiological levels in OVX rats that received E_2 replacement (OVX+E_2), E_2 levels were measured in plasma with a commercially available estradiol ELISA kit (Cayman Chemicals, Ann Arbor, MI) according to manufacturer's instructions.

2.11 Heart rate variability (spectral analysis) studies

In this study, spectral analysis of blood pressure data obtained from healthy, vehicle dosed diabetic and AdipoRon dosed diabetic females were recorded by ML870 (PowerLab 8/30; Colorado Springs, CO) and analyzed by LabChart (v.7) pro software (AD Instruments, Colorado Springs, CO). Frequency domain analysis using FFT algorithms of RR data series was followed (El-Mas and Abdel-Rahman, 2007). Spectra were integrated into 2 specific frequency bands, low-frequency (LF) (0.25–0.75 Hz) and high-frequency (HF) (0.75–3 Hz) bands. To reflect cardiac sympathetic and parasympathetic dominance, respectively, the data was expressed as LFnu and HFnu. The LF/HF ratio was computed and taken as a measure of cardiac sympathovagal balance (El-Lakany et al., 2018).

2.12 Measurements of Asymmetric Dimethylarginine

For indication of cardiovascular risk, Asymmetric Dimethylarginine (ADMA) levels were measured in serum, in duplicates, with a commercially available ADMA ELISA rat specific kit (MyBioSource, San Diego, CA) according to manufacturer's instructions.

2.13 Reactive Oxygen Species (ROS) Measurement

Oxidative stress level was measured using 2', 7'-dichlorofluorescein diacetate (DCFH-DA), a detector of ROS (Korystov et al., 2009). In summary, a stock solution of DCFH-DA (20 mM, Molecular Probes, Grand Island, NY) in DMSO (protected from light) was freshly diluted with PBS to prepare a 150 µM working solution. After fresh preparation, 10 µl of left ventricular cardiac tissue homogenate supernatant was pipetted into a 96-well plate to give a final concentration of 25 µM DCFH-DA to produce

fluorescent 2′,7′-Dichlorofluorescein (DCF) in the incubation medium at 37°C. Fluorescence intensity was measured after reaction initation using a microplate fluorescence reader set at excitation 485 nm/emission 530 nm. The level of ROS was determined as relative fluorescence units (RFU) of generated DCF using standard curve of DCF production (Fouda and Abdel-Rahman, 2017; Fouda et al., 2018).

2.14 Intravenous Glucose Tolerance Testing (IV GTT)

The day before terminal surgery, rats were fasted overnight (approximately 16 h) according to approved animal use protocol (AUP). On the morning of terminal surgery, animals were anesthetized (as outlined in OVX section) and catheterized for hemodynamic measurements. Each rat was given fifteen minutes for continuous data recordings to stabilize, and then injected intravenously with a 20% bolus glucose dose (2g/10ml/kg; I.V.). Femoral artery blood was analyzed with a blood glucose monitoring system (Freestyle, Precision Neo, Abbott, Alameda, CA), and blood glucose levels (BGL) were recorded at intervals of baseline (5 min prior to bolus), and post bolus—1 min, 3 min, 5 min, 15 min and 30 min as reported (Dimitrova et al., 2008).

Table 1 Diet Information

Diet	Protein	Fat	Carbohydrates	Energy
57W5 (AIN-93G) Control Diet	18.80 %	16.40 %	64.90%	3.90 (kcal/g)[2]
58V8 High Fat Diet	18.00 %	45.70 %	35.50 %	4.65 (kcal/g)[2]

CHAPTER THREE: ESTROGEN DEPENDENT DISRUPTION OF ADIPONECTIN-CONNEXIN43 SIGNALING UNDERLIES EXACERBATED MYOCARDIAL DYSFUNCTION IN DIABETIC FEMALE RATS

3.1 Abstract

The reasons for the higher severity of type 2 diabetes (T2DM)-associated cardiomyopathy in women, despite their inherent estrogen (E_2)-dependent cardioprotection, remain unknown. We hypothesized that the reliance of the healthy females' hearts on augmented adiponectin (APN)-connexin43 (Cx43) signaling becomes paradoxically detrimental when disrupted by T2DM in an E_2-dependent manner. We tested this hypothesis in high fat-low dose streptozotocin diabetic rats and their controls with the following designations: (a) sham operated (SO); (b) ovariectomized (OVX); (c) ovariectomized with E_2 supplementation (OVX+E_2); and (d) male. E_2-replete (SO or OVX+E_2) diabetic rats exhibited higher mortality and greater increases in left ventricular (LV) mass, and reduced LV developed pressure, LV contractility and fractional shortening, but preserved ejection fraction. Further, compared to respective nondiabetic counterparts, the hearts of these E_2-replete diabetic rats exhibited greater upregulation of cardiac estrogen receptor α (ERα) and reductions in Cx43 expression and in the phosphorylation levels of the survival molecules pERK1/2 and pAKT. While serum APN was reduced independent of sex and ovarian hormone status in all DM, cardiac APN was most drastically reduced in DM SO rats. The present translational findings are the first to implicate ovarian hormones/E_2 in the exacerbated myocardial dysfunction in diabetic females, and to suggest a pivotal role for malfunctioning cardiac APN-Cx43 signaling in this sex/E_2-specific clinical problem.

3.2 Introduction

There is mounting interest to further understand the role of estrogen (E_2) regulation in cardiovascular function (Gros et al., 2016; Dworatzek and Mahmoodzadeh, 2017). Specifically, there is a need to understand why cardiovascular morbidity and mortality are higher in women with type-2 diabetes mellitus (T2DM), compared with age-matched diabetic men (Regensteiner et al., 2015). Paradoxically, premenopausal women not only lose their inherent E_2-mediated cardioprotection, but exhibit exacerbated T2DM-induced cardiac anomalies, compared to T2DM men (Juutilainen et al., 2004). New evidence for a lack of negative cardiovascular consequences of E_2 replacement therapy (ERT) (Manson et al., 2017) raises the potential for a rise in cases of exacerbated T2DM associated cardiac anomalies.

Alterations in E_2 receptor (ER) expression (upregulation of ERα and GPER) may contribute to sex differences in left ventricular (LV) hypertrophy and heart failure (Regitz-Zagrosek et al., 2010; Lee et al., 2014). The complex interplay between ER subtypes highlights the need to study their expression in the hearts of diabetic females in the absence or presence of estrogen. Notably, while ERα knockout mice exhibit disrupted adiponectin (APN) signaling (Mauro et al., 2014), there were no studies on cardiac levels of ER subtypes or APN in diabetic females.

APN, an adipokine with anti-inflammatory and antidiabetic properties (Guo et al., 2007; Fisman and Tenenbaum, 2014), is reduced in T2DM, and coronary artery disease (Zhu et al., 2008; Tian et al., 2009). Higher APN levels and greater protection of LV function from oxidative stress in eNOS deficient mice (Durand et al., 2012) suggest a cardioprotective role for APN in female mice. Further, APN supplementation in aged female

rats and porcine models reduced myocardial infarct size (Tomicek et al., 2015) and oxidative stress (Tao et al., 2007). However, it remained unknown if this sexually dimorphic cardioprotection, conferred by APN or its downstream effector connexin43 (Cx43), is compromised in DM females.

Connexins are widely distributed transmembrane gap junction proteins and Cx43, the primary connexin expressed in cardiomyocytes, has emerged as a promising therapeutic target for cardioprotection (Bikou et al., 2011; Stauffer et al., 2011). The reduction or disorganization of LV Cx43 in heart diseases (Michela et al., 2015) and cardiac dysfunction (Lin et al., 2005) support its protective role against cardiac injury. Importantly, while female cardiomyocytes exhibit higher levels of Cx43 and its phosphorylated form (Stauffer et al., 2011) and E_2 upregulates Cx43 expression (Moinfar et al., 2016), there are no studies on cardiac Cx43 expression, and E_2 regulation in diabetic females.

The major goal of the present study was to determine if diabetes-evoked myocardial dysfunction is more pronounced in female, than male, rats in an E_2-dependent manner. Further, we tested the hypothesis that disruption of the E_2-APN-Cx43 axis underlies this sex/E_2-specific hypersensitivity to T2DM-evoked myocardial dysfunction. The outcome of these findings will identify potential molecular targets for developing novel therapeutics for alleviating myocardial dysfunction in DM females.

3.3 Protocols and experimental groups

Eight groups of rats (n=8 surviving) divided into 4 pairs (diabetic and control) of sham operated (SO), ovariectomized (OVX), ovariectomized with E_2 replacement (OVX+E_2), and male rats. Baseline echo was obtained before treatments and biweekly in all groups. Four

weeks after the second STZ injection were allowed for the development of the diabetic/hyperglycemic state before the terminal invasive catheterization surgery for hemodynamic measurements, and ex vivo studies (Fig. 1).

3.4 Data analysis and statistics

Initially, statistical analysis consisted of two-way ANOVA. Post hoc testing of significant findings included the F-test for comparison of diabetic groups vs. control groups, and Tukey's unpaired t-test for evaluation of the estrogen variant groups (Sham, OVX, OVX+E_2 and male). Kaplan-Meier survival curves equality was assessed using the Wilcoxon's log-rank test. Values are expressed as means ± SEM with probability levels less than 0.05 to be considered significant. Prism 5 software (Graphpad Software Inc., San Diego, CA) was used to perform statistical analysis.

Figure 1

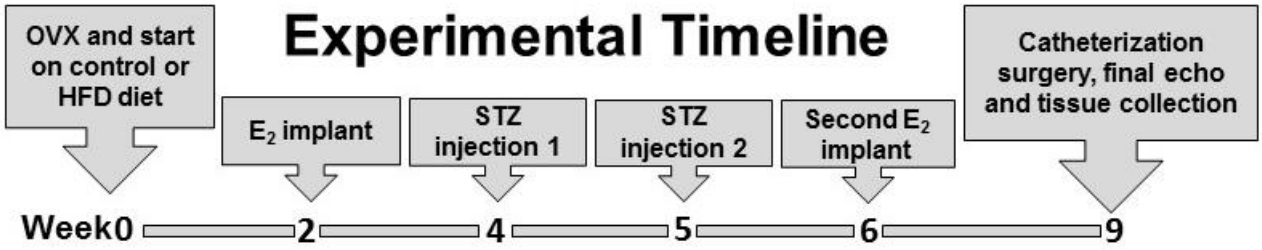

Experimental Timeline
OVX and start on control or HFD diet
E2 implant
STZ injection 1
STZ injection 2
Second E2 implant
Catheterization surgery, final echo and tissue collection
Week0
2
4
5
6
9

Figure 1. A schematic presentation of high fat diet regimen, diabetes induction, and the biochemical and molecular cardiovascular measurements in sex/estrogen variant groups of Wistar rats. OVX, ovariectomy; STZ, streptozotocin; E_2, estradiol.

3.5 Results

3.5.1 Higher mortality and heart weight to body weight ratio in diabetic E_2 replete females

Serum E_2 levels were lower ($P<0.0001$) in healthy OVX and male rats compared with healthy SO and OVX+E_2 rats, and was not affected by TDM in any of the groups (Table 2). At the conclusion of the study, diabetic SO and diabetic OVX+E_2 exhibited the most pronounced mortality (33%) followed by diabetic male (20%) and diabetic OVX (8%) rats vs. no mortality in healthy SO, OVX+E_2 or OVX groups, and a 10% mortality in healthy male rats. The higher mortality in T2DM male rats is consistent with preclinical (Guo et al, 2007) and clinical (Regensteiner et al., 2015) findings. Final body weights were similar in diabetic and healthy SO or OVX+E_2 rats, but lower ($P=0.02$ and $P=0.008$, respectively) than those of age-matched OVX or male rats (Table 3). The heart weight (HW) to body weight (BW) ratio was higher in the diabetic SO ($P<0.0001$) and OVX+E_2 ($P=0.02$), when compared to respective healthy controls. The HW/BW ratio was also significantly higher when compared to E_2 depleted diabetic groups. Diabetic sham vs. diabetic OVX ($P=0.04$) or diabetic male ($P=0.02$), and diabetic OVX+E_2 vs. diabetic OVX ($P=0.02$) or diabetic male ($P=0.02$) (Table 3).

At the conclusion of the ten weeks (see Fig. 1), echocardiography findings showed no change in LV mass or major LV function indices in OVX rats, compared to their healthy counterparts (Figs. 2B, 2C and 3) and ejection fraction was not influenced by diabetes or the hormonal status (Fig 2A). However, LV mass was higher in diabetic SO ($P=0.003$), diabetic OVX+E_2 ($P=0.04$) and diabetic male ($P=0.02$) rats, compared with their respective controls (Fig. 2C). The findings in the SO and OVX+E_2 groups were verified by higher heart

weight/body weight ratios (P<0.05, see first paragraph for specific P values) in the healthy rat groups, compared to their non-diabetic counterparts (Table 3). Further, echocardiography and directly measured LV function findings reflected significant myocardial dysfunction in diabetic E_2 replete vs. health groups via two-way ANOVA (Figs. 2B, 3A and 3B). Diabetic SO (P=0.03) and diabetic OVX+E_2 (P=0.02) displayed reduced fractional shortening, compared with their respective controls (Fig. 2B). In Fig. 3a, left ventricular developed pressure (LVDP) was decreased in diabetic SO vs. healthy SO (P=0.009) or OVX+E_2 (P=0.04) rats. Fig. 3B shows reduced (P=0.007) dP/dt_{max} in the diabetic OVX+E_2 vs. OVX+E_2 rats. The study also showed higher (P=0.02) Tau values in diabetic vs. healthy SO groups (Fig. 3C). Echocardiography findings displayed a reduced cardiac contractility index (Fig. 3D) in diabetic vs. healthy SO (P=0.003) or OVX+E_2 (P=0.04) rats, which overlaps with the fractional shortening findings from echocardiography (Fig. 2B).

3.5.2 Sex- and diabetes-dependent ER subtype, APN, AdipoR1 responses

DM upregulated cardiac ERα (Fig. 4A) in all groups when compared to respective controls (SO: P=0.04; OVX: P=0.04; OVX+E_2: P=0.01 and male: P=0.02), and upregulated GPER (Fig. 4B) in all female groups (SO: P=0.04; OVX: P=0.03 and OVX+E_2: P=0.04), but not in male rats. DM did not change (2-way ANOVA) cardiac ERβ (Fig. 4C) or AdipoR1 (Fig. 4D) expression. In additon to reduced serum APN in all diabetic vs. healthy groups (SO: P=0.007; OVX: P=0.02; OVX+E_2: P=0.04 and male: P=0.002), the reduction was most pronounced in the SO group (Fig. 5A). Notably, cardiac APN was only reduced (P=0.02) in SO diabetic vs. control groups (Fig. 5B).

3.5.3 Myocardial Cx43, pERK/ERK and pAKT/AKT expressions are reduced in diabetic estrogen replete female rats

E_2-replete diabetic rats exhibited significant reduction in the phosophorylated levels of cardiac ERK1/2 (pERK1/2; Fig. 5C) when compared to respective controls (SO: P=0.04 and OVX+E_2: P=0.01) as well as reduced cardiac levels of the cell phosphorylated survival molecule AKT, pAKT (Fig. 5D; SO: P=0.02 and OVX+E_2: P=0.04). Finally, cardiac Cx43 was reduced in diabetic SO (P=0.0002) or OVX+E_2 (P=0.04) vs. their respective control rats, but not in the E_2 deficient (OVX and male) groups (Figs. 6A and B). The reduction in cardiac Cx43 expression was corroborated by the reduced colocalization of Cx43 with the scaffolding protein caveolin-3 (cav3) in diabetic vs. SO healthy rats (Fig. 6C). Collectively, these molecular findings paralleled the reductions in cardiac function in the same E_2-replete diabetic vs. healthy female rats (Figs. 2 and 3).

Table 2. Serum E_2 levels in diabetic and control rats (n=8 surviving/group)

Group	Estradiol (pg/mL)
Sham Operated (SO)	**26.72 ± 1.35**
OVX	**3.02 ± 0.52***
OVX+E_2	**28.47 ± 2.67**
Male	**1.22 ± 0.27***
Diabetic SO	**25.74 ± 0.87**
Diabetic OVX	**2.54 ± 0.58***
Diabetic OVX+E_2	**31.78 ±4.63**
Diabetic Male	**1.46 ± 0.26***

*Denotes significant difference ($P<0.05$) compared with healthy sham-operated female rats.

Table 3. Heart weight, body weight, the heart weight/body weight ratio and left ventricular (LV) mass in diabetic and control groups (n=8 surviving/group)

Group	Heart weight (HW) (g)	Body weight (BW) (g)	HW/BW Ratio	LV mass (mg/kg)
Sham	0.44 ± 0.02	278.22 ± 8.00	1.60 ± 0.07	1554.00 ± 64.73
OVX	0.57 ± 0.04	324.00 ± 9.06	1.74 ± 0.09	1736.00 ± 113.30
OVX+E_2	0.54 ± 0.03	293.50 ± 6.40	1.83 ± 0.14	1772.00 ± 150.90
Male	0.95 ± 0.01$^{\#}$	475.11 ± 10.88$^{\#}$	1.99 ± 0.20	1591.00 ± 101.70
Diabetic Sham	0.74 ± 0.05	253.40 ± 16.00	3.01 ± 0.21$^{*\#}$	2531.00 ± 315.90$^{*\#}$
Diabetic OVX	0.56 ± 0.04	313.89 ± 10.37	1.87 ± 0.11	1789.00 ± 103.70
Diabetic OVX+E_2	0.67 ± 0.04	278.13 ± 12.68	2.43 ± 0.19$^{*\#}$	2037.00 ± 67.86$^{*\#}$
Diabetic Male	0.85 ± 0.07	451.17 ± 21.23$^{\#}$	1.95 ± 0.12	1978.00 ± 96.64

# denotes intergroup significant differences ($P<0.05$). The HW/BW ratio and LV mass are higher in the diabetic estrogen replete groups, when compared to estrogen depleted groups.
* denotes significant ($P<0.05$) differences between a diabetic and its respective healthy group.

Figure 2

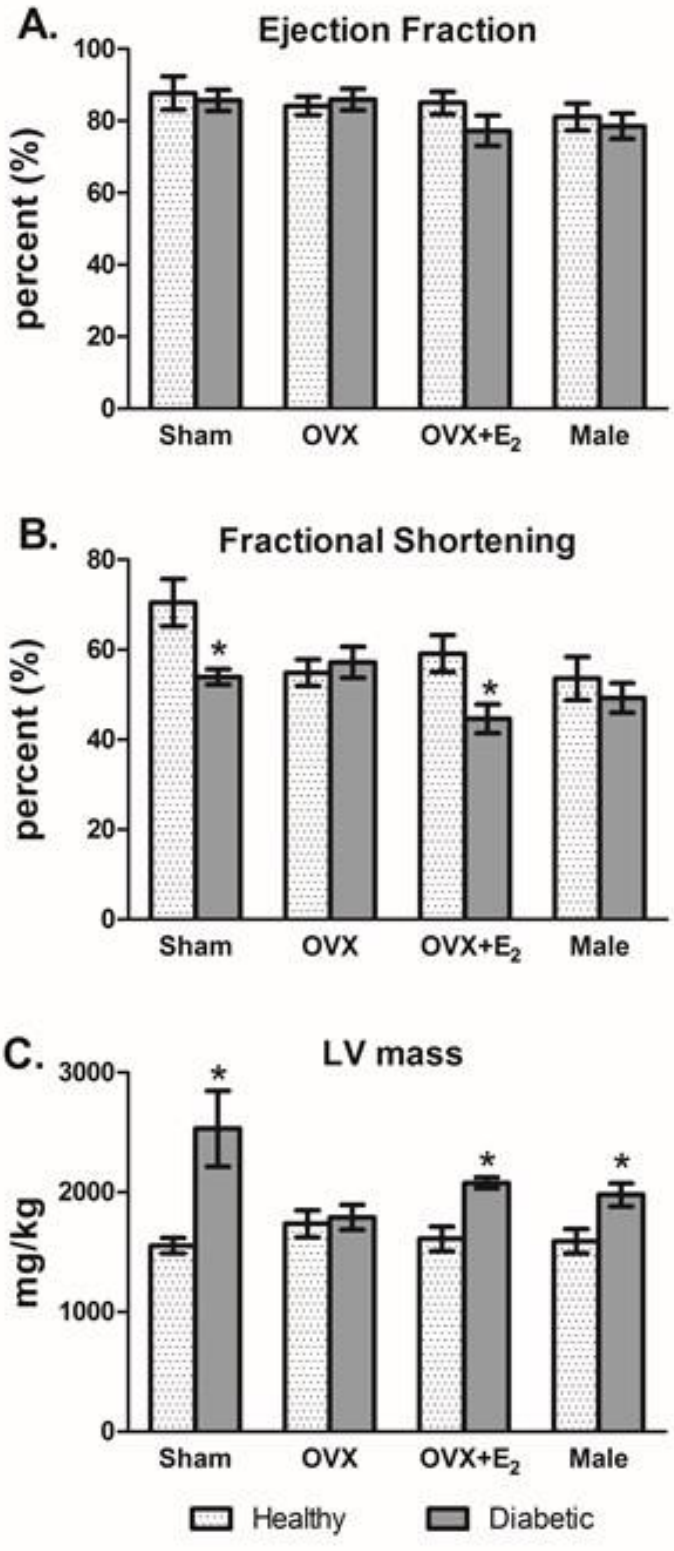

A.
Ejection Fraction
percent (%)
100
80
60
40
20
0
Sham
OVX
OVX+E2
Male
B.
Fractional Shortening
percent (%)
80
60
40
20
0
*
*
Sham
OVX
OVX+E2
Male
C.
LV mass
mg/kg
3000
2000
1000
0
*
*
*
Sham
OVX
OVX+E2
Male
Healthy
Diabetic

Figure 2. Effect of diabetic state, after four weeks, on echocardiography-derived ejection fraction (A), fractional shortening (B), and left ventricular mass (C). Values are means ± SEM (n=8 or 9 surviving/group). *P<0.05 when comparing diabetic vs. respective healthy groups (actual P values are included in the Results section).

Figure 3

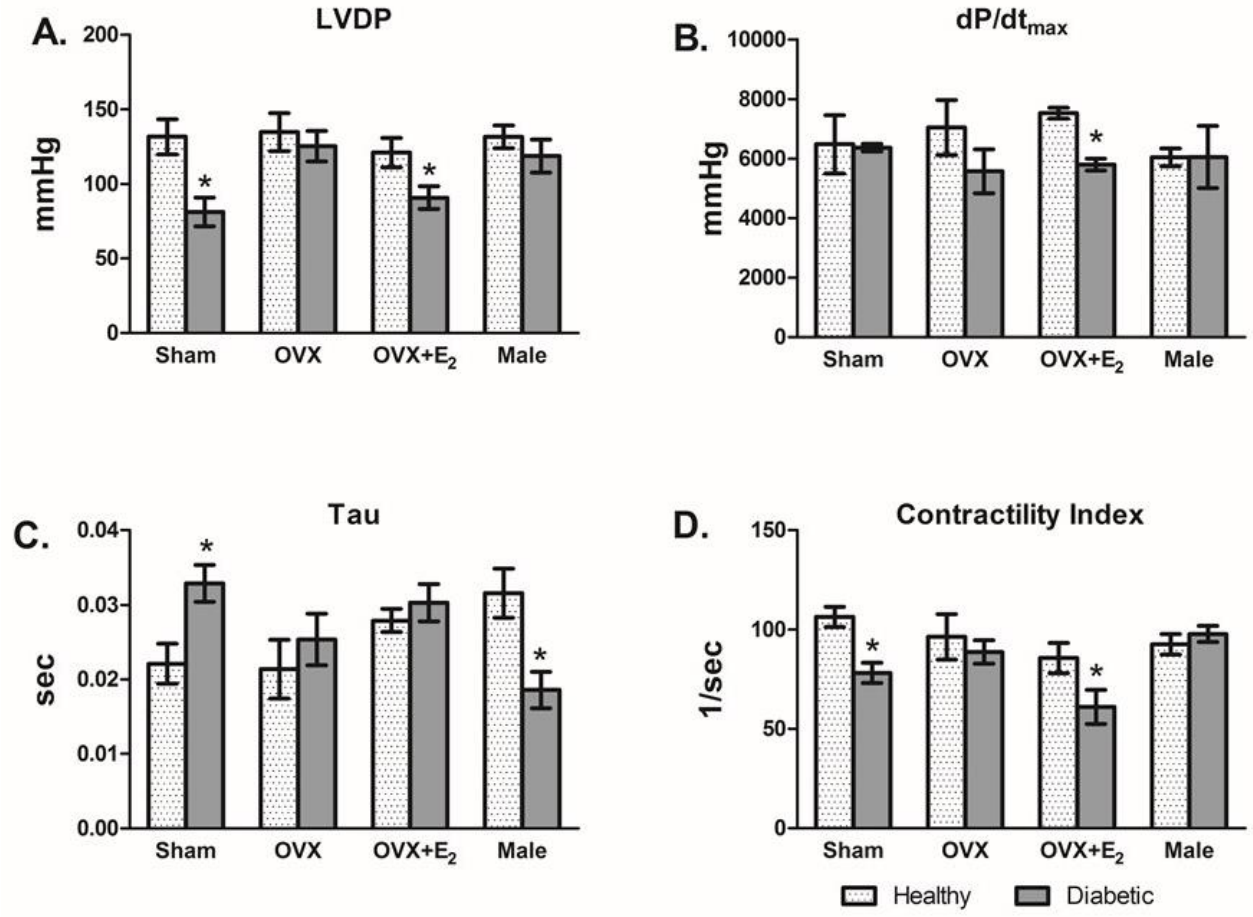
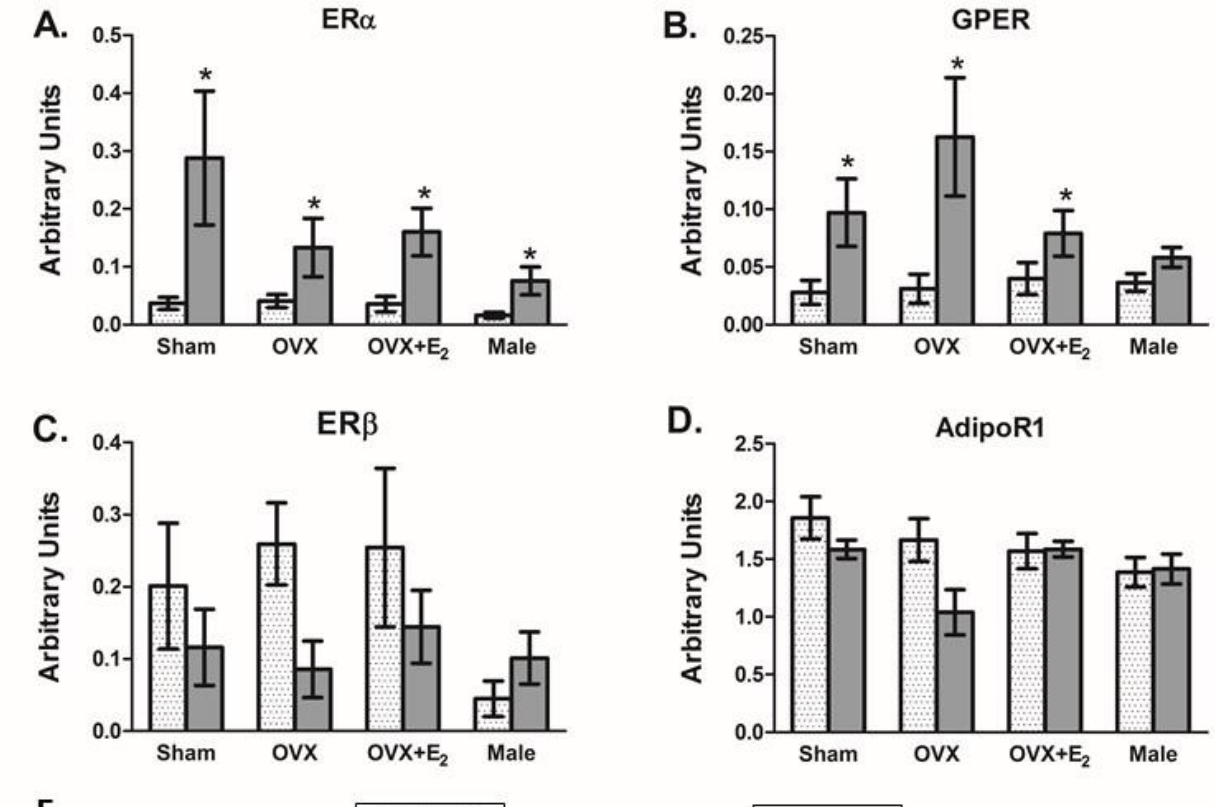

A.
LVDP
mmHg
200
150
100
50
0
Sham
OVX
OVX+E2
Male
B.
dP/dtmax
mmHg
10000
8000
6000
4000
2000
0
C.
Tau
sec
0.04
0.03
0.02
0.01
0.00
D.
Contractility Index
1/sec
150
100
50
Healthy
Diabetic

Figure 3. Effect of diabetic state, after four weeks, on directly measured hemodynamic variables via femoral and left ventricular catheterization. Diabetic vs. healthy estrogen variant groups display differences in left ventricular developed pressure (LVDP) (A), dP/dt_{max} (B), Tau (C) and contractility index (D). Values are means ± SEM (n=8 surviving/group). *P<0.05 when comparing diabetic vs. respective healthy groups (actual P values are included in the Results section).

Figure 4


A.
ERα
Arbitrary Units
0.5
0.4
0.3
0.2
0.1
0.0
Sham
OVX
OVX+E2
Male
B.
GPER
Arbitrary Units
0.25
0.20
0.15
0.10
0.05
0.00
Sham
OVX
OVX+E2
Male
C.
ERβ
Arbitrary Units
0.4
0.3
0.2
0.1
0.0
Sham
OVX
OVX+E2
Male
D.
AdipoR1
Arbitrary Units
2.5
2.0
1.5
1.0
0.5
0.0
Sham
OVX
OVX+E2
Male

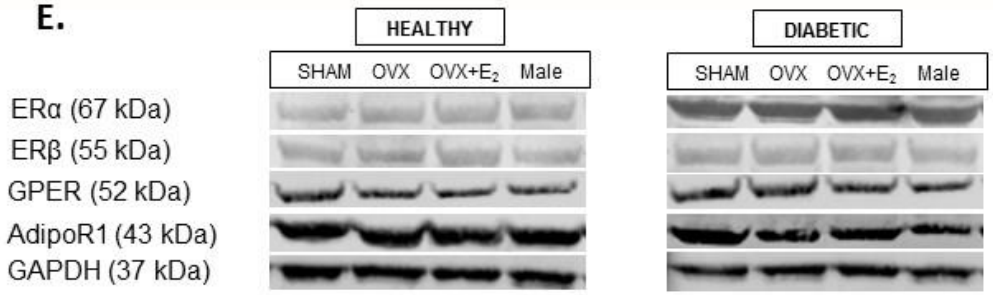

E.
HEALTHY
SHAM OVX OVX+E2 Male
DIABETIC
SHAM OVX OVX+E2 Male
ERα (67 kDa)
ERβ (55 kDa)
GPER (52 kDa)
AdipoR1 (43 kDa)
GAPDH (37 kDa)

Figure 4. Expression of cardiac estrogen and adiponectin receptors in diabetic and control rats. Shown are alterations due to estrogen or sex variations in estrogen receptor alpha; ERα (A), G-protein coupled receptor; GPER (B), estrogen receptor beta; ERβ (C), and adiponectin receptor 1; AdipoR1 (D). Homogenized left ventricular tissue was analyzed via western blot (n=8 surviving/group) and presented as the means ± SEM. Representative blots (E) for a particular protein in healthy and diabetic groups were run on the same gel and "arbitrary units" is a normalization of the target band to the GAPDH band on the same gel. *P<0.05 when comparing diabetic vs. respective healthy groups (actual P values are included in the Results section). Molecular weights of proteins are given in kDa.

Figure 5

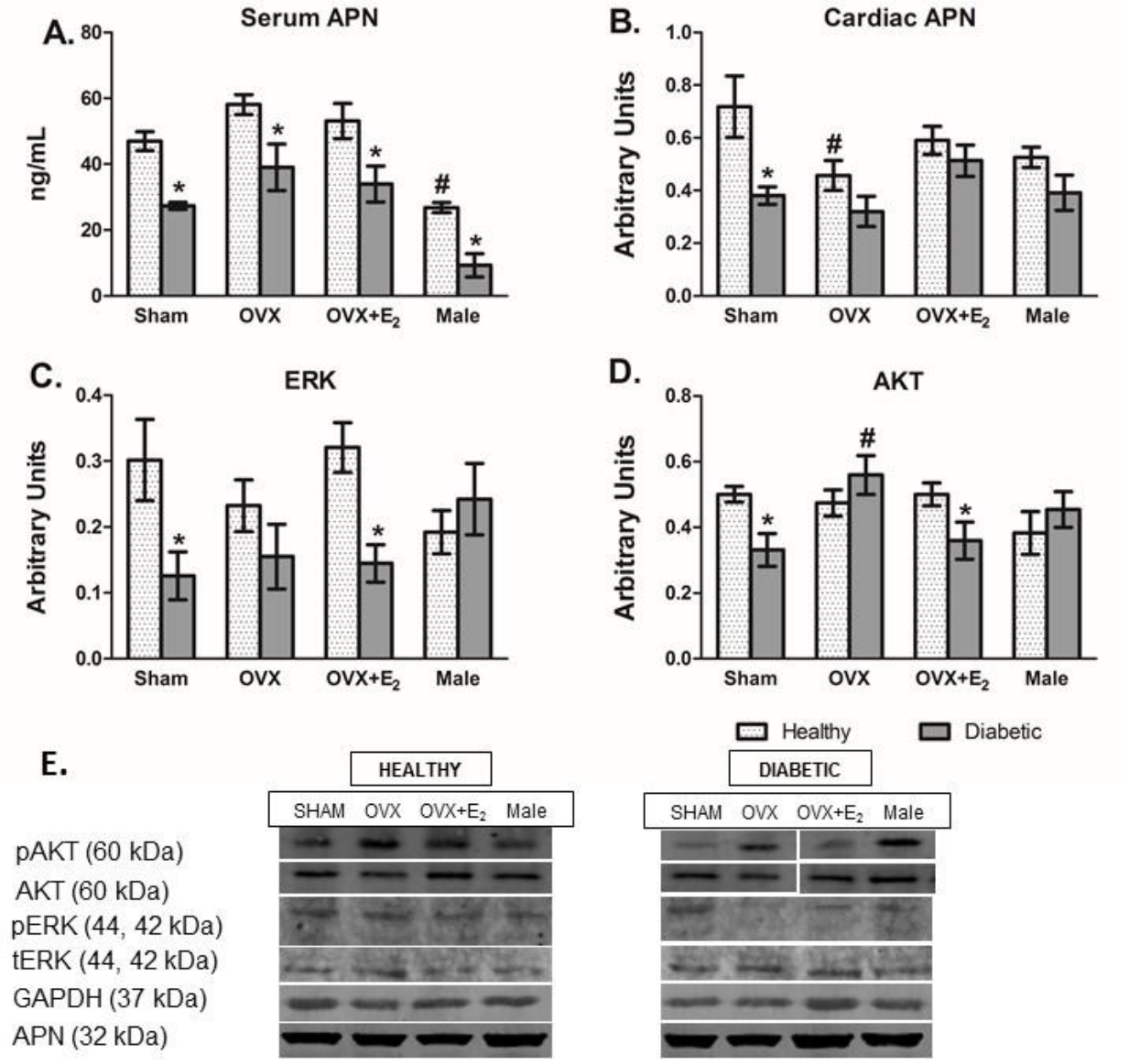

A.
Serum APN
ng/mL
80
60
40
20
0
Sham
OVX
OVX+E2
Male
B.
Cardiac APN
Arbitrary Units
1.0
0.8
0.6
0.4
0.2
0.0
C.
ERK
D.
AKT
Healthy
Diabetic
E.
HEALTHY
DIABETIC
SHAM OVX OVX+E2 Male
pAKT (60 kDa)
AKT (60 kDa)
pERK (44, 42 kDa)
tERK (44, 42 kDa)
GAPDH (37 kDa)
APN (32 kDa)

Figure 5. Diabetes reduces serum adiponectin (APN) in all groups (A) and cardiac APN in sham-operated (SO) rats (B) and causes estrogen dependent decreases in ERK1/2 (C) and AKT (D) phosphorylation. Measurements in left ventricular tissues were made using ELISA (A) and western blot (B-D). Representative blots (E) for a particular protein in diabetic and healthy groups were run on the same gel and "arbitrary units" is a normalization of the target band to the GAPDH (B), pERK to total ERK (C) and pAKT to total AKT (D). Data presented as the means ± SEM (n=8 surviving/group). *P<0.05 when comparing diabetic vs. respective healthy groups. $^{\#}$P<0.05 when comparing sex/estrogen variant groups within the diabetic or control factors (actual P values are included in the Results section).

Figure 6

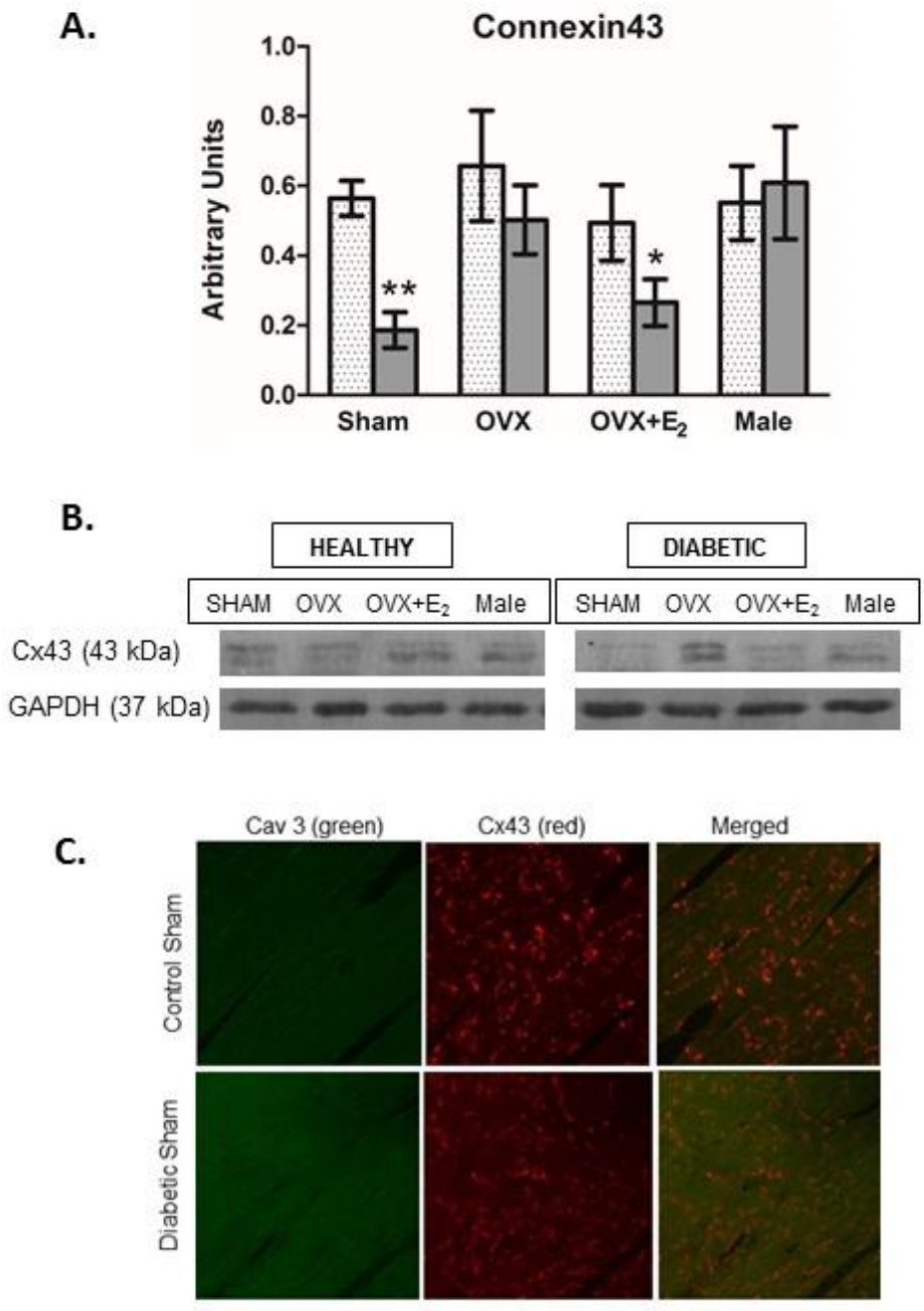

A.
Connexin43
Arbitrary Units
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Sham
OVX
OVX+E2
Male
B.
HEALTHY
DIABETIC
SHAM OVX OVX+E2 Male
SHAM OVX OVX+E2 Male
Cx43 (43 kDa)
GAPDH (37 kDa)
C.
Cav 3 (green)
Cx43 (red)
Merged
Control Sham
Diabetic Sham

Figure 6. Sex/E_2-dependent reduction in cardiac connexin43 (Cx43) expression in diabetic rats (A) and representative Western blots are shown (B). Representative confocal dual label immunofluorescence images of cardiac tissue show reduced expression and colocalization of Cx43 (*red*) with the scaffolding protein Cav3 (*green*) in diabetic SO vs. healthy SO (C) and corroborate the western blot findings. Values are presented as means ± SEM (n=8 surviving/group). *P<0.05 and **P<0.001 when comparing diabetic vs. respective healthy groups (actual P values are included in the Results section).

3.6 Discussion

It is currently accepted that T2DM-induced cardiac dysfunction, even in the presence of preserved ejection fraction, is associated with higher cardiac morbidity and mortality in women than men (Murphy et al., 2017), and our current findings agree with this premise. However, the mechanisms of this women's cardiovascular health problem remained unknown most likely due to the lack of preclinical studies that replicated this clinical problem. While the increased LV mass is linked to the severity of hyperglycemia, the higher obesity in T2DM women might have confounded this sex difference (Rutter et al., 2003). The present study is the first to show that diabetic female rats exhibited higher LV mass despite their lower body weights, to mimic the greater sensitivity of female rats to diabetes-evoked cardiac dysfunction, and the dependence of these detrimental cardiac effects on ovarian hormones, particularly estrogen (E_2). Our novel findings support the hypothesis that disruption of the salutary cardiac APN-Cx43 signaling underlies this sex/E_2-dependent problem.

The high-fat diet and low STZ dose regimen produces clinically relevant slowly developing myocardial dysfunction after 10-12 weeks in male rats (Hoit et al., 1999). Therefore, it was not surprising that applying the same regimen for only 4 weeks had no significant effect on cardiac function in male rats (Figs. 2 and 3). Nonetheless, parallel longitudinal echocardiography studies revealed significant myocardial dysfunction in young age-matched female rats (8 weeks old at the initiation of the study) subjected to the same 4 week regimen (Figs. 2 and 3). These findings, along with higher mortality findings are the first to mimic the higher sensitivity of women to T2DM-related myocardial dysfunction and mortality (Pradhan, 2014; Regensteiner et al., 2015) and support one of the overall study

goals. Next, we showed that this clinically relevant problem is dependent on ovarian hormones because OVX, similar to male, rats did not exhibit the short-term diabetes-evoked myocardial dysfunction and E_2 replacement uncovered diabetes-evoked myocardial dysfunction (Figs. 2 and 3) and higher mortality in OVX rats. We focused on E_2 because it paradoxically transforms into a proinflammatory hormone in the presence of oxidative stress (White et al., 2005) and diabetes induces oxidative stress (Rochette et al., 2014).

Some limitations of echocardiography might have precluded linking increases in LV mass to fractional shortening in a clinical study (Rutter et al., 2003). Here, our echocardiographic findings, validated and complemented by direct hemodynamic measurements of multiple cardiac indices (LVDP, contractility, fractional shortening, Tau, dP/dt_{max}), confirmed the myocardial dysfunction and its dependence on E_2 in diabetic female rats. Notably, decreased LVDP, contractility index and fractional shortening are signs of cardiac dysfunction, while increased LV mass indicates detrimental cardiac remodeling (Nagueh et al., 2009; Regitz-Zagrosek et al., 2010).

Our findings might reflect the DM-associated heart failure with preserved ejection fraction, which is more frequently diagnosed in women (Regitz-Zagrosek et al., 2010; Murphy et al., 2017), perhaps due to sex differences in cardiac remodeling processes (Dworatzek and Mahmoodzadeh, 2017). We acknowledge that a constant physiological plasma E_2 level (Table 2) is not identical to the phasic release of endogenous E_2 and could explain the more exaggerated cardiac dysfunction in diabetic SO vs. OVX+E_2 rats. Nonetheless, these detrimental cardiac effects in diabetic SO rats reflect the pathological outcomes in diabetic women (Pradhan, 2014). While some laboratory tests that confirm heart failure without reduced ejection fraction in humans are precluded in rodents due to

their high heart rate, the clinical use of BNP for this purpose (Bosseau et al., 2015) can be adopted in our future studies. Further, our novel preclinical findings in diabetic OVX+E_2 rats may be pertinent to diabetic surgical menopause women of child bearing age who are usually placed on E_2 replacement therapy (Baeza et al., 2010). Also notable as a study limitation is that while the diabetic rodent model utlilized in this study resembles, but not identical to, human T2DM, and results should be interpreted accordingly.

Findings that concomitant ERα downregulation and GPER upregulation inhibit vascular remodeling more in females than in males (Lee et al., 2014; Gros et al., 2016) indirectly infer detrimental consequences of higher ERα expresssion or its ratio to GPER. While the higher cardiac ERα in diabetic rats (Fig. 4A) is consistent with this premise, two seemingly discordant findings must be discussed. First, albeit more prominent in SO rats, cardiac ERα expression was higher in all diabetic rats irrespective of sex or the hormonal status (Fig. 4A) or myocardial (dys)function (Fig. 2). It is likely that the activation of the upregulated ERα by endogenous (SO) or exogenous E_2 (OVX+E_2) expedited cardiac dysfunction. Our findings that activation of ERα by E_2 or its highly selective agonist mediates alcohol-evoked cardiac dysfunction (El-Mas and Abdel-Rahman, 2000; Yao and Abdel-Rahman, 2017), and the elevated ERα espresssion in gestational diabetes and heart failure (Mahmoodzadeh et al., 2006; Kleiblova et al., 2010) support this premise. Second, the activation of the upregulated GPER by E_2 might play an additive detrimental role via increased LV mass in E_2-replete rats (Fig. 2C) because GPER-mediated inhibition of cardiomyocyte apoptosis likely expedites cardiac remodeling and heart failure (Gros et al., 2016). Nonetheless, it is possible that upregulations of ERα and GPER constitute a compensatory protective mechanism in diabetes given the recent recognition of GPER as a

major player in sex dependent cardioprotection (Lenhart et al., 2013). More studies in ER knockout mice or pharmacological loss or gain of ER subtype function studies (Yao and Abdel-Rahman, 2017) in diabetic rats are needed to address this important issue. Collectively, our findings that E_2 expedites the progression of diabetes-evoked cardiac dysfunction raised the possibility that disruption of E_2-dependent cardioprotective molecules might contribute to this clinically important problem.

We focused on the cardioprotective adipokine, adiponectin (APN), and its downstream effector Cx43 because their levels are higher in females (Stauffer et al., 2011), and they induce cell survival molecules such as AKT (Fujio et al., 2000; Muslin, 2011). APN and its receptors AdipoR1 and AdipoR2 (Ding et al., 2007) regulate a local cardiac-specific APN system to maintain normal myocardial energy homeostasis (Ding et al., 2007; Guo et al., 2007). We hypothesized that disruption of this local APN-Cx43 regulated cardiac homeostasis might play a critical role in the sex/E_2-dependent exacerbation of DM-evoked myocardial dysfunction. This premise is supported by AdipoR1 mediation of the APN cardioprotective action, and the abrogation of this cardioprotection in diabetes (Li et al., 2016), at least partly via reductions in AdipoR1 expression or APN level (this study). Clinically, while more studies involving adolescents and young women are needed, early cardiopulmonary dysfunction has been correlated with low adiponectin levels in adolescents with type 2 diabetes (Bjornstad et al., 2016).

Physiologically, Cx43 protects cardiomyocyte function and its downregulation, under pathological conditions such as diabetes, contributes to heart failure (Michela et al., 2015). Notably, remodeling after cardiac pressure overload downregulated cardiac Cx43 expression via the AMPK pathway (Alesutan et al., 2015) and APN activates the AMPK

pathway (Fisman and Tenenbaum, 2014). Therefore, the reduction in cardiac Cx43 expression (Fig. 6A-C) as a consequence of the reduction in circulating and myocardial APN levels (Figs. 4D and 5) likely contributed to the suppressed LV contractility (Fig. 3d) and higher mortality in E_2-replete diabetic rats. This E_2/sex-specific mechanism is supported by the lack of similar reductions in cardiac Cx43 expression or in cardiac function in diabetic male or OVX rats under the same conditions (Fig. 5). Importantly, prolonged (>10 weeks) exposure to diabetes caused cardiac Cx43 disorganization and dysfunction in male rats (Hoit et al., 1999; Regensteiner et al., 2015). These findings support the higher sensitivity of E_2-replete rats to diabetes-evoked myocardial dysfunction, and implicate the dysfunction in cardiac APN-Cx43 signaling in this clinically relevant problem.

Cx43 confers cardioprotection, at least partly, via activation (phosphorylation) of the cell survival molecule AKT and its downstream cardioprotective effecctor, ERK1/2 (Fujio et al., 2000; Muslin, 2011). Therefore, we hypothesized that reductions in the phosphorylation of AKT or ERK1/2, secondary to reduced Cx43, contribute to the E_2-dependent myocardial dysfunction in diabetic rats. As ERK activation protects cardiomyocytes from apoptosis under oxidative stress (Gong et al., 2015), the reduced phosphorylation of cardiac ERK in diabetic E_2-replete female rats (Fig. 5C) might contribute, at least partly, to the associated cardiac dysfunction (Fig. 2B and Fig. 3). The hypothesis is also supported by the reduction in phosphorylated (p)-AKT levels in the hearts of these rats (Fig. 5D) and by the association of reduced cardiac p-AKT with disrupted gap junction proteins and cardiac contractile dysfunction (Ock et al., 2018).

In conclusion, we represented the higher predisposition to, and severity of, diabetes-evoked cardiac anomalies in women. Our novel findings implicate E_2 in the accelerated

disruption of cardiac APN-Cx43 signaling as a molecular mechanism for the exacerbated cardiac dysfunction in diabetic females. Further, our findings yielded new insights into potential therapeutic targets for mitigating the sex-specific exacerbation of cardiac dysfunction in diabetic females.

CHAPTER FOUR: RESTORATION OF ADIPONECTIN-CONNEXIN43 SIGNALING AXIS REVERSES MYOCARDIAL DYSFUNCTION IN DIABETIC FEMALE RATS

4.1 Abstract

A cause-and-effect relationship between disrupted cardiac adiponectin (APN)-connexin43 (Cx43) signaling and the hypersensitivity to type 2 diabetes (T2DM)-evoked cardiomyopathy in estrogen (E_2)-replete rats has remained elusive. We hypothesized that restoration of the APN-Cx43 signaling would alleviate the E_2-dependent cardiac dysfunction in diabetic female rats. To test this hypothesis, we administered the adiponectin receptor 1 (AdipoR1) agonist AdipoRon (30 mg/kg/d) or its vehicle during the last 10 days of an 8-week diabetes mellitus (DM) regimen, comprised of a high fat diet and low dose streptozotocin treatment, in sham operated (SO) or ovariectomized (OVX) rats; control SO and OVX rats received control diet and vehicle for streptozotocin. In DM SO rats, which exhibited left ventricular (LV) and autonomic dysfunction, AdipoRon alleviated the: (i) shift to sympathetic dominance (increased LF band and LF/HF ratio), (ii) LV hypertrophy, (iii) the reductions in fractional shortening (FS), LV developed pressure, dP/dt_{max}, dP/dt_{min} and Tau. T2DM had no effect on any of these variables in OVX rats. In LV tissues, AdipoRon reversed the reduction in Cx43 and the elevations in TNFα, hemeoxygenase-1 (HO-1) and circulating Asymmetric Dimethylarginine (ADMA) levels. These novel findings yield new insight into a causal role for compromised APN-Cx43 signaling in the E_2-dependent hypersensitivity to DM-evoked cardiac inflammation and dysfunction. Equally important the findings identify restoration of Cx43 signaling as a viable therapeutic modality for alleviating this sex/E_2-specific clinical problem.

4.2 Introduction

More studies are needed to understand the mechanisms of the paradoxical hypersensitivity of women (Regensteiner et al., 2015) and female rats (Leffler and Abdel-Rahman, 2019) to type-2 diabetes mellitus (T2DM) associated cardiac anomalies despite their inherent estrogen (E_2)-mediated cardioprotection (Juutilainen et al., 2004). Our recent preclinical findings are the first to link E_2-dependent disruption of the adiponectin (APN)-connexin43 (Cx43) signaling to cardiac dysfunction in a model of T2DM (Leffler and Abdel-Rahman, 2019). However, it remains unknown if such disruption plays a causal role in E_2-regulated molecular responses and the subsequent autonomic and cardiac dysfunction in our model system.

E_2 regulation of its receptor (ER) subtypes contributes to sex differences in cardiovascular health and anomalies via modulation of redox enzymes and anti-inflammatory modulators (Lee et al., 2014). Specifically, while ERα upregulation contributes to the higher antioxidant catalase and $ALDH_2$ activities in healthy E_2-replete rats (Steagall et al., 2017), ERα is paradoxically upregulated in dysfunctional cardiac myocytes of E_2-replete diabetic female rats (Leffler and Abdel-Rahman, 2019). Further, E_2 escalates cardiac G-protein coupled estrogen receptor (GPER) upregulation in female diabetic rats (Leffler and Abdel-Rahman, 2019).

The levels of the anti-inflammatory adipokine APN (Guo et al., 2007) are higher in healthy (Kadowaki et al.; Zhu et al., 2008), but substantially lower in T2DM rats (Leffler and Abdel-Rahman, 2019), in the presence of E_2. While the APN receptors (AdipoR1, AdipoR2 and T-cadherin) exhibit cardiac protective qualities and display gender differences in certain

tissues (Iglesias, et al., 2006), their roles are poorly understood. It is noteworthy that reduced APN receptor signaling may constitute an important missing link in our understanding of the mechanisms of the adverse outcomes of T2DM (Schernthaner and Stangl, 2013) likely due to a compromised cardioprotective cell signaling.

APN induces the primary cardiomyocyte connexin, Cx43 (Ruiz-Meana et al., 2008), which constitutes a promising therapeutic target (Bikou et al., 2011; Stauffer et al., 2011) for alleviating heart diseases (Michela et al., 2015) (Lin et al., 2005). Our recent findings that Cx43 is reduced in T2DM female rats along with cardiac dysfunction (Leffler and Abdel-Rahman, 2019) provided the framework for investigating the potential therapeutic benefits of restoring the APN-Cx43 signaling axis in our model system.

AdipoRon, an orally active small molecule agonist for AdipoR1 and AdipoR2 has been identified as a possible therapeutic for DM (Okada-Iwabu et al., 2013). AdipoRon reproduces the effects of endogenous full molecular weight APN, such as activation of AMPK signaling, in both male and female mice (Okada-Iwabu et al., 2013) and attenuates: (i) post-ischemic myocardial apoptosis (Zhang et al., 2015), (ii) pressure overload-evoked cardiac remodeling (Lin et al., 2005; Zhang et al., 2018), and (iii) diabetic nephropathy (Choi et al., 2018). None of these studies, however, dealt with E_2-dependent hypersensitivity of female animals to DM-evoked cardiac inflammation and dysfunction.

The goal of the present study was to determine if AdipoRon mitigates the E_2-dependent exacerbation of cardiac dysfunction in diabetic females. These findings may discern a causal role for the cardiac APN-Cx43 disruption in the hypersensitivity to diabetic cardiomyopathy and may identify a novel therapeutic modality for alleviating these anomalies, in E_2-replete diabetic females.

4.3 Protocols and experimental groups

Six groups, 3 sham operated (SO) and 3 ovariectomized (OVX) groups, of rats (n=7 surviving) were divided into 3 pairs as follows (Fig. 1): i. DM SO with AdipoRon or its vehicle. ii. DM OVX with AdipoRon or its vehicle. iii control/healthy SO and OVX groups. Baseline echocardiography was obtained prior to the initiation of the studies and performed biweekly in all groups. After the second STZ dose, four weeks were allowed for the development of the diabetic state and AdipoRon (30 mg/kg/d) or its vehicle was administered orally during the last 10 days. Thereafter, terminal arterial and LV catheterizations were performed for hemodynamic measurements and concluded with euthanasia and tissue collection (see Figure 1).

4.4 Data analysis and statistics

Statistical analysis consisted of two-way ANOVA with post hoc testing of significant findings including the F-test for comparison and Tukey's unpaired t-test for evaluation of the estrogen variant groups (Control SO, DM SO and DM OVX) and vehicle vs. AdipoRon dosing. Values are expressed as means ± SEM with probability levels below 0.05 considered significant. Prism 7 software (Graphpad Software Inc., San Diego, CA) was used to perform statistical analysis.

4.5 Results

4.5.1 Overall mortality of diabetic female rats was not significantly altered with AdipoRon dosing

Consistent with our previous results (Leffler and Abdel-Rahman, 2019), DM SO rats had higher mortality compared to DM OVX rats (P=0.01 for OVX vs. DM OVX and P<0.001 SO vs. DM SO) (Figure 2A). AdipoRon (30 mg/kg/d; P.O.) had no effect on mortality when the drug was administered during the last 10 days of the 8-week DM regimen (Figure 2B).

4.5.2 AdipoRon mitigates the hypersensitivity to autonomic dysregulation in DM SO rats

A physiologically lower (P=0.01) low frequency (LF) nu (Figure 3A) and LF/HF ratio (P=0.002) (Figure 3C) in healthy SO than OVX females was negated and transformed into higher LFnu (P=0.008) and LF/HF ratio (P=0.004) by DM only in SO rats. These findings reflect a shift toward sympathetic dominance in DM SO when compared to DM OVX rats (Figure 3). AdipoRon reversed this autonomic abnormality to a level that was not different (P>0.05) from SO control levels (Figure 3).

4.5.3 AdipoRon ameloriated LV hypertrophy in diabetic SO female rats

OVX (healthy and DM) rats were heavier (P=0.04) than SO (healthy and DM) rats, respectively, and AdipoRon (30 mg/kg/d; P.O.; 10 days) had no effect on body weights of DM SO or OVX rats (Fig. 4A). LV mass was higher (P=0.002) in DM SO, but not DM OVX, rats, when compared to their healthy controls, and AdipoRon partially, but significantly

(P=0.04) reversed this hypertrophy (Figure 4B). Ejection fraction was not affected by DM in SO or OVX rats but was decreased (P=0.03) with AdipoRon in DM SO rats (Figure 4C).

4.5.4 AdipoRon mitigates cardiac dysfunction in diabetic SO female rats

AdipoRon reversed the decrease in fractional shortening (FS) in DM SO rats while the lower (P=0.02) FS in OVX rats was not affected by DM in the absence or presence of AdipoRon (Figure 4D). Similiarly, the reductions in LV developed pressure (P=0.03) (LVDP; Fig. 5A), dP/dt_{max} (Figure 5C) and dP/dt_{min} (P=0.001) (Figure 5D) along with the increase (P=0.04) in Tau (Figure 5B), observed in DM SO, but not in DM OVX, rats were reversed by AdipoRon. Finally, LV systolic and diastolic end volume, end diastolic pressure (EDP) and contractility index (CI) were not altered (P>0.05) with 10 days of AdipoRon administration when compared to vehicle-treated DM animals (Table 1).

4.5.5 AdipoRon reverses ERα upregulation, heightens GPER upregulation and restores cardiac Cx43 levels in DM SO females

DM upregulated cardiac ERα in SO and OVX rats (P=0.007 and P=0.008; respectively), compared to respective controls, consistent with our recent findings (Leffler and Abdel-Rahman, 2019), and AdipoRon reversed this upregulation (P=0.02 and P=0.02) (Figure 6A). AdipoRon further upregulated GPER (P=0.02) (Figure 6B) and restored Cx43 to control levels (P=0.04 SO vs. DM SO and P=0.01 DM SO vehicle vs. DM SO AdipoRon dosed) (Figure 6C) only in DM SO females. Interestingly, while these studies showed similar downregulation in cardioprotective molecule AKT in DM SO when compared to DM OVX, AdipoRon did not alter cardiac levels of AKT in DM SO rats, and further lowered them in DM OVX rats (P=0.02) (Figure 6D).

4.5.6 AdipoRon suppresses cardiac expression of AdipoR1 and APN

AdipoRon, an agonist for AdipoR1, reduced (P=0.002) the LV expression of AdipoR1 in DM SO rats (Figure 7A) when compared to healthy SO or vehicle-treated DM SO rats. While DM had no effect, combining DM and AdipoRon reduced (P=0.03) AdipoR1 in OVX females (Figure 7A). Consistent with previous studies (Leffler and Abdel-Rahman, 2019) cardiac APN was reduced in DM SO and DM OVX females, regardless of hormonal status and, here we show further cardiac APN reduction caused by AdipoRon in both groups (Figure 7B, C).

4.5.7 AdipoRon reversed the elevations in ADMA and TNFα in DM SO females

Serum asymmetric dimethylarginine (ADMA; Figure 7D) and cardiac TNFα (Figure 8A) were higher in healthy OVX vs. SO rats (Fig. 8C) (P=0.02 for TNFα; trend for ADMA; P=0.06). The greater elevation (P=0.001, SO vs. DM SO and P=.02 OVX vs. DM OVX) in ADMA and the increase (P=0.002, SO vs. DM SO) in TNFα in DM SO, compared with DM OVX (P=0.04), rats were reversed by AdipoRon (P=0.04, DM SO vehicle vs. DM SO AdipoRon treated for ADMA levels; Figure 7D) (P=0.03, DM SO vehicle vs. DM SO AdipoRon treated for TNFα; Figures 8A). In contrast, the similar increases (P<0.05) in heme-oxygenase 1 (HO-1) in DM SO and DM OVX rats were reversed by AdipoRon (P=0.02 both; Figure 8B). HO-1 was increased (P=0.03) in healthy OVX vs. healthy SO. DCF kinetics (Figure 8D) revealed higher cardiac ROS levels in both T2DM female groups, regardless of hormone status, when compared to healthy controls (P=0.03 both).

Table 1. Cardiac Functional Parameters from Echocardiography (Effect of 4 weeks of diabetes on directly measured hemodynamic variables via femoral catheterization). No significant differences were found between the estrogen depleted and replete female groups after treatment. Values are means ± SEM; n=7 surviving/group). s=systolic; d=diastolic.

	LV (s) end volume	LV (d) end volume	EDP	Contractility Index
Sham Vehicle	34.62 ± 5.02 µL	135.52 ± 12.77 µL	69.46 ± 10.08 mmHg	13.69 ± 1.44 sec^{-1}
Sham AdipoRon	42.48 ± 6.42 µL	134.92 ± 8.90 µL	75.90 ± 12.09 mmHg	14.41 ± 2.85 sec^{-1}
OVX Vehicle	44.00 ± 4.07 µL	185.33 ± 9.50 µL	85.37 ± 13.72 mmHg	9.40 ± 2.42 sec^{-1}
OVX AdipoRon	37.35 ± 5.04 µL	163.77 ± 10.41µL	71.11 ± 2.36 mmHg	12.68 ± 1.06 sec^{-1}

Figure 1. Experimental Timeline

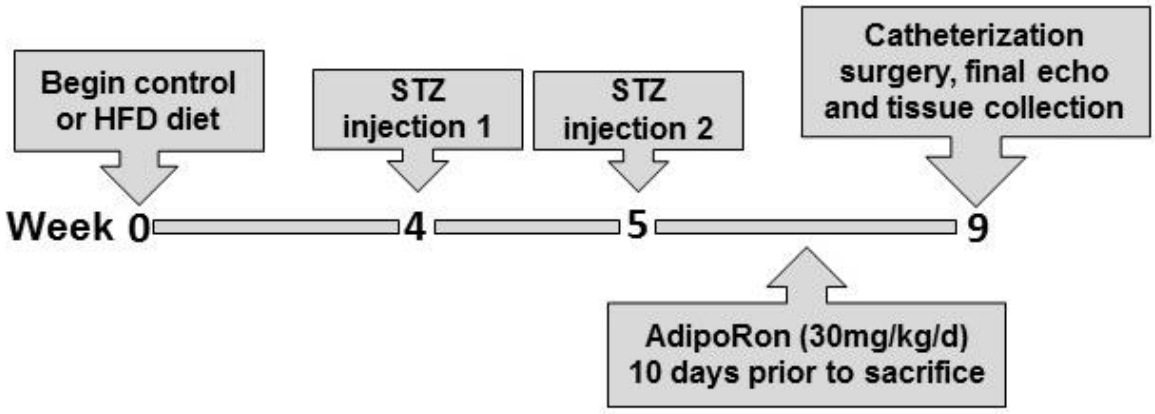
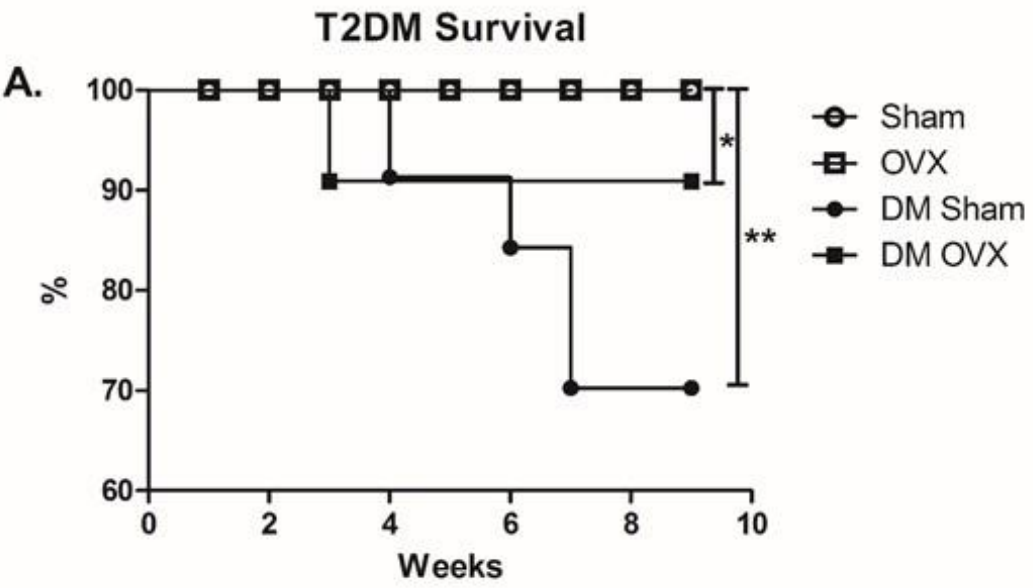


Figure 1. A schematic presentation of experimental diet regimen, T2DM induction, dosing schedule and the biochemical and molecular cardiovascular measurements in sex/estrogen variant groups of Wistar rats. OVX, ovariectomy; STZ, streptozotocin.

Figure 2


T2DM Survival
A.
100
90
80
70
60
%
0
2
4
6
8
10
Weeks
*
**
Sham
OVX
DM Sham
DM OVX

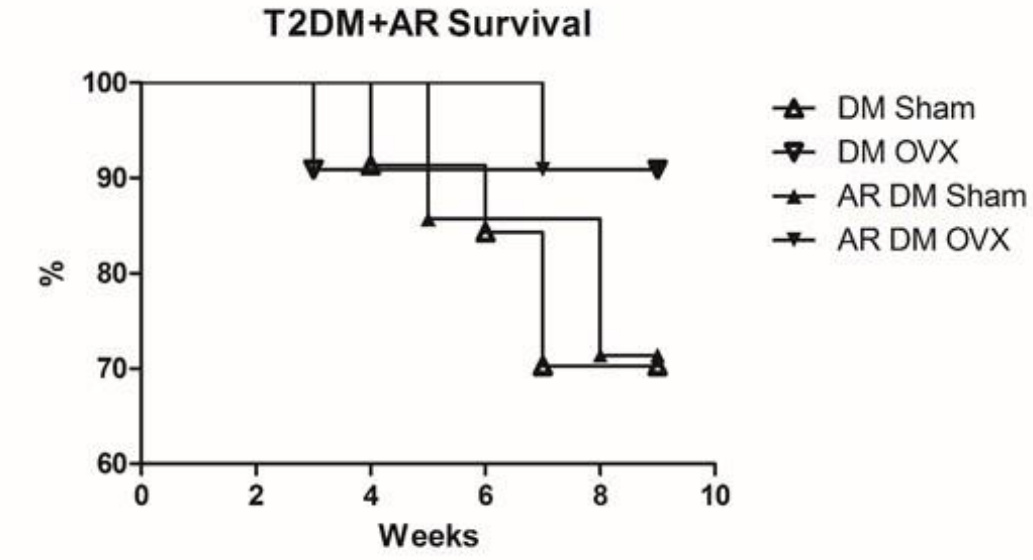

B.
T2DM+AR Survival
100
90
80
70
60
%
0
2
4
6
8
10
Weeks
DM Sham
DM OVX
AR DM Sham
AR DM OVX

Figure 2. Kaplan-Meier survival curves for the 9-week study timeline, comparing healthy and diabetic SO and OVX females (A) and diabetic SO and OVX females treated with either vehicle or AdipoRon (B). *P<0.05 when compared to respective control; **P<0.001 when compared to respective control. Actual P-values are in the results section.

Figure 3

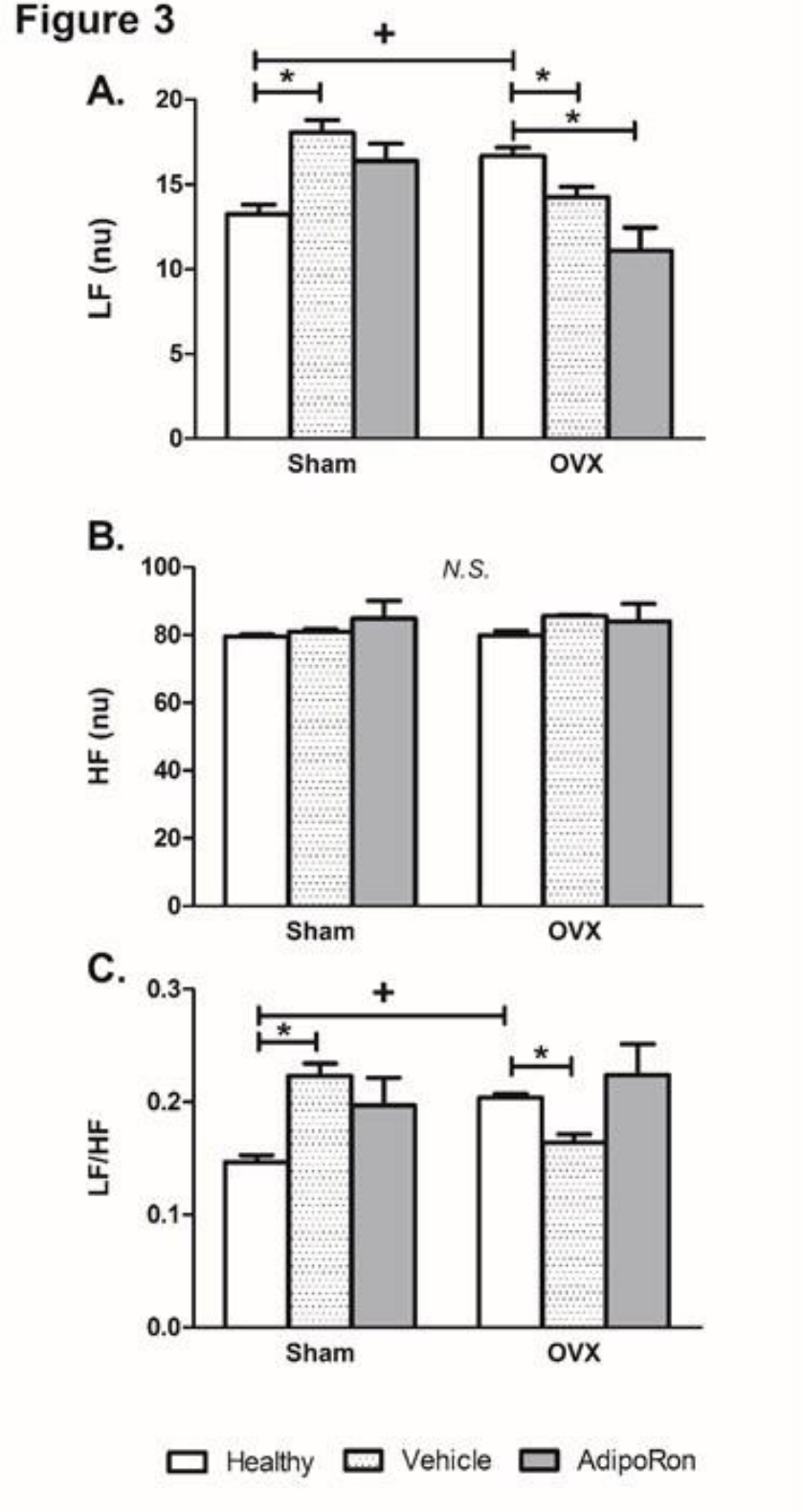

A.
LF (nu)
20
15
10
5
0
+
*
*
*
Sham
OVX
B.
HF (nu)
100
80
60
40
20
0
N.S.
Sham
OVX
C.
LF/HF
0.3
0.2
0.1
0.0
+
*
*
Sham
OVX
Healthy
Vehicle
AdipoRon

Figure 3. Spectral indices of heart rate variability measured at the conclusion of the study in sham operated (SO) and ovariectomized (OVX) females in the following groups: healthy controls, vehicle dosed DM and AdipoRon treated DM. (A) low-frequency (LFnu) (0.25–0.75 Hz) bands. (B) High-frequency (HFnu) (0.75–3 Hz) bands. (C) The LF/HF ratio depicting cardiac sympathovagal balance. Values are means ± SEM of 7 observations. *P<0.05 when compared to respective control and ⁺P<0.05 when compared to control sham operated (SO). Actual P-values are in the results section.

Figure 4

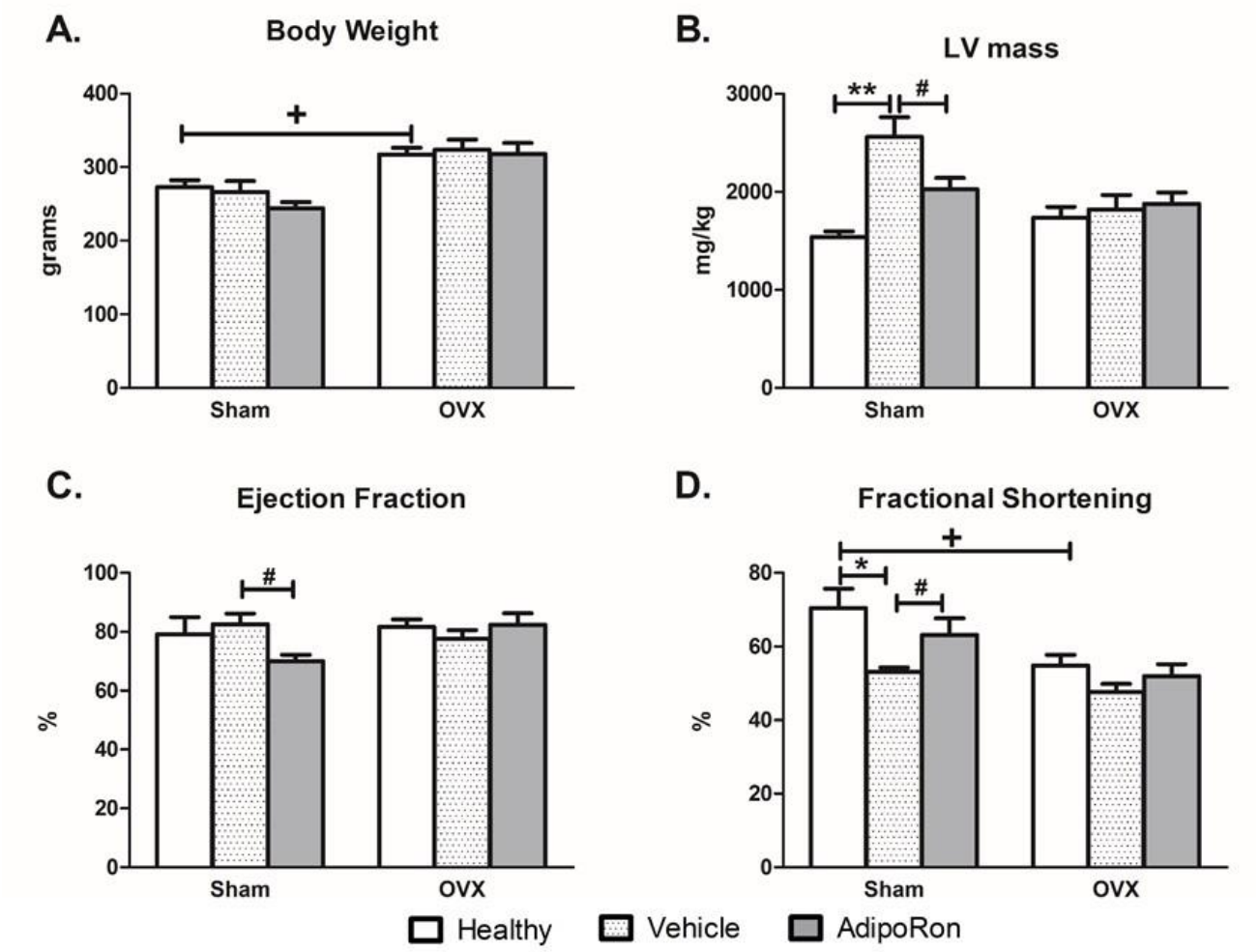

A.
Body Weight
grams
400
300
200
100
0
+
Sham
OVX
B.
LV mass
mg/kg
3000
2000
1000
0
**
#
Sham
OVX
C.
Ejection Fraction
%
100
80
60
40
20
0
#
Sham
OVX
D.
Fractional Shortening
%
80
60
40
20
0
+
*
#
Sham
OVX
Healthy
Vehicle
AdipoRon

Figure 4. Effect of four weeks of diabetes, on animal body weight (A), echocardiography-derived left ventricular mass (B), ejection fraction (C) and fractional shortening (D). Diabetic E_2 replete females (DM SO) display attenuation of detrimental cardiac findings after AdipoRon treatment. Values are means ± SEM (n=7 surviving/group). *P<0.05 when compared to respective control, **P<0.001 when compared to respective control, $^{\#}$P<0.05 when compared to DM vehicle-dosed group and $^{+}$P<0.05 when compared to control sham operated (SO). Actual P-values are in the results section.

Figure 5

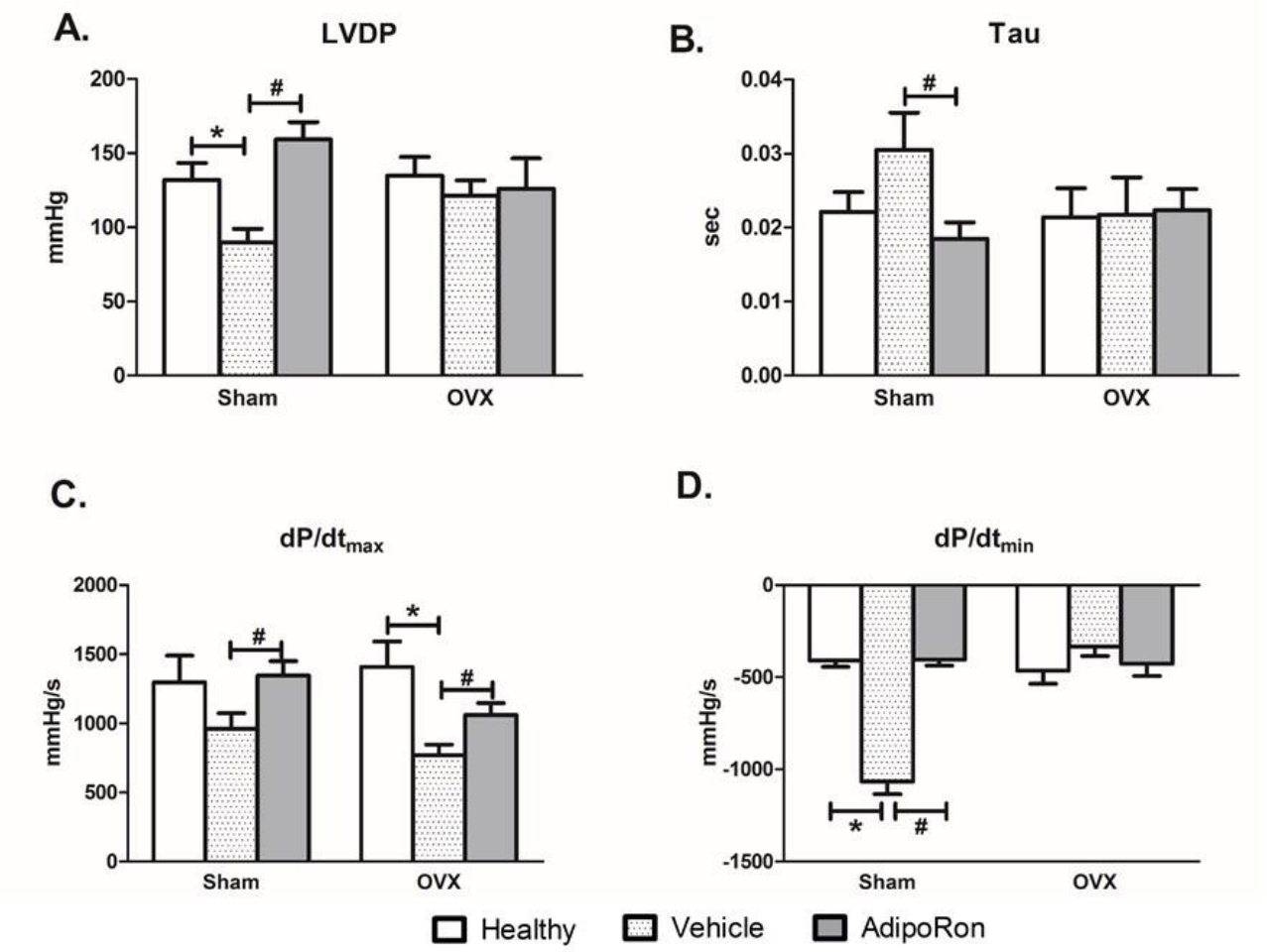

A.
LVDP
mmHg
200
150
100
50
0
Sham
OVX
B.
Tau
sec
0.04
0.03
0.02
0.01
0.00
C.
dP/dtmax
mmHg/s
2000
1500
1000
500
D.
dP/dtmin
0
-500
-1000
-1500
Healthy
Vehicle
AdipoRon

Figure 5. Effect of four weeks of diabetes, on directly measured hemodynamic variables via femoral catheterization. DM SO females display significant differences in left ventricular developed pressure (LVDP) (A), Tau (B), dP/dt_{max} (C), dP/dt_{min} (D) with AdipoRon treatment reversal. Values are means ± SEM (n=7 surviving/group). *P<0.05 when compared to respective control; $^{\#}$P<0.05 when compared to DM vehicle-dosed group. Actual P-values are in the results section.

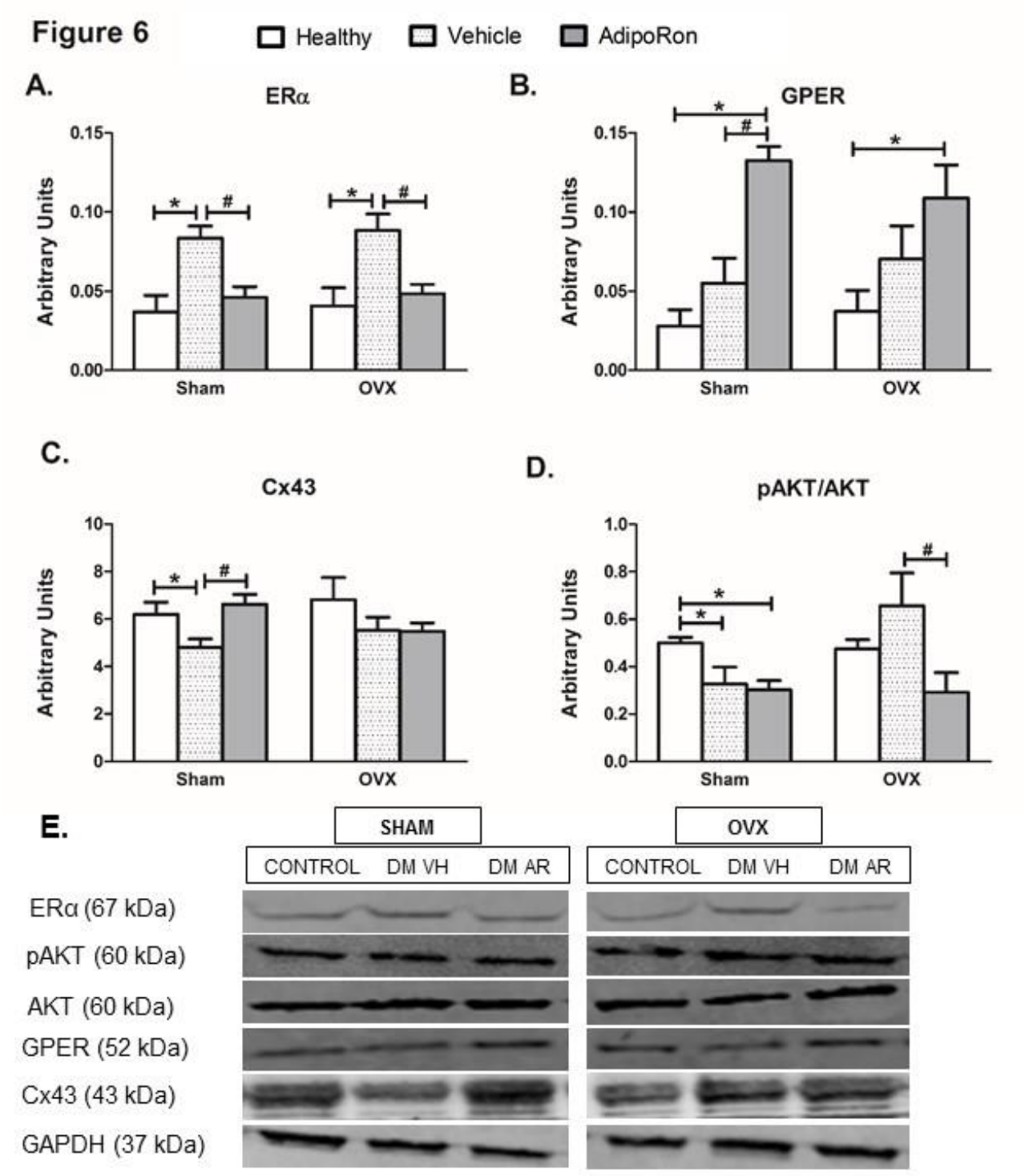


Figure 6
Healthy
Vehicle
AdipoRon
A.
ERα
Arbitrary Units
0.15
0.10
0.05
0.00
Sham
OVX
B.
GPER
C.
Cx43
10
8
6
4
2
0
D.
pAKT/AKT
1.0
0.8
0.6
0.4
0.2
0.0
E.
SHAM
OVX
CONTROL
DM VH
DM AR
ERα (67 kDa)
pAKT (60 kDa)
AKT (60 kDa)
GPER (52 kDa)
Cx43 (43 kDa)
GAPDH (37 kDa)

Figure 6. Expression of cardiac ERs, connexin43 (Cx43) and AKT in control and diabetic (vehicle and AdipoRon treated) female rats. Shown are alterations estrogen receptor alpha; ERα (A), G-protein coupled receptor; GPER (B), Cx43 (C), and pro-survival molecule pAKT (D). Homogenized left ventricular tissue was analyzed via western blot (n=7 surviving/group) and presented as the means ± SEM. Representative blots (E) for a particular protein in all experimental groups were run on the same gel and "arbitrary units" is a normalization of the target band to the GAPDH band or AKT. Molecular weights of proteins are given in kDa. *P<0.05 when compared to respective control; $^{\#}$P<0.05 when compared to DM vehicle-dosed group. Actual P-values are in the results section.

Figure 7

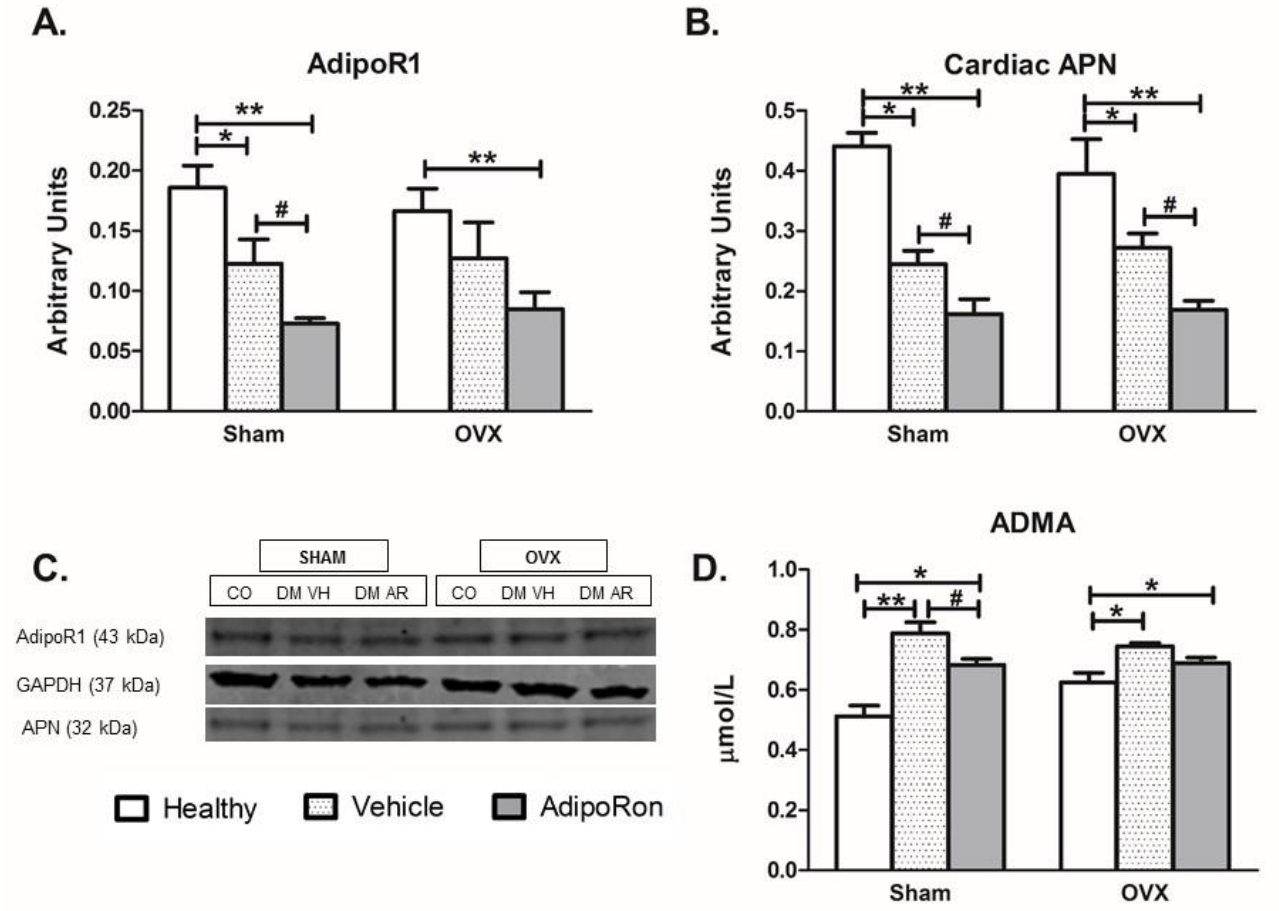

A.
AdipoR1
Arbitrary Units
0.25
0.20
0.15
0.10
0.05
0.00
**
*
#
**
Sham
OVX
B.
Cardiac APN
Arbitrary Units
0.5
0.4
0.3
0.2
0.1
0.0
**
*
#
**
*
#
Sham
OVX
C.
SHAM
OVX
CO
DM VH
DM AR
CO
DM VH
DM AR
AdipoR1 (43 kDa)
GAPDH (37 kDa)
APN (32 kDa)
Healthy
Vehicle
AdipoRon
D.
ADMA
µmol/L
1.0
0.8
0.6
0.4
0.2
0.0
*
**
#
*
*
*
Sham
OVX

Figure 7. Diabetes and AdipoRon treatment reduce cardiac adiponectin receptor 1 (AdipoR1) (A) and adiponectin (APN) (B) expression assessed via western blot in both female groups. ADMA levels in serum showed elevations in diabetic animals and attenuation in treatment groups via ELISA (C). Representative blots (D) for a particular protein in diabetic and healthy groups were run on the same gel and "arbitrary units" is a normalization of the target band to the GAPDH. Data presented as means ± SEM (n=7 surviving/group). *P<0.05 when compared to respective control, **P<0.001 when compared to respective control and $^{\#}$P<0.05 when compared to DM vehicle-dosed group. Actual P-values are in the results section.

Figure 8

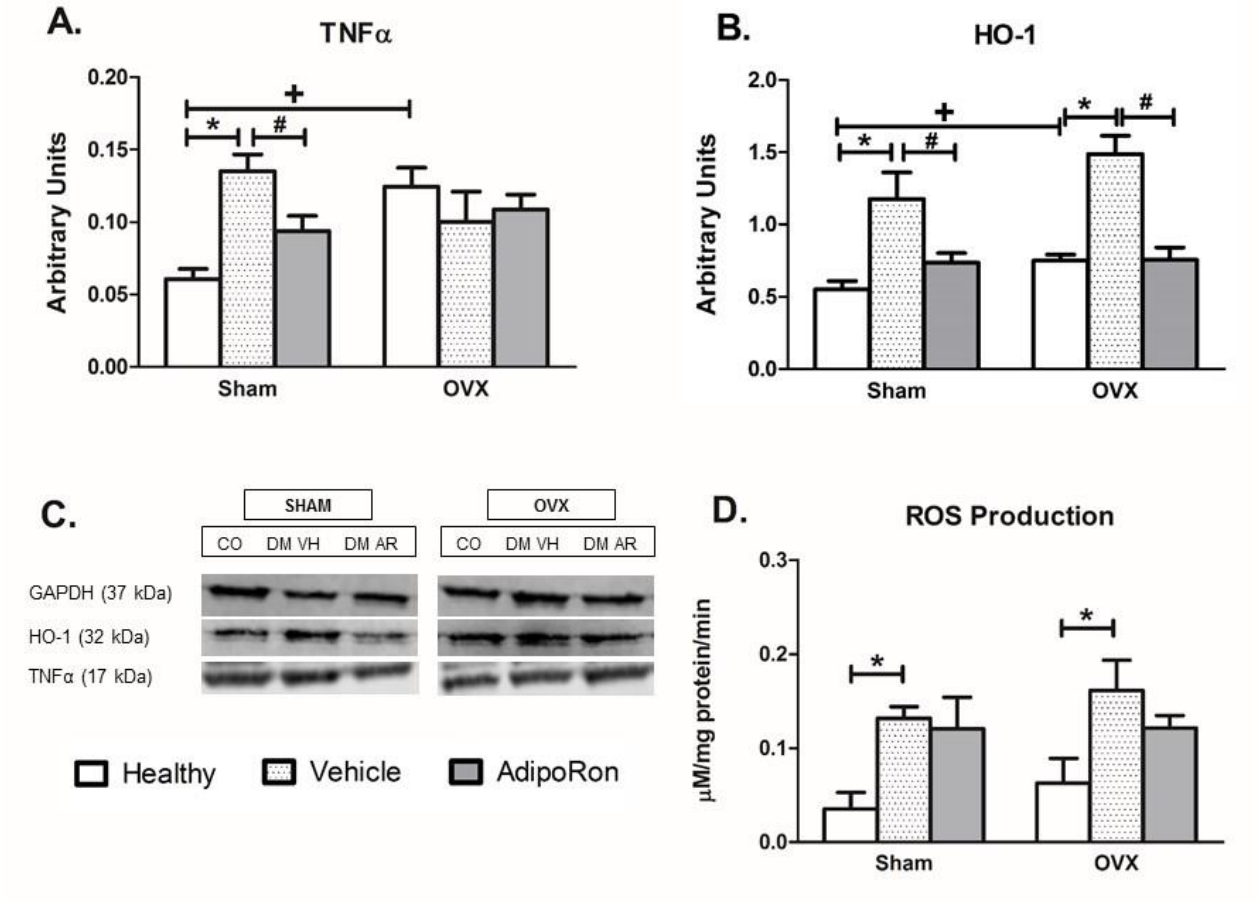

A.
TNFα
Arbitrary Units
0.20
0.15
0.10
0.05
0.00
+
*
#
Sham
OVX
B.
HO-1
Arbitrary Units
2.0
1.5
1.0
0.5
0.0
+
*
#
*
#
Sham
OVX
C.
SHAM
OVX
CO
DM VH
DM AR
CO
DM VH
DM AR
GAPDH (37 kDa)
HO-1 (32 kDa)
TNFα (17 kDa)
Healthy
Vehicle
AdipoRon
D.
ROS Production
μM/mg protein/min
0.3
0.2
0.1
0.0
*
*
Sham
OVX

Figure 8. Cardiac expression of TNFα (A) and HO-1 (B). Quantification of the DCF biochemical assay of ROS generation (C), measured by 2′,7′-dichlorofluorescein, in cardiac tissue shows the slopes (regression coefficients) of the regression lines in the respective healthy and diabetic groups. Values are expressed as means ± SEM (n=7 surviving/group). *P<0.05 when compared to respective control, **P<0.001 when compared to respective control, $^{+}$P<0.05 when compared to control sham operated (SO) and $^{\#}$P<0.05 when compared to DM vehicle-dosed group. Actual P-values are in the results section.

4.6 Discussion

While cardiac remodeling is initially a compensatory mechanism to decrease ventricular wall stress and maintain cardiac function during cardiac stress, over the long term, it is detrimental to heart health and inhibition remains a promising therapeutic goal (Nagueh 2009 and Regitz-zagrosek 2010). Sex differences in cardiac remodeling have also been recognized as a limitation to male specific studies (Dworatzek and Mahmoodzadeh, 2017; Murphy et al., 2017) and ERs play a crucial role in female cardiac health (Steagall et al., 2017; Yao and Abdel-Rahman, 2017).

APN has been shown to ameliorate cardiac remodeling in a pathological state (Takemura et al., 2007; Tian et al., 2009) and delivery of APN has been suggested as a repressor of deteriorating cardiac function (Tao et al., 2007; Zhu et al., 2008; Fisman and Tenenbaum, 2014). However, use of exogenous APN as a therapeutic drug in T2DM is obstructed clinically due to its high molecular weight, short half-life and inability to administer orally (Kadowaki et al.; Zhang et al., 2018). However, AdipoRon, an orally available small molecule agonist for AdipoR1 and AdipoR2, is more clinically feasible and has been shown to alleviate post-ischemic cardiac remodeling and apoptosis in APN KO male mice (Zhang et al., 2015) and to attenuate cardiac hypertrophy (Zhang et al., 2018). Additionally, AdipoRon ameliorated diabetic nephropathy and oxidative stress in male mice (Choi et al., 2018). However, AdipoRon has not been evaluated in a female model for cardiac function preservation or attenuation in diabetes mellitus.

While this study reproduced the original findings of female hypersensitivity to cardiovascular anomalies resulting in higher mortality among E_2 replete female diabetic

rats (Figure 2A), it did not show a significant reduction in overall mortality when comparing vehicle treated to AdipoRon treated groups (Figure 2B). The 10 day course of treatment was chosen from supporting literature (Okada-Iwabu et al., 2013; Zhang et al., 2018) to address therapeutic investigation (as opposed to prevention). However, it is probable that the length of treatment, only 10 days prior to the termination of the study, was insufficient to reduce overall mortality. Several animals were lost from the study prior to the initiation of dosing, resulting in a lack of sufficient time to reduce mortality.

When investigating exacerbated female cardiac effects in diabetes, autonomic dysregulation must be considered because autonomic dysregulation/sympathoexcitation prediposes to cardiac dysfunction (De Angelis et al., 2009). To address this question, we conducted spectral analysis of inter-beat data obtained the experimental groups used in our study. The occurrence of autonomic dysregulation only in E_2-replete DM rats (DM SO) during the course of our dosing study (Figure 3A and C) supports the premise of sympathetic imbalance, linked to E_2 modulation. This DM-evoked autonomic dysreguation was partially reversed with the administration of AdipoRon (Figure 3A and C) to restore APN-Cx43 signaling. Heart rate variability suppression contributes to cardiovascular morbidity and mortality (Gonzalez-Clemente et al., 2007) and to cardiac dysfunction in diabetic male rats rats (El-Sayed et al., 2016), yet this study is the first to show the drastic hypersensitivity of females to DM-induced cardiac oxidative stress in E_2-dependent manner and possibe reversal by APN signaling restoration (Figure 3).

Here, we showed that 10 days of AdipoRon treatment (30mg/kg/d; P.O.) had no effect on body weight (Figure 4A) or serum APN (not shown), which is consistent with

several separate independent study findings (Okada-Iwabu et al., 2013; Choi et al., 2018; Zhang et al., 2018). However, the increased LV mass shown in the utilized T2DM model (Leffler and Abdel-Rahman, 2019) was attenuated with AdipoRon treatment in T2DM estrogen replete rats (Figure 4B). Additionally, cardiac functional parameters, such as fractional shortening (Figure 4D), LVDP (Figure 5A), and Tau (Figure 5B) were significantly improved with activating the APN signaling axis in the E_2 replete female groups (DM SO), when compared to control animals. These findings were not replicated in the E_2 depleted females (DM OVX). An independent study in male mice was consistent with these findings, and showed decreased cardiac hypertrophy post aortic banding, decreased fibrosis and decreased BNP levels after AdipoRon treatment (Zhang et al., 2018). In contrast to these E_2 specific findings, dP/dt_{max} (Figure 5C) and dP/dt_{min} (Figure 5D) showed improve functional values in both female groups—some reaching significance and some trending, suggesting therapeutic value for AdipoRon in all females, regardless of E_2 status.

Increased ERα expression has been linked to detrimental cardiac effects in diabetic humans (Mahmoodzadeh et al., 2006; Kleiblova et al., 2010) and rats (Chung et al., 2009) and this was replicated in this study. The AdipoRon dosing reversed ERα expression upregulation in both female groups, returning levels to those of healthy controls (Figure 6A). It is probable that reduction in ERα expression slowed the progression of cardiac dysfunction in all groups and to an even greater degree in E_2 replete females (DM SO). The findings that estrogen expedites the progression of diabetes-evoked cardiac dysfunction raised the question regarding whether disruption of physiological E_2 cardioprotection may contribute to this clinical problem. Interestingly,

while ERα expression decreased, GPER expression was further increased with AdipoRon treatment. Increased GPER is cardioprotective (Deschamps and Murphy, 2009; Li et al., 2015), and may play a role in the attenuation of cardiac dysfunction seen in AdipoRon treated DM SO groups (Figure 5). An imbalance in the ratio of ERα to GPER has also been implicated in vascular and cardiac dysfunction (Lee et al., 2014; Gros et al., 2016). It is probable that alteration of the ERs is involved in the attenuation process.

While Cx43 is physiologically higher in females when compared to males (Stauffer et al., 2011) and it induces pro-survival AKT (Fujio et al., 2000; Muslin, 2011), both are downregulated in the diabetic female rat model (Leffler and Abdel-Rahman, 2019). Restoration of APN-Cx43 signaling via the small molecule agonist increased and restored cardiac levels of Cx43 in the DM estrogen replete female (DM SO) (Figure 6C), accompanied by decreased LV mass (Figure 4B) and restored cardiac function (Figures 4D and 5). This was not the case in the E_2 deprived females (DM OVX) (Figures 4D, 5A-D and 6C). These findings reinforce the link between estrogen and modulation of the APN-Cx43 signaling axis. On the contrary, while these expansion studies confirmed a decrease in pro-survival cardiac AKT in DM SO when compared to DM OVX, AdipoRon dosing did not increase AKT in cardiac tissue (Figure 6D). This lack of activated (phosphorylated) AKT may be explained by a diabetic nephropathy study in human glomerular endothelial cells following AdipoRon treatment where increases in AKT in vitro were found at a concentration of 50 µM daily, but not observed with AdipoRon treatment at 10 µM daily (Choi et al., 2018), suggesting that the concentration was not high or long enough to produce alterations in cardiac AKT levels.

This study was the first to investigate cardiac APN and AdipoR1 expression in AdipoRon treated females. AdipoR1 levels (Figure 7A) and APN (Figure 7B) were decreased in LV tissue in both female groups, when compared to healthy or DM vehicle treated animals, which would be expected based on receptor downregulation in the presence of increased availability of an agonist.

Asymmetric Dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) synthesis (Böger et al., 2009). ADMA has been identified as a strong indicator of high cardiovascular risk and overall mortality across broadly defined global human populations (Böger et al., 2009; Nemeth et al., 2015; Schlesinger et al., 2016). ADMA causes endothelial dysfunction, vasoconstriction, blood pressure elevation and aggravates atherosclerosis and has been identified as a key risk marker clinically to assess cardiovascular outcomes (Böger et al., 2009). This study analyzed ADMA levels across the six experimental groups and found that while the healthy SO females had lower levels of circulating ADMA when compared to control OVX, T2DM more drastically raised circulating levels in the SO vs. OVX females (Figure 7C). While quantitative values are still debatable, it has been suggested that ADMA plasma concentrations above 0.7μmol/L indicate substantially increased negative cardiovascular outcomes (Böger et al., 2009), our studies found that both diabetic female groups had circulating levels above this threshold (μmol/L for DM SO and μmol/L for DM OVX). In groups where APN signaling was restored, circulating levels were reduced below this threshold (Figure 7C), particularly in E_2 replete females.

Oxidative stress-evoked activation of cardiovascular regulating nuclei underlies cardiac sympathetic dominance and subsequent autonomic dysregulation (Kishi and

Hirooka, 2012). The latter contributes to cardiac dysfunction in models of human diseases including DM although most, if not all, of these studies were conducted in male animals (Ceriello and Motz, 2004; Fouda et al., 2018). TNFα was decreased in AdipoRon treated females, when compared to vehicle treated females, suggesting decreased oxidative stress in the myocardium. AdipoRon treatment in male mice also decreased Angiotensin II induced TGF-β1 expression and cardiac fibrosis (Zhang et al., 2018), further supporting decreased oxidative stress with APN signaling activation.

Exaggerated oxidative stress and proinflammatory molecules (Figures 8A-C) likely explain the E_2-dependent cardiac sympathetic dominance (Figure 3A and C) and cardiac dysfunction (Figure 5) in DM female rats as well as the poor cardiac outcomes in DM premenopausal women (Regensteiner et al., 2015). Consistent with a physiologically suppressed central sympathetic tone in premenopausal women (Weitz et al., 2001), and a neuroprotective role for E_2 (Brann et al., 2007), healthy SO rats exhibited lower LF domain and LF/HF ratio (Figure 3A and C), indicative of reduced sympathetic dominance (Ori et al., 1992), compared to control OVX females. This physiologically/clinically relevant finding is likely explained by lower basal levels of ROS (Figure 8B and C) and proinflammatory molecules (Figure 8A) in E_2-replete rats.

While more studies are needed in females and women, it is becoming clear that the protective action of APN is lost in diabetes (Li et al., 2016) and support for the E_2-APN-Cx43 signaling axis involvement is increasing. Activation of APN receptors via AdipoRon treatment enhances the expression of Cx43 in the heart (Figure 6C), improves cardiac function (Figures 4D and 5), and attenuates autonomic dysfunction (Figure 3). These findings: cardiac functional data, autonomic regulation data,

biochemical alterations and oxidative stress markers all support the E_2 modulation of the APN-Cx43 signaling axis underlying heightened sensitivity of female rats to diabetes-evoked myocardial dysfunction. Coupled to the findings of a significant overall mortality and cardiovascular risk factor in both rats and humans, ADMA, the evidence is mounting to support development of a novel therapuetic targeting APN signaling.

CHAPTER FIVE: ADDITIONAL RESULTS AND FINDINGS

5.1 Autonomic Dysfunction in DM SO and OVX+E_2 female rats

Investigation of autonomic regulation via spectral analysis of heart rate variability revealed sex/E_2-specific adverse cardiac autonomic effects in T2DM rats. DM SO or OVX+E_2 rats exhibited significant ($P<0.05$) increases in the R-R oscillations in the LFnu range (0.25–0.75 Hz; Figure 1A) and no change in R-R oscillations in the HFnu range (0.75–3 Hz; Figure 1B). The resultant higher ($P<0.05$) LF/HF ratio reflected a shift in the cardiac sympathovagal balance toward sympathetic dominance in E_2-replete DM female, compared to diabetic male or OVX rats (Figure 1C). Importantly, healthy control SO and OVX+E_2 female rats exhibited lower ($P<0.05$) LFnu and LF/HF ration (reduced sympathetic dominance), compared to male or OVX control rats (Figure 1). These clinically relevant findings over all eight experimental groups yield complementary insight into central mechanisms for an E_2-dependent autonomic dysregulation, which likely contributes to the exacerbated cardiac dysfunction in T2DM females.

5.2 Mean Arterial Pressure (MAP), Glucose Tolerance Test (GTT) and Survivability

As part of the research project, blood pressure was recorded in all groups. Findings revealed that while healthy males and OVX females had higher baseline blood pressures ($P<0.05$; Figure 2) than estrogen replete females (SO and OVX+E_2), end point MAP between the control and T2DM groups was not different ($P>0.05$) (Figure 2). Additionally, intravenous glucose tolerance testing (IV GTT), at the conclusion of the study, ruled out a role for hyperglycemia in LV hypertrophy (Table 1). While all DM

groups had hyperglycemia, the estrogen replete females (DM SO or OVX+E_2) had improved glycemic control (Table 1) and LV hypertrophy when compared to OVX females or males (Leffler and Abdel-Rahman, 2019). We concluded that glycemic control was not an underlying contributing factor to the hypersensitivity of females to DM-evoked cardiac dysfunction. Finally, Kaplan-Meier survival curves for groups (SO, OVX, OVX+E_2 and Male) in the absence (Figure 3A) or presence of T2DM (Figure 3B) revealed the highest all-cause mortality in DM E_2-replete rats.

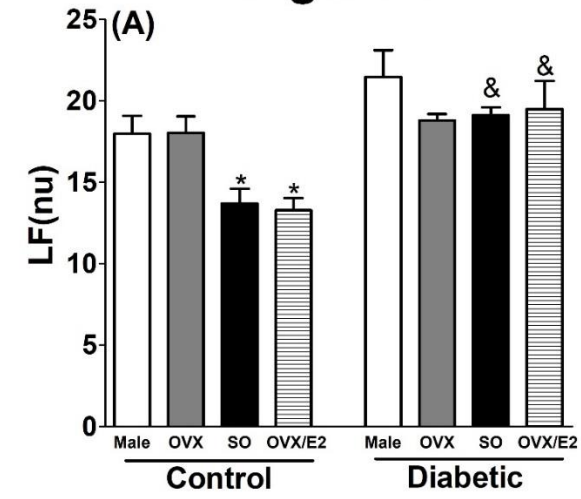
5.3 Cx43 vs. Cav-3

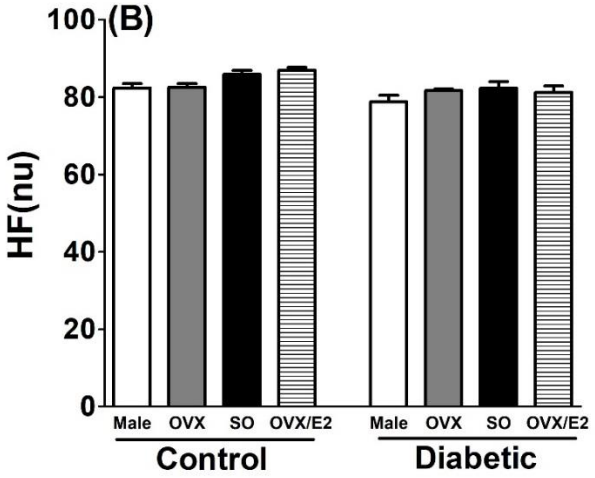
Finally, a preliminary study identified a therapeutic potential for targeting caveolin-3 (Cav-3) reduced DM-evoked cardiomyopathy in females based on findings DM males (Murfitt et al., 2015; Li et al., 2016). Cav-3 was used as a scaffolding protein in confocal immunofluorescent studies in the first phase of this research project. As a follow up, LV Cav-3 was quantified (western blot). Cav-3 was only downregulated ($P<0.05$) in DM male rats (Figure 4), but not in females regardless of their hormonal status or cardiac dysfunction (Leffler and Abdel-Rahman, 2019). In contrast, Cx43 was downregulated in estrogen replete DM females (Leffler and Abdel-Rahman, 2019) in agreement with findings in DM men and male rats (Ruiz-Meana et al., 2008; Ock et al., 2018) along with cardiac dysfunction. These findings complemented the study conclusions of identifying Cx43 as a more promising molecular target for drug development.

Table 1. Intravenous Glucose Tolerance Test Results

	Group							
Time	**Sham**	**OVX**	**OVX+E_2**	**Male**	**DM Sham**	**DM OVX**	**DM OVX+E_2**	**DM Male**
0 min	97.40 ± 5.41	125.8 ± 13.01	104.75 ± 9.88	97.75 ± 10.02	253.00 ± 12.00	199.67 ± 45.76	261.00 ± 95.00	250.67 ± 19.79
1 min	201.50 ± 9.24	361.00 ± 60.23	282.75 ± 72.76	286.25 ± 17.60	450.67 ± 19.67	356.33 ± 30.67	242.36 ± 68.30	314.31 ± 30.23
3 min	219.00 ± 7.15	220.40 ± 20.37	207.67 ± 11.62	258.25 ± 12.84	390.67 ± 8.35	314.00 ± 28.00	306.53 ± 79.53	316.00 ± 20.64
5 min	163.25 ± 16.02	212.40 ± 26.53	156.67 ± 29.38	196.50 ± 18.58	333.67 ± 16.42	263.96 ± 24.00	250.50 ± 72.00	289.44 ± 47.56
15 min	139.75 ± 9.45	189.00 ± 29.11	168.33 ± 3.71	178.50 ± 15.58	318.33 ± 9.33	234.667 ± 5.10	272.89 ± 63.21	282.78 ± 34.43
30 min	123.25 ± 19.53	191.00 ± 19.53	140.00 ± 19.47	167.00 ± 10.90	275.68 ± 22.10	171.10 ± 17.33	260.41 ± 36.74	240.32 ± 63.41

Figure 1


(A)
LF(nu)
25
20
15
10
5
0
*
*
&
&
Male
OVX
SO
OVX/E2
Male
OVX
SO
OVX/E2
Control
Diabetic


(B)
HF(nu)
100
80
60
40
20
0
Male
OVX
SO
OVX/E2
Male
OVX
SO
OVX/E2
Control
Diabetic

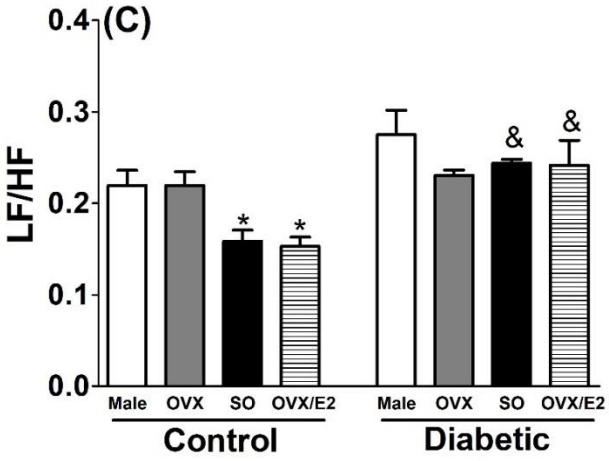

(C)
LF/HF
0.4
0.3
0.2
0.1
0.0
*
*
&
&
Male
OVX
SO
OVX/E2
Male
OVX
SO
OVX/E2
Control
Diabetic

Figure 1. Spectral indices of heart rate variability measured at the conclusion of the study in the following DM and respective control groups: male, ovariectomized (OVX), sham operated (SO), and OVX with E_2 supplementation (OVX+E_2). **(A)** low-frequency (LFnu) (0.25–0.75 Hz) bands. **(B)** High-frequency (HFnu) (0.75–3 Hz) bands. **(C)** The LF/HF ratio depicting cardiac sympathovagal balance. Values are means ± SEM of 10 observations. *P < 0.05 vs. male control values. $^{\&}$P < 0.05 vs. sham control values.

Figure 2.

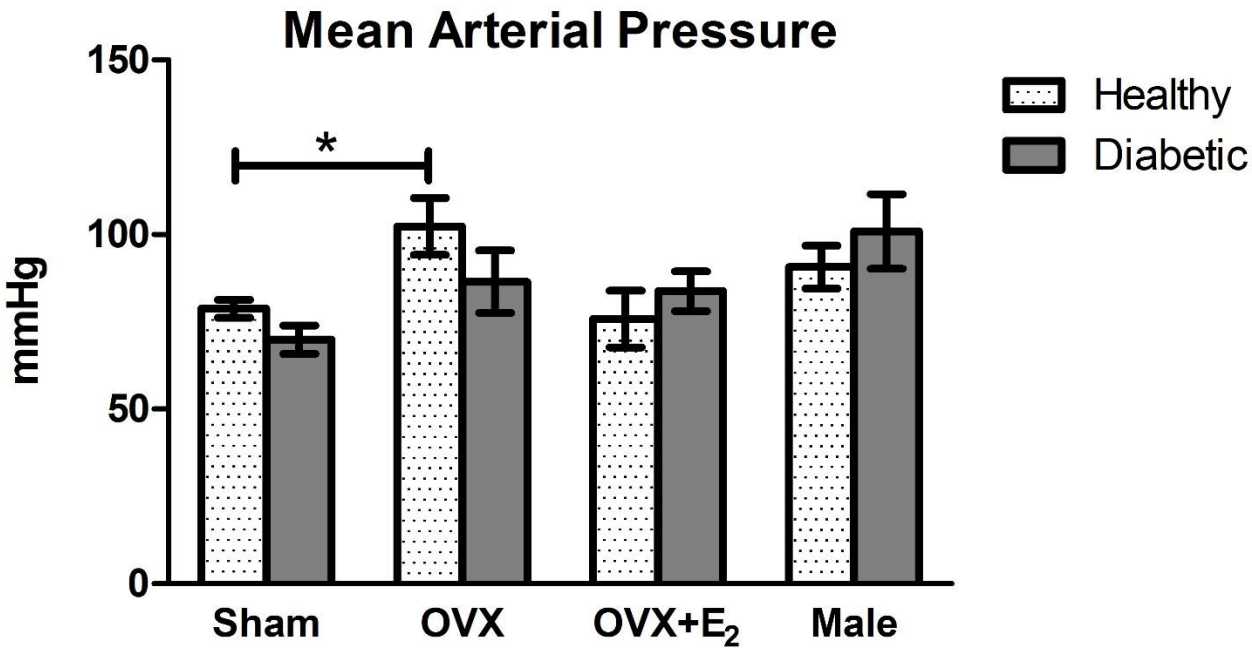

Mean Arterial Pressure
150
100
50
0
mmHg
*
Sham
OVX
OVX+E2
Male
Healthy
Diabetic

Figure 2. Endpoint mean arterial pressure (MAP) via invasive direct hemodynamic monitoring at the conclusion of the study; no significance ($P>0.05$) between diabetic and respective healthy/control groups, *$P<0.05$.

Figure 3.

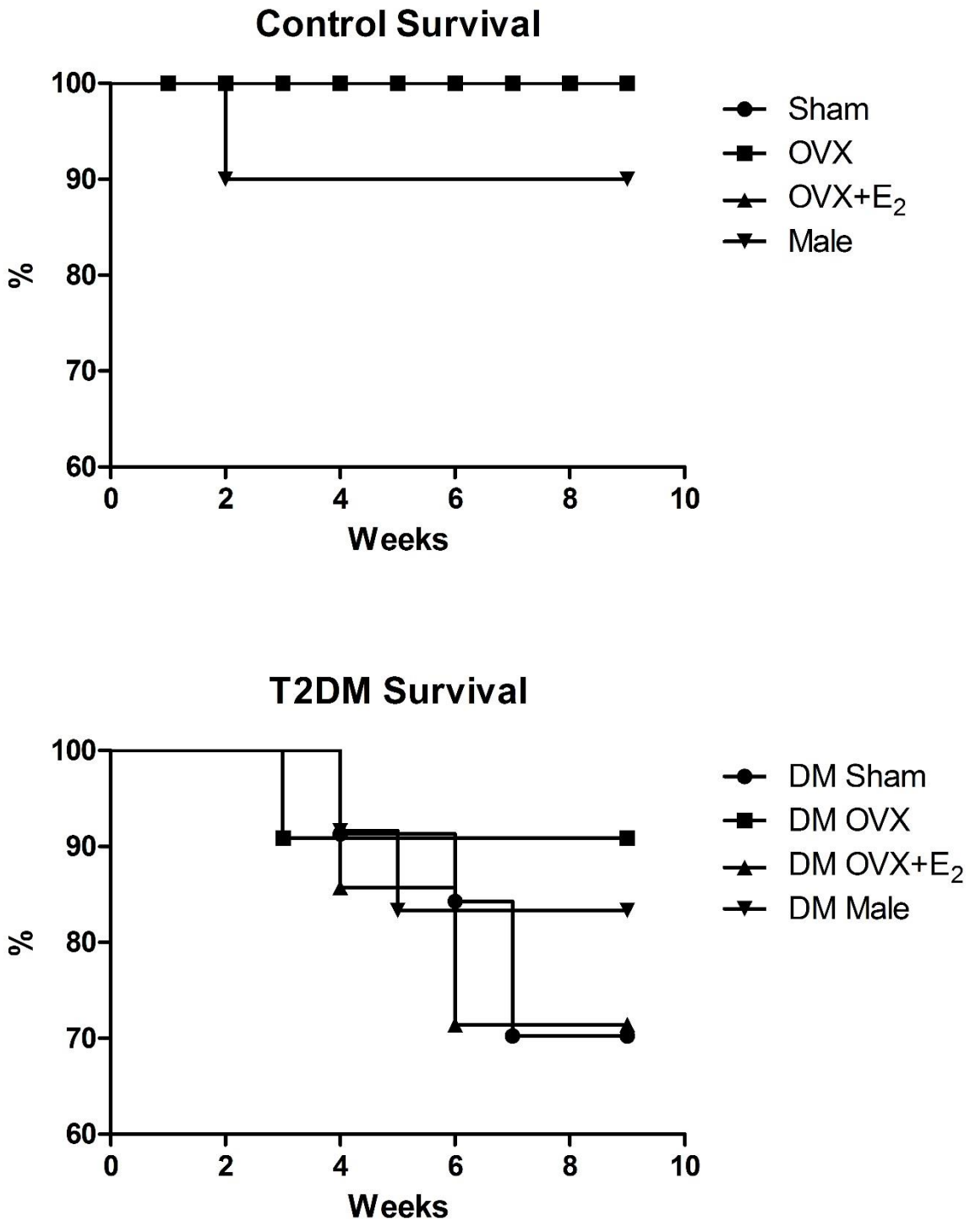

Control Survival
100
90
80
70
60
%
0
2
4
6
8
10
Weeks
Sham
OVX
OVX+E2
Male
T2DM Survival
100
90
80
70
60
%
0
2
4
6
8
10
Weeks
DM Sham
DM OVX
DM OVX+E2
DM Male

Figure 3. Kaplan-Meier survival curves for the 9-week study timeline, comparing healthy control (top) and diabetic (bottom) SO, OVX, OVX+E_2 and male rats.

Figure 4.

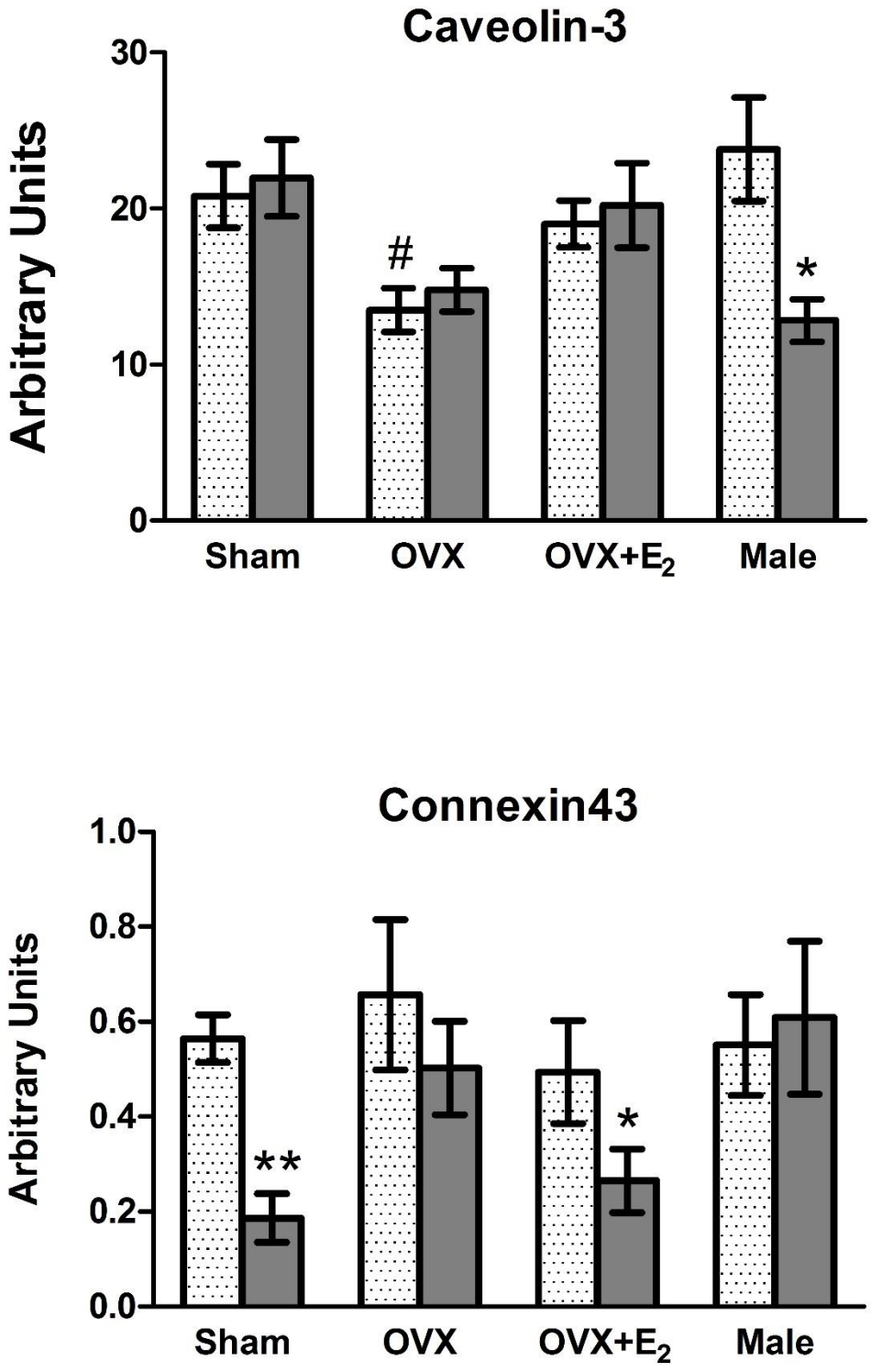

Caveolin-3
Arbitrary Units
30
20
10
0
#
*
Sham
OVX
OVX+E2
Male
Connexin43
Arbitrary Units
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Sham
OVX
OVX+E2
Male

Figure 4. Comparison of Cardiac Expression of Cav-3 and Cx43 with a quantitation of cardiac Cav-3 (top) and Cx43 (bottom) in diabetic (dark bars) and control rats. Homogenized left ventricular tissue was analyzed via western blot and normalized to GAPDH. Data are presented as means ± SEM (n=8 surviving/group). *P<0.05 when compared to respective control, **P<0.001 when compared to respective control, #P<0.05 when compared to control sham operated (SO).

CHAPTER SIX: GENERAL DISCUSSION AND SUMMARY

6.1 Phase I (Chapter 3)

In the first part of this research project, the goal of creating a female rodent model that mimicked the higher cardiac morbidity and mortality in T2DM women than in T2DM men (Murphy et al., 2017) was achieved. The research revealed novel data suggesting that ovarian hormones, particularly estrogen (E_2), accelerate the disruption of physiologically protective cardiac APN-Cx43 signaling as a underlying molecular mechanism for the exacerbated cardiac dysfunction in diabetic females. The second phase of the study built on the establishment of heightened cardiac dysfunction to elucidate the role of the inflammatory microenvironment in this problem using pharmacological and molecular approaches.

6.1.1 Replication of women's hypersensitivity to DM-evoked cardiac anomalies in a female rodent model

The selected high-fat diet and low STZ dose regimen produces clinically relevant T2DM associated cardiac dysfunction after 8-10 weeks in male rats (Hoit et al., 1999). This study reproduced this cardiac dysfunction in female rats after only 4 weeks (Figure 2 and 3). These findings, along with higher mortality (Figure 5.3) are the first to mimic the higher sensitivity of women to T2DM-related cardiac morbidity and mortality (Pradhan, 2014; Regensteiner et al., 2015) and support one of the overall research projects goals.

6.1.2 Ovarian hormones mediate hypersensitivity to DM-evoked cardiac dysfunction

Collectively, study of longitudinal and endpoint echocardiographic findings and terminal direct hemodynamic measurements of multiple cardiac indices (LVDP, contractility, fractional shortening, Tau, dP/dt_{max}), confirmed DM-evoked myocardial dysfunction and its estrogen dependence in diabetic female rats. Notably, decreased LVDP, contractility index and fractional shortening are signs of cardiac dysfunction, while increased LV mass indicated detrimental cardiac remodeling (Nagueh et al., 2009; Regitz-Zagrosek et al., 2010). Convincingly, these novel results were replicated in the second phase of the research project.

6.1.3 Disruption of APN-Cx43 signaling is a critical ovarian hormone dependent molecular mechanism for heightened diabetic cardiac anomalies in females

While all molecular ER expression alterations (Figure 4) can not be overlooked, the central focus was on the ERα upregulation seen in all DM groups (Figure 4A). It is likely that the activation of the upregulated ERα by endogenous (SO) or exogenous (OVX+E_2) E_2 expedited and explained why cardiac dysfunction was only observed in the estrogen replete experimental groups (Figures 2 and 3). These studies focused on the cardioprotective adipokine, APN, and its downstream effector Cx43 because their levels are higher in females (Stauffer et al., 2011), and they induce cell survival molecules such as AKT (Fujio et al., 2000; Muslin, 2011). We hypothesized that disruption of APN-Cx43 regulated cardiac homeostasis plays a critical role in the sex/E_2-dependent exacerbation of DM-evoked myocardial dysfunction.

APN reduction in T2DM patients and rodent models (Kadowaki et al.; Bjornstad et al., 2016) was replicated in this study (Figure 5). Physiologically, Cx43 protects cardiomyocyte function and its downregulation, under pathological conditions such as T2DM, contributes to

heart failure (Michela et al., 2015). Therefore, the reduction in cardiac Cx43 expression (Fig. 6A-C) as a consequence of the reduction in circulating and myocardial APN levels (Figs. 4D and 5) likely contributed to the suppressed LV contractility (Fig. 3D) and higher mortality in E_2-replete diabetic rats. The lack of similar reductions in cardiac Cx43 expression or in cardiac function in diabetic male or OVX rats under the same conditions (Figure 5) supports this hypothesis. Cx43 confers cardioprotection, at least partly, via activation (phosphorylation) of the cell survival molecule AKT and its downstream cardioprotective effector, ERK1/2 (Fujio et al., 2000; Muslin, 2011). The reductions in the phosphorylation of AKT or ERK1/2 (Figure 5C and 5D), secondary to reduced Cx43, contribute to the E_2-dependent myocardial dysfunction in diabetic rats.

6.2 Phase II (Chapter 4)

In the second part of this research project, the studies reproduced the original findings of female hypersensitivity to cardiovascular anomalies resulting in higher mortality among E_2 replete female diabetic rats (Figure 2A). Notably, the chosen 10 day AdipoRon regimen (Okada-Iwabu et al., 2013; Zhang et al., 2018) had no effect on overall mortality (Figure 2B). It is probable that the length of treatment, only 10 days prior to the termination of the study, was insufficient to reduce overall mortality or occurred after mortality reached its peak (Figure 2B). Nonetheless, it is noteworthy that the study achieved its goal by demonstrating the potential for AdipoRon therapeutic utility via reversing (rather than preventing) DM-evoked functional and molecular cardiac anomalies.

The following are the major findings of the second phase of research studies. First, ovarian hormone-dependent reduction in cardiac Cx43 expression is the major

cause for the heightened proinflammatory milieu and cardiac dysfunction in DM SO rats. Second, AdipoRon restoration of cardiac Cx43 is pivotal for alleviating, at least partly, the ovarian hormone-dependent LV inflammation, hypertrophy, and dysfunction. Third, while ovarian hormone-independent adverse responses were not sufficient to cause autonomic/cardiac dysfunction, AdipoRon also alleviated these adverse effects in DM OVX rats. These findings suggest that additive ovarian hormones dependent and independent molecular events additively contribute to the SO rats' hypersensitivity to DM-evoked cardiac and autonomic anomalies. Further, the findings identified restoration of cardiac Cx43 as a potential therapeutic option for alleviating cardiac anomalies in premenopausal or postmenopausal ERT diabetic women.

6.2.1 Ovarian hormone-dependent reduction of cardiac Cx43 underlies inflammation and cardiac dysfunction in E_2 replete female diabetic rats

These critical studies focused on female rats with (healthy and DM SO rats) and without (healthy DM OVX rats) ovarian hormones. Hypersensitivity to cardiac dysfunction four weeks post DM induction in DM SO rats included autonomic dysfunction (Figure 3A and C), LV hypertrophy (Figure 4B), reduced fractional shortening (Figure 4D), LVDP (Figure 5A), dP/dt_{max} (Figure 5C), dP/dt_{min} (Figure 5D) and increased Tau (Figure 5B). These functional deficits were accompanied by the signficant reduction in cardiac Cx43 (Figure 6C) and increases in a proinflammatory environment (see below). These findings were not observed in the DM OVX rats, with the exception of reduced dP/dt_{max} (Figure 5C), perhaps suggesting its progression into cardiac dysfunction in this group at a future timepoint.

Tumor Necrosis Factor (TNF) α is potent pro-inflammatory cytokine involved in diabetic pathogenesis (Yamakawa et al., 2011). TNFα is elevated in the diabetic patients,

particularly those with progressively worsening complications (Preciado-Puga et al., 2014) and rodent models, and its inactivation ameliorates the associated pathologies (Yamakawa et al., 2011). Further, the TNFα pathway has sex-dependent molecular signaling alterations in diabetic mice (Delgado et al., 2019). These sex-dependent molecular studies were performed with young, ovarian hormone replete female rodents, whereas our novel studies examined SO and OVX female groups. DM SO females had elevated cardiac TNFα (Figure 8A and C) when compared to DM OVX. The mitigation of this proinflammatory environment and cardiac dysfuncction by AdipoRon suggest a greater therapeutic effect in DM SO rats. In healthy control animals, the OVX females had higher LV levels of the proinflammatory molecule, when compared to SO females (Figure 8A and C). Additionally, elevations in asymmetric dimethylarginine (ADMA)(Figure 7D), heme oxygenase 1 (HO-1) (Figure 8B), and ROS (Figure 8D) biochemically validate the proinflammatory state of DM SO rats. These findings corrobrate and build on the first phase of this research project (Leffler and Abdel-Rahman, 2019).

6.2.2 AdipoRon ameliorates ovarian hormone-dependent LV inflammatory markers, hypertrophy and dysfunction

APN ameliorates cardiac remodeling in a pathological state (Takemura et al., 2007; Tian et al., 2009); however, delivery of exogenous APN as a therapy is hindered by its short half-life, high molecular weight and oral inactivity (Kadowaki et al.; Zhang et al., 2018). AdipoRon is more clinically feasible and alleviates post-ischemic cardiac remodeling and apoptosis in APN KO mice (Zhang et al., 2015), attenuates LV hypertrophy (Zhang et al., 2018) and ameliorates diabetic nephropathy and oxidative stress (Choi et al., 2018). However, AdipoRon therapeutic potential has not been

evaluated in a model of female hypersensitivity to DM-evoked cardiac anomalies. These studies examined AdipoRon treatment in DM SO and OVX female rats and demonstrated mitigation of hormone-dependent LV inflammatory markers, hypertrophy and dysfunction (both autonomic and cardiac).

The heightened susceptibility of DM SO females to cardiovascular anomalies was mitigated by AdipoRon. Specifically, AdipoRon trended toward improvement in autonomic dysfunction (Figure 3A and C), attenuates LV hypertophy (Figure 4B), reverses decreases fractional shortening (Figure 4D), LVDP (Figure 5A), dP/dt_{max} (Figure 5C), dP/dt_{min} (Figure 5D) and Tau (Figure 5B) in DM SO females. At a molecular level, DM SO females displayed restorations of cardiac ERα (Figure 6A and 6E) and Cx43 (Figure 6C and 6E) levels. The LV microenvironment indicated decreases in inflammation with AdipoRon reversing elevations in TNFα (Figures 8A and 8C), asymmetric dimethylarginine (ADMA)(Figure 7D), and HO-1 (Figure 8B).

6.2.3 AdipoRon alleviates ovarian hormone-independent detrimental molecular LV alterations

Importantly, and perhaps even more convincingly, AdipoRon alleviated both ovarian hormones-dependent, and independent, adverse molecular responses. While cardiac dysfunction in male diabetic rodent models develops between 8-10 weeks (Hoit et al., 1999), its occurrence after 4 weeks in the presence of estrogen suggests female hypersensitivity to this anomaly (Leffler and Abdel-Rahman, 2019). While DM OVX females in this study lacked clinical evidence of cardiac dysfunction, the findings point to an onset at a later timepoint, perhaps more in line with the DM timeline in males. Preceding the cardiac dysfunction are biochemical changes at the molecular level, as evidenced by the increases

in ERα (Figure 6A) and GPER (Figure 6B) along with a trend toward reduced Cx43 (Figure 6C) in cardiac tissues. Additional results showed the LV tissue transforming to a proinflammatory environment with increased circulating ADMA (Figure 7D), HO-1 (Figure 8B) and ROS production (Figure 8D) in DM OVX perhaps resulting from escalating the consequences of OVX. These pre-dysfunctional molecular alterations were ameloriated by AdipoRon, as evidenced by reversal in ERα upregulation (Figure 6A), trending reductions (P=0.05) in circulating ADMA (Figure 7D), and reversal of HO-1 (Figure 8B and 8C). These exciting findings illustrate the feasability of the proposed novel target in all diabetic females, regardless of ovarian hormone status.

6.2.4 Physiological Implications: Sex/Ovarian Hormones-dependent differences

The adopted multilevel experimental approach permitted the generation of novel molecular findings on ovarian hormones, particularly estrogen, regulation of cardiac function. While the overall objective of this research project was to examine the paradox of a diabetic pathology, it is important to not overlook key physiologial differences in the healthy control (non-diabetic) groups. Chapter 3 highlighted the differences between control and diabetic groups, based upon the research hypothesis investigation. However, there were significant differences ($P<0.05$) in a variety of integrative (body weight, heart weight, etc.) and biochemical (APN, AKT, HO-1, etc.) data sets within the healthy control animal groups. These significant findings are summarized in Table 1.

Table 1. Physiological Implications: Sex/Ovarian Hormone Dependent Differences.

(P<0.05 when compared to **sham** operated within healthy controls. ↑ indicates an increased value, ↓ indicates a decreased value and a blank box indicates no significant difference with that experimental group)

		SIGNIFICANT FINDINGS		
VARIABLE	**FIGURE**	**OVX**	**OVX+E_2**	**Male**
Fractional Shortening	Fig 3.2B	↓		↓
Tau	Fig 3.3C			↑
ERβ	Fig 3.4C			↓
Serum APN	Fig 3.5A			↓
Cardiac APN	Fig 3.5B	↓		↓
pErk/Erk	Fig 3.5C			↓
pAKT/AKT	Fig 3.5D			↓
MAP	Fig 5.2	↑		↑
Body weight	Table 3.3 Fig 4.4A	↑		↑
Heart weight	Table 3.3			↑
LV (d) end volume	Table 4.1	↑		
LF(nu)	Fig 4.3A Fig 5.1A	↑		↑
LF/HF	Fig 4.3C Fig 5.1C	↑		↑
Cardiac TNFα	Fig 4.8A	↑		
Cardiac HO-1	Fig 4.8B	↑		
Survival	Fig 5.3			↓
Cav-3	Fig 5.4 (top)	↓		

6.3 Conclusions

While more studies are needed in female animals and women, it is becoming clear that the protective action of APN is lost in diabetes (Li et al., 2016) and support for the E_2-APN-Cx43 signaling axis involvement is increasing. The suggested overall signaling schematic is presented in Figure 6.1. There are many novel functional and molecular findings of this project that support the E_2 modulation of the APN-Cx43 signaling axis as an underlying mechanism for the heightened sensitivity in female rats to diabetes-evoked myocardial dysfunction. The pharmacological evidence obtained with AdipoRon suggests that activation of this signaling axis has potential therapeutic value in all females although the effect is more evident in the presence of ovarian hormones. The evidence is mounting to support development of a novel therapuetic targeting restoration of cardiac Cx43.

Figure 1. Overall Schematic

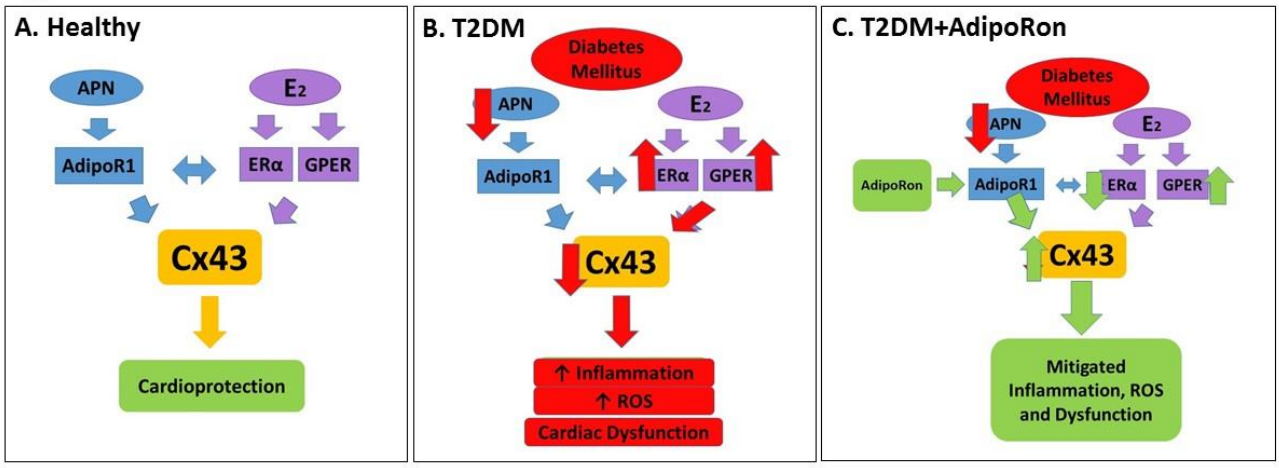


Figure 1. Suggested Overall Signaling Schematic. Healthy female signaling in the cardiac E_2-APN-Cx43 axis (A). The loss of this cardioprotection with the introduction of a diabetic pathology, leading to exacerbated myocardial anomalies (B) and the amelioration of exacerbated dysfunction with the restoration of APN-Cx43 signaling by AdipoRon in the diabetic female (C).

LIMITATIONS

1 All results should be interpreted with the understanding that the adopted T2DM rodent model mimics human type 2 diabetes, but neither the physiology of a rodent, nor the pathology of the disease are identical to the human condition.

2 The in vivo and ex vivo studies are crucial for understanding the molecular mechanisms in an established DM model system, particularly when linking biochemical changes to functional alterations. However, our studies may need be complemented with in vitro cell culture techniques because in vivo studies decrease the ability to intricately isolate and examine cell signaling pathways with precision.

3 While the ten-day course of treatment with AdipoRon was chosen to examine its feasibility as a therapeutic modality and elucidate underlying pathological mechanisms, a longer course of treatment could possibly alter overall study mortality—a result not found in this research project.

4 There are limitations for all equipment and this must be acknowledged for the echocardiography machine (VEVO 2100-3100) as much of the acquired cardiac function data relied heavily on the echo imaging. This methodology, however, was validated by direct invasive hemodynamic monitoring, strengthening the reliability of the data.

5 We acknowledge that a constant physiological plasma E_2 level from the subcutaneous implantation of an estradiol supplement is not identical to the phasic release of endogenous E_2. However, this regimen is clinically relevant to women undergoing surgical or natural menopause.

FUTURE DIRECTIONS

Future research projects will build on these findings of this to expand expertise in two important areas. First, studies on molecular myocardial signaling of Cx43 and APN in healthy and diabetic females. In addition to the gap junction function, Cx43 undergoes a mitochondrial import cycle that appears to play an independent role in cardioprotection (Ruiz-Meana et al., 2008). Future studies will examine the signaling mechanism of the Cx43 mitochondrial import cycle and mitochondrial ROS. Additionally, APN regulates mitochondria (Bugger and Abel, 2010; Koentges et al., 2015) and the mitochondrial and Cx43 content of the heart is higher in females (Juutilainen et al., 2004; Stauffer et al., 2011; Pradhan, 2014). This important mitochondrial link suggests that expanded studies are needed to investigate the role the E_2-APN-Cx43 signaling axis plays on cardiac mitochondria. Second, recent evidence suggests that adult cardiac myocytes release miRNAs that mediate signal transduction to target cells and circulating miRNAs are remarkably stable (Iaconetti et al., 2015). The identification of several miRNAs linked independently to Cx43, estrogen, and DM (see Table 1), such as miR1, miR22, miR126, and miR130a (Klinge, 2009; Iaconetti et al., 2015; Le+¦n et al., 2016), asks the question if the combination further contributes to the clinical problem. Future studies including miRNAs could shed new light on both clinical diagnostic tools for diabetic women with cardiac pathologies and possible novel therapeutic targets.

Table 1. Dysregulation of exosomal miRNAs between cardiac cells in female T2DM

miRNA-	Associated with	E2 influence	T2DM	Released from	Source
22	Cardiomyocyte survival	Decreases	Unknown	Cardiomyocytes	(Wang, et al., 2015)
126	Cell survival	Decreases	Decreases	Cardiac EC	(Dai, et al., 2008;Iaconetti, et al., 2015)
146a	Inflammation and metabolism regulation	Decreases	Decreases	Cardiac EC, monocytes	(Halkein, et al., 2013;Freire, et al., 2014;Klinge, 2009;Iaconetti, et al., 2015)
155	Inflammation	Unknown	Decreases	Monocytes, macrophages	(Freire, et al., 2014)
221	ER expression and ox stress	Unknown	Inhibits AdipoR1	Cardiomyocytes	(Lustig, et al., 2014;Klinge, 2009)
223	GLUT4 uptake, ox stress	Increases	Increases	Cardiomyocytes	(Klinge, 2009;Lu, et al., 2010;Dai, et al., 2008)
320	T2DM, heart failure	Unknown	Increases	Cardiomyocytes	(Iaconetti, et al., 2015)
451	Cardiac hypertrophy	Increases	Increased on HFD/unclear	Monocytes, macrophages	(Klinge, 2009;Dai, et al., 2008;Leon, et al., 2016)

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APPENDIX

ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER

East Carolina University
Tomorrow starts here.

Animal Care and
Use Committee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834-4354

252-744-2436 office
252-744-2355 fax

June 7, 2017

Abdel Abdel-Rahman, Ph.D.
Department of Pharmacology
Brody 6S-10
East Carolina University

Dear Dr. Abdel-Rahman:

The Amendment to your Animal Use Protocol entitled, "Mechanisms of Binge Alcohol Evoked Myocardial Injury/Dysfunction in Hypertension" (AUP # W250) was reviewed by this institution's Animal Care and Use Committee on June 7, 2017. The following action was taken by the Committee:

"Approved as amended"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure