

CARDIAC DYSFUNCTION IN THE ZDF RAT:
POSSIBLE MECHANISMS AND BENEFITS OF EXERCISE

BY

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CARDIAC DYSFUNCTION IN THE ZDF RAT:
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Abstract

Cardiovascular disease has resulted in an increased risk of premature deaths for the 104 million Americans with prediabetes or diabetes and has accounted for approximately 65% of total diabetic deaths annually. Clinical manifestations of diabetic heart disease include left ventricular hypertrophy, diastolic and systolic dysfunction, and diabetic cardiac autonomic neuropathy, which are regularly observed at varying severities in persons with type 2 diabetes. The Zucker diabetic fatty (ZDF) rat has shown promise as a model of diabetic heart disease since it resembles the blending of cardiac diseases seen in humans and as such can be utilized to investigate diabetic heart disease and therapeutic interventions. We chose to investigate the impact of exercise on diabetic heart disease in the ZDF rat, and to explore a novel mechanism. **The objectives of the dissertation were to investigate the cardiac dysfunction in the ZDF model, determine whether aerobic exercise training can reverse electrocardiographic (ECG) and hemodynamic changes induced by diabetes, and identify whether cardiac edema may be one of the factors contributing to diabetic heart disease and a possible target of exercise.**

Myocardial edema is an imbalance between vascular permeability, lymphatic vessels, lymph flow, and cardiac function. It is unknown if diabetes causes myocardial edema. Little is known about the impact of diabetes on the lymphatic system and its receptors, vascular endothelial growth factor receptor 3 (VEGFR-3) and lymphatic endothelial receptor 1 (LYVE-1). These receptors are responsible for the uptake of their respective ligands, VEGF-C and hyaluronan. Each receptor's expression is regulated by prospero homeobox protein 1 (PROX-1), which is the master switch for the

lymphangiogenesis. Myocardial fluid imbalances have been implicated in the fibrosis and hypertrophy associated with common cardiovascular diseases, which makes edema a suitable target for possible interventions.

Diabetes in the ZDF rat caused crucial changes in R wave amplitudes ($p < 0.001$), heart rate variability ($p < 0.01$), QT intervals ($p < 0.001$) and QT_c intervals ($p < 0.001$). R wave amplitude augmentation in sedentary diabetic rats from baseline to termination was ameliorated by exercise, resulting in R wave amplitude changes in exercised diabetic rats similar to control rats. Of these changes, aerobic exercise training was only able to correct R wave amplitude changes. In addition, exercise had beneficial effects in this diabetic rat model with regards to ECG correlates of left ventricular mass.

Of the 24 hemodynamic parameters tested, 15 were negatively affected by diabetes. The debility of diabetic heart disease was evident in the diastolic filling, isovolumic contraction, ejection, and isovolumic relaxation phases. Importantly, exercise training restored 13 of the 15 hemodynamic parameters affected by diabetes. However, we did not observe differences in left ventricular weights, a direct measure of myocardial edema, or alterations in the levels of VEGF-C, VEGFR-3, LYVE-1, or hyaluronan. We were able to observe systemic differences in plasma interleukin (IL)-2 levels, reductions in dP/dt_{max} , and differences in PROX-1 protein levels and DNA binding activity that were suggestive of the presence of myocardial edema in the ZDF rat. However, these alterations are indirect measures of myocardial edema, therefore we were unable to conclude in the 19 week old ZDF rat if myocardial edema exists and plays a role in diabetic heart disease.

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List of Abbreviations

AGE	Advanced glycation end products
AVT	Anterior interventricular trunk
CLN	Cardiac lymph nodes
COI	Cardiac output index
CO	Cardiac output
CVD	Cardiovascular disease
CHS	Cardiovascular Heart Study
cDNA	Complementary DNA
CAD	Coronary artery disease
C _t	Cycle threshold
DAN	Diabetic cardiac autonomic neuropathy
DEPC	Diethylpyrocarbonate
ECG	Electrocardiogram/electrocardiography
ELISA	Enzyme-linked immunosorbent assay
EC	Exercised control
ED	Exercised diabetic
FFA	Free fatty acids
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HRV	Heart rate variability
HbA1C	Hemoglobin A1C
IFN- γ	Interferon gamma
IL	Interleukin
IJV	Internal jugular vein
LSD	Least significant difference
LCC	Left coronary channel
LV	Left ventricle/ventricular
LYVE-1	Lymphatic vessel endothelial receptor 1
MSC	Main supracardiac channel
P _{max}	Maximum pressure
P _{min}	Minimum pressure
MRFIT	Multiple Risk Factor Intervention Trial
NF- $\kappa\beta$	Nuclear factor kappa beta
OMT	Obtuse marginal trunk
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
PVT	Posterior interventricular trunk
PROX-1	Prospero homeobox protein 1
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RCC	Right coronary channel
RLD	Right lymphatic duct
RT-PCR	Reverse transcription polymerase chain reaction
SC	Sedentary control
SD	Sedentary diabetic

SA	Sinoatrial
SEs	Standard errors
STZ	Streptozotocin
SVI	Stroke volume index
SV	Stroke volume
SWI	Stroke work index
SW	Stroke work
ScV	Subclavian vein
ARIC	The Artherosclerosis Risk in Communities Study
TNF- α	Tumor necrosis factor-alpha
T1D	Type 1 diabetes
T2D	Type 2 diabetes
U.S.	United States
VEGF-C	Vascular endothelial growth factor C
VEGFR-3	Vascular endothelial growth factor receptor 3
WHO	World Health Organization
ZDF	Zucker diabetic fatty

Chapter 1

Introduction

1.1 Diabetes Mellitus

1.1.1 Epidemiology of diabetes mellitus

The diabetes explosion has resulted in significant global public health concerns regarding its impact on lifespan and health care costs. Over 347 million people worldwide have diabetes (Tobias, 2011), and the United States (U.S.) accounts for at least 7% of all cases (ADA, 2011). Diabetes is classified as type 1 (T1D), type 2 (T2D), or gestational (onset during pregnancy). T1D, predominately diagnosed in children, is characterized by the lack of insulin production. T2D is identified as poor insulin utilization, and is commonly associated with obesity and a sedentary lifestyle. T2D is reported as the predominant classification for Americans diagnosed with diabetes, and an additional 79 million people are estimated to have prediabetes (ADA, 2011). Prediabetes is recognized as an intermediate phase of elevated blood glucose levels, which have not met the clinical diagnosis of diabetes, i.e. fasting blood glucose levels greater than 110 mg/dL (WHO, 2011). Classically considered adult diseases, prediabetes and T2D have exponentially increased in children in the last two decades (CDC, 2011b). The World Health Organization (WHO) estimated that diabetic deaths will double by 2030 (WHO, 2011) for adults and children affected by the disease. The cost associated with the management of diabetes related complications, comorbidities, and premature death has presented as a surmounting challenge for several countries, including the U.S. where medical costs are the highest in the world (OCED, 2011).

The U.S. has experienced an outstanding financial burden due to a third of its population having diabetes or prediabetes. Approximately 200 billion dollars are spent annually for direct and indirect costs associated with diabetic care (ADA, 2011). Direct

and indirect costs included medical management of the disease and its complications and individual and societal losses of production. Prediabetes has been associated with the presentation of complications commonly identified with the disease (Schaefer et al., 2010; Singleton and Smith, 2006). Those complications include retinopathy, neuropathy, nephropathy, and cardiovascular disease. **Cardiovascular disease is associated with an increased risk of premature death for the 104 million Americans with prediabetes or diabetes (ADA, 2011) and accounts for approximately 65% of total diabetic deaths annually (Stamler et al., 1993); (Clearinghouse, 2011).**

1.2 Diabetic Heart Disease

1.2.1 Classification of diabetic heart disease

Cardiovascular disease is stratified into three categories based on the site of injury: cardiovascular, cerebrovascular, and peripheral vascular disease (Clearinghouse, 2005) as noted in Figure 1. Cardiovascular disease is defined as conditions that affect the muscles of the heart or the coronary vessels.

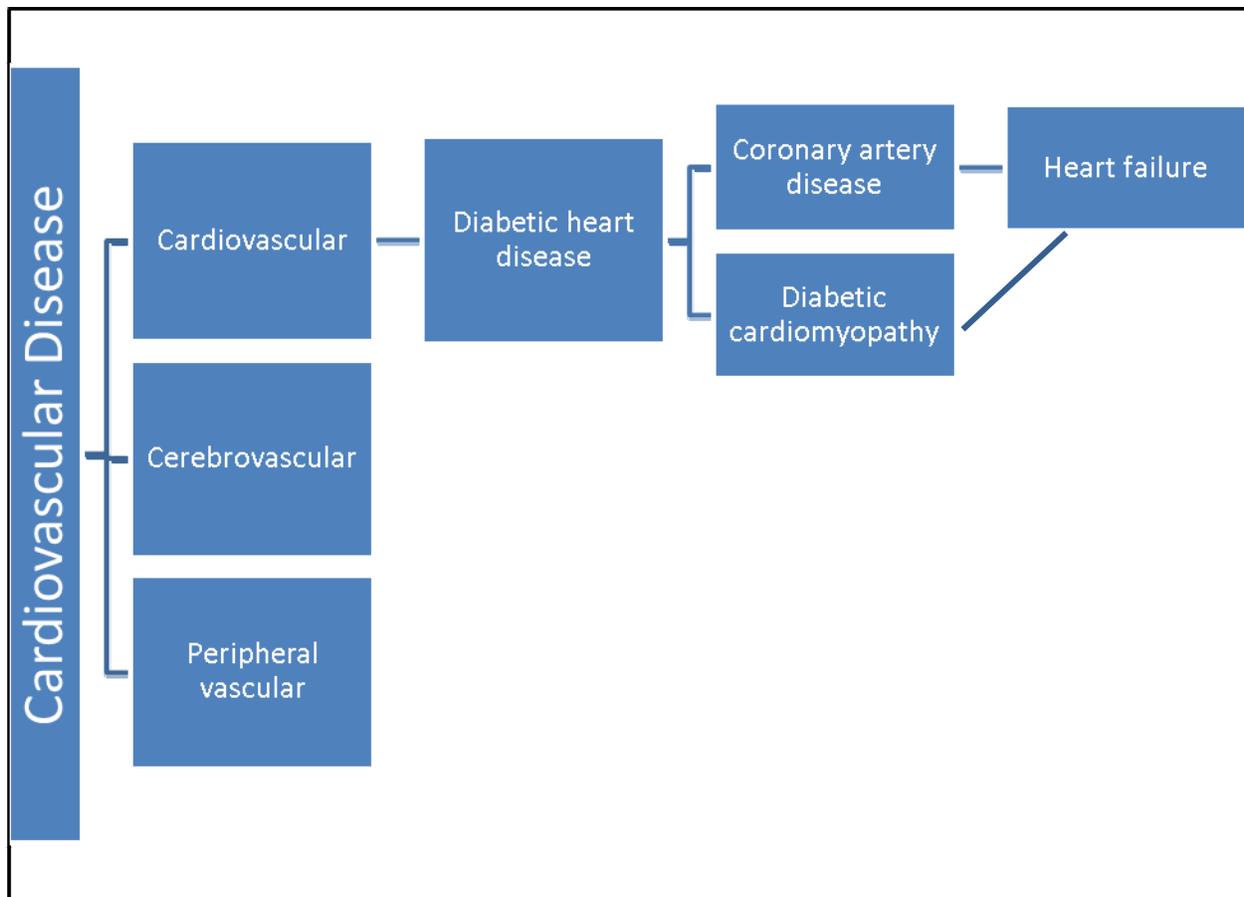


Figure 1 Classification of cardiovascular disease and related diabetic heart diseases. The clinical progression of cardiovascular disease under the backdrop of diabetes is outlined with the common endpoint of heart failure.

The term diabetic heart disease has typically been described as coronary artery disease (CAD), diabetic cardiomyopathy, or a combination of the two diseases. CAD encompasses the sequelae that occur from the narrowing and dysfunction of the coronary arteries that can ultimately result in a myocardial infarction (El-Sherif et al., 2010). Diabetic cardiomyopathy, or disease of the heart muscle, is considered an independent risk factor for the development of CAD (Ueda et al., 2011; Picchi et al., 2010). Diabetic cardiomyopathy can be defined as ventricular abnormalities under diabetic conditions in the absence of hypertension, CAD, or other cardiac diseases (Boudina and Abel, 2010; Maisch, Alter, and Pankuweit, 2011). Clinical manifestations

of diabetic cardiomyopathy include left ventricular (LV) hypertrophy, diastolic and systolic dysfunction, and diabetic cardiac autonomic neuropathy (DAN), which are regularly observed at varying severities in persons with T2D (Fang, Prins, and Marwick, 2004; Murarka and Movahed, 2010). Often diabetic heart disease in humans and animals presents as a complex amalgam of multiple cardiovascular diseases, not as a solitary condition. Nevertheless, the common endpoint for CAD and diabetic cardiomyopathy, independently or as comorbidities, is clinical heart failure (Schainberg, Ribeiro-Oliveira Jr, and Ribeiro, 2010).

1.2.2 Epidemiology of diabetic heart disease

Population based studies have firmly established the association between T2D and CAD. The Atherosclerosis Risk in Communities Study (ARIC) (Folsom et al., 1997), Cardiovascular Heart Study (CHS) (Psaty et al., 1999), Multiple Risk Factor Intervention Trial (MRFIT) (Stamler et al., 1993), and the Framingham Heart Study (Kannel and McGee, 1979; Garcia et al., 1974) reported the concurrent nature of the two diseases. The ARIC study showed that the risk of developing CAD was 3.45 times higher for women with diabetes and 2.52 for men (Folsom et al., 1997) compared to healthy peers. The CHS study showed a significant association between blood glucose levels and myocardial infarction. The risk of a myocardial infarction was 10% higher in those with diabetes (Psaty et al., 1999). The CHS and ARIC study suggested that diabetes may increase the risk of the development of CAD and associated adverse cardiac events such as myocardial infarctions. The MRFIT and Framingham studies supported these findings, but also emphasized the relationship of diabetes and fatal cardiac events. In the MRFIT study, 603 cardiovascular deaths occurred in the sample of 5163

men with diabetes. The 12% mortality rate indicated the increased risk in those with diabetes compared to the 3% cardiovascular death rate in men without diabetes (Stamler et al., 1993). The Framingham Study reinforced these findings with higher cardiovascular mortality rates for persons with diabetes compared to the non-diabetic group and reported higher mortality risk for diabetic women compared to affected men (Kannel and McGee, 1979).

Interestingly, diabetes is recognized as a “CAD risk equivalent” confirming the deleterious, independent impact on the cardiovascular system (NCEP, 2002). People with diabetes and no CAD have an equivalent absolute 10 year risk of CAD events as those with CAD and no diabetes (NCEP, 2002). Therefore, the American College of Cardiology and the American Heart Association have recognized diabetes as a precursor for heart failure. This acknowledgement mandated that patients with diabetes be classified as having Stage A heart failure (Hunt et al., 2005). The mandate and the epidemiological studies have validated the impact of diabetes on cardiovascular health independently or in combination with other cardiac diseases.

1.2.3 Phenotypes of diabetic heart disease

Multiple detection methods are currently used for phenotyping diabetic heart disease (Folsom et al., 1997; Faglia et al., 2002, MiSAD, 1997). **The classic characteristics of diabetic heart disease include LV hypertrophy, diastolic and systolic dysfunction, and DAN.** The clinical manifestations are routinely presented as a group of abnormalities.

1.2.3.1 Left ventricular hypertrophy

LV hypertrophy is defined as increases in LV mass and is commonly adjusted by gender, body surface area, or height (Levy et al., 1990). This augmentation is typically due to extracellular matrix alterations (Deschamps and Spinale, 2006). Hypertrophy is classified as physiological or pathological (Figure 2). Physiological hypertrophy can develop secondary to increased volume in the LV chamber from pregnancy or exercise demands. This remodeling is known as eccentric hypertrophy, in which the sarcomeres contribute to increased cellular length (Heineke and Molkentin, 2006). The modifications result in a uniformed increase in ventricular dimension, which maintains or enhances cardiac function. LV hypertrophy can also occur as a pathological response to diabetic complications.

An association between hypertension, obesity, and diabetes is indicative of pathological hypertrophy according to clinical research (Sharma et al., 2011). The compensatory thickening of the ventricular wall has been shown to maintain cardiac function by countering pressure demands related to local or systemic hypertension in humans (Lalande and Johnson, 2008). However, this compensatory mechanism will convert to a pathological response over time with losses in LV compliance and diastolic function. Pathological remodeling is primarily described as concentric hypertrophy with greater increases in the width of the cardiomyocyte. Cardiomyocyte remodeling in animal models has been shown to increase the thickness of the ventricular wall and septum due to collagen accumulation and fibrosis (Heineke and Molkentin, 2006). Such pathological changes in ventricular mass coupled with fibrosis are associated with the substantial loss of the ventricular chamber volume and advanced stages of diabetic

heart disease. The Framingham study reported poor prognosis for individuals with LV hypertrophy (Levy et al., 1990). Regardless of the etiology, thickening of the LV wall has a direct effect on the diastolic and systolic phases of the cardiac cycle.

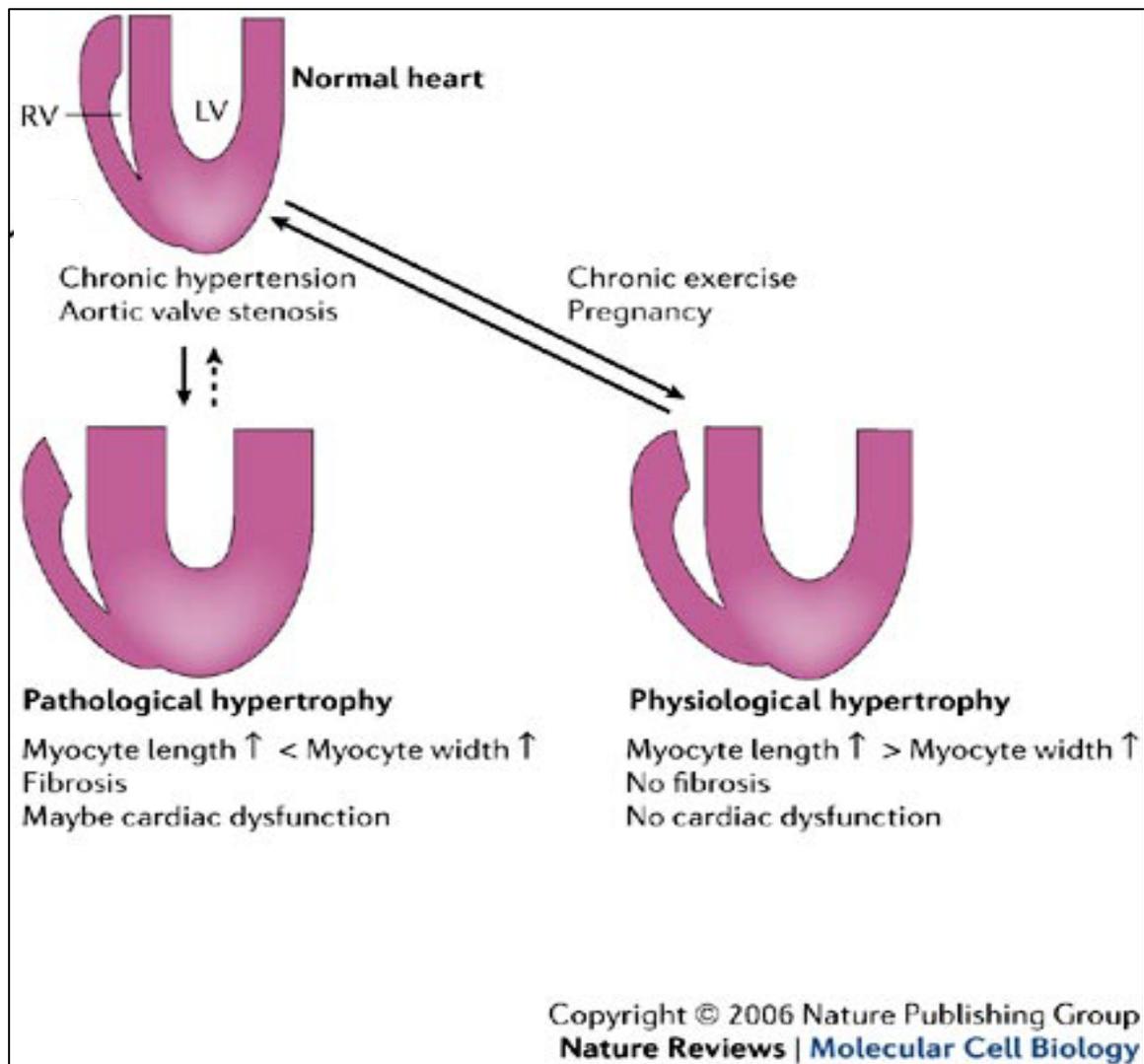


Figure 2 Different types of hypertrophy (Heineke and Molkenin, 2006).

1.2.3.2 Diastolic and systolic dysfunction

Diastolic dysfunction typically precedes systolic dysfunction. Decreased filling or relaxation of the ventricle is often reported to be a measure of diastolic dysfunction (Boudina and Abel, 2010; Lalande and Johnson, 2008). The diastolic phase accounts for the time period when the ventricle is at rest. During this phase, the healthy ventricle can fill to capacity depending on its compliance. Compliance is defined as the comparison of the change in volume of the ventricular chamber to the pressure applied to the ventricle or, simply stated, the ability of the chamber to stretch. However the fibrotic, thickened, diabetic ventricle cannot adequately fill due to the loss in compliance. Diastolic dysfunction can impede the contractile potential of the ventricle, because the ability to produce tension depends on the stretching and release of myocardial fibers. Elastance is described as the ability of the ventricle to recoil after ejection (Chang, Lo, and Tseng, 2002). Ventricular remodeling, such as that observed with diabetes, can impair compliance and elastance and result in reduced stroke volume.

Stroke volume is the amount of blood the ventricle can eject and is typically referred to as the expulsion of blood into the body for circulation. The impact of diabetes on ventricular contractility or systolic function has been debated in the literature (Mbanya et al., 2001; Borow et al., 1990; Palmiero, Macello, and De Pascalis, 2006; Alvarez et al., 2004). A strong, coordinated contraction is necessary for modulating the stroke volume needed for cardiovascular demand. Systolic dysfunction in diabetic humans and animals has been reported as attenuations in fractional shortening, velocity of shortening, force production, and cardiac output (Vinereanu et al., 2003; Friedman et

al., 1982). Impaired systole has been suggested to be a combination of contractility deficits and a lack of sensory regulation (Valensi et al., 2001).

1.2.3.3 Diabetic cardiac autonomic neuropathy

DAN is a neuropathy affecting the cardiac afferent nerves that presents as contractility and conduction abnormalities (Adamson and Vanoli, 2001; Khoharo et al., 2009). Electrocardiographic (ECG) assessments have suggested the presence of DAN (Valensi et al., 2001) and LV hypertrophy (Ciardullo et al., 2004) in people with diabetes. The loss of sympathetic response impairs conduction and contractility. In depth observations have shown DAN as regional sympathetic denervation of ventricles in humans (Schnell et al., 1995). Positron emission tomography studies have mapped proximal hyperinnervation in these regions with distal denervation under diabetic conditions in humans and animals (Stevens et al., 1998b; Schmid et al., 1999). Deficits in blood flow are also associated with these sensory alterations, but presented primarily in the proximal segments in human studies (Stevens et al., 1998a). These reports suggested that diabetes cause losses in cardiac sensory response and blood flow proximal to the denervated site. While the body attempts to respond with limited hyperinnervation, the heterogeneity of innervation predisposes those with diabetes to arrhythmias and sudden cardiac death. The increased risk of DAN may be revealed through multiple detection methods in people with diabetes and animal models of diabetic heart disease.

1.2.4 Murine models of diabetic heart disease

Murine models have proven beneficial in the study of diabetic heart disease. Both mouse and rat models mimic the characteristics of the human diabetic process. Recent

studies have indicated that mechanisms responsible for diabetic heart disease may differ between T1D and T2D animal models (Boudina et al., 2007; Bugger and Abel, 2008). However, diastolic and systolic dysfunction has varied in severity in all models. The streptozotocin (STZ) injected rodent has remained a widespread model of T1D (Deeds et al., 2011). Yet, the model that resembles human T1D the closest is the BioBreeding Diabetes Resistant rat with its autoimmune component (Mordes et al., 2004). Models of T2D include the ob/ob mouse, db/db mouse, and the Zucker diabetic fatty (ZDF) rat. Each of these T2D models stems from alterations in leptin or its receptor. Leptin is a polypeptide hormone that controls hunger and metabolism. Elevated levels of circulating leptin are associated with obesity (Myers et al., 2010). These animal models have the concomitant presentation of obesity and T2D.

KK mice (Bugger and Abel, 2009), Goto-Kahizaki (Bugger and Abel, 2009) and Kob (Igarashi et al., 1994) rats offer alternative models of T2D based on various mechanisms. The KK mouse is a polygenic model of obesity and T2D (Portha et al., 2010). The Goto-Kahizaki rat has genetic alterations related to beta cell metabolism (Suto et al., 1998). Hemosiderin deposition may be the mechanism responsible for the spontaneous development of diabetes in the Kob rat (Igarashi et al., 1994). The larger size and hemodynamic parameters of the rat have made it the preferred murine model for cardiac physiology experiments. Although multiple models are available, the ZDF rat has had increased use in cardiovascular research in the last decade due to its phenotype (Young et al., 2002; Sidell et al., 2002; Boudina and Abel, 2010).

The ZDF rat has a leptin receptor deficit due to a mutation in the Fa gene (fa/fa). The uptake of leptin by the leptin receptor has been shown to signal satiety (Satoh et

al., 1997), but the uptake is diminished in the ZDF rat. The receptor mutation is linked to hyperphagia and subsequent obesity as observed in the Zucker fatty rat (Phillips et al., 1996). For consistent development of diabetes, the male ZDF rat is fed a commercially available high fat and protein diet; while the female ZDF rat requires a specialized high fat diet for the induction of diabetes. At 8 weeks of age, male ZDF rats develop hyperglycemia and hyperinsulinemia by 12 weeks. The rodents have many of the same characteristics as humans with T2D, including increased serum free fatty acids (FFAs) and triglycerides along with cardiac dysfunction (Clark, Palmer, and Shaw, 1983; Golfman et al., 2005; Wang et al., 2005). In 19 week old ZDF rats, cardiomyocytes are enlarged and extensive perivascular fibrosis is observed the ventricles (Fredersdorf et al., 2004). However, both the absence (Chatham and Seymour, 2002) and the presence (Golfman et al., 2005) of cardiac hypertrophy have been reported in the ZDF rat.

Some research publications have referred to diabetic heart disease in the ZDF model as diabetic cardiomyopathy (van den Brom et al., 2010; Forcheron et al., 2009; Boudina and Abel, 2007). At the same time, both hypertension (Tikellis et al., 2004; Oltman et al., 2006; Toblli et al., 2010) and normotension (Cosson et al., 2009) have been reported in the ZDF rat. Of interest, despite being on a high fat diet, ZDF rats do not appear to develop atherosclerosis (Oltman et al., 2006); although coronary artery dysfunction has been noted (Oltman et al., 2006). Regardless, **the ZDF rat has shown promise as a model of diabetic heart disease** (Russell and Proctor, 2006; Poornima, Parikh, and Shannon, 2006); since it has resembled the amalgamation of cardiac diseases seen in humans and as such can be utilized **to investigate diabetic heart disease and therapeutic interventions.**

1.3 Electrocardiographic Assessment of Diabetic Heart Disease

1.3.1 Normal heart electrical conduction system

Figure 3 provides a visual representation of the electrical activity of the heart, known as the ECG. As the action potential migrates through the heart, the atria and ventricles depolarize and repolarize, which is captured as the ECG waveform.

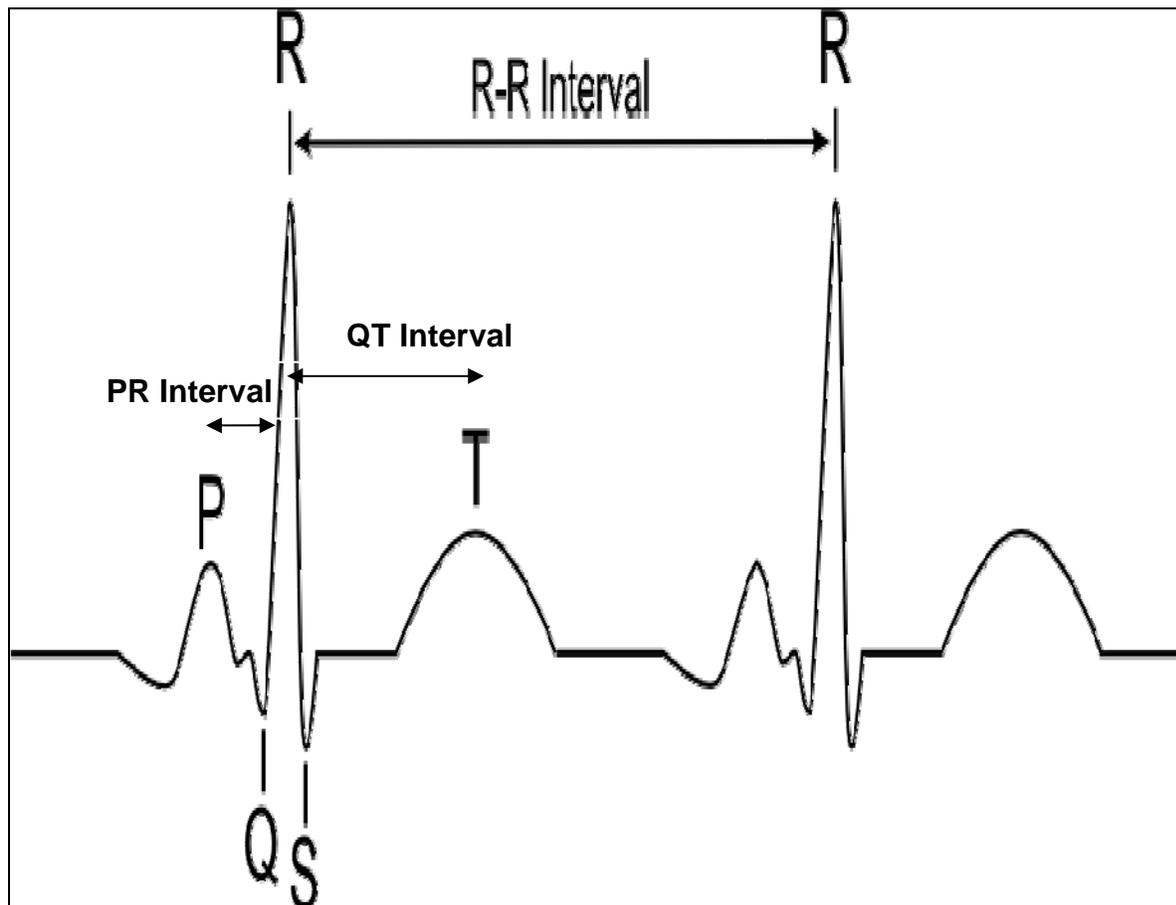


Figure 3 ECG waveform Modified from (MacLeod, 2010). The ECG waveform depicting atrial depolarization (P wave), followed by ventricular contraction (QRS complex), and ventricular repolarization (T wave). The corresponding intervals are identified in the figure.

Atrial changes are masked in the typical ECG, except for the P wave, due to the disparities in size and force production compared to the ventricles. The sinoatrial (SA)

node serves as the pacemaker of the heart. When the SA node fires, the atria depolarize and are noted electrically as a P wave. In male, healthy Sprague Dawley rats, the average P wave amplitude is 0.028 ± 0.012 mV (Normann, Priest, and Benditt, 1961). As the impulse travels from the SA node to the ventricles, the average elapsed time of 51 ± 5 msec (Normann, Priest, and Benditt, 1961) was reported as the PR interval in these normal rats.

As the action potential moves through the ventricles, the QRS complex is recorded. The typical R wave amplitude was 0.138 ± 0.006 mV (Normann, Priest, and Benditt, 1961) in adult male rats with an average heart rate of 388 ± 29 beats per minute (Normann, Priest, and Benditt, 1961). The time between two consecutive R waves is measured as the RR interval, which provides the heart rate measurement. Heart rate variability (HRV) indicates the balance between sympathetic and parasympathetic activity and is calculated by the standard deviation of the RR intervals. The QT interval represents a complete cycle of depolarization and repolarization of the ventricles and was measured as 54 ± 5 msec in Sprague Dawley rats (Normann, Priest, and Benditt, 1961). The QT interval varies inversely with heart rate, for example a lower heart rate presents with a longer QT interval. Therefore, QT intervals must be corrected to heart rate. Corrected QT (QT_c) can be calculated with mean values and the Bazett's formula ($QT_c = QT \text{ Interval} / \sqrt{RR \text{ interval}}$) (Heffernan, Jae, and Fernhall, 2007). The resting phase of the ventricles is represented by the T wave amplitude and signifies the end of the normal cardiac cycle.

1.3.2 Common ECG abnormalities

Conduction irregularities can occur within any ECG wave, complex, or interval from acute and chronic conditions. Sympathetic and parasympathetic input can alter the spontaneous depolarization of the SA node located in the right atrial wall. The sympathetic induced release of noradrenaline at the SA node leads to tachycardia (Mlynarska et al., 2006). In contrast, bradycardia results from increased parasympathetic input and the release of acetylcholine (Mlynarska et al., 2006). As electrical activity is recorded from the atria, abnormalities in the shape, duration, or amplitude of the atrial waveform may indicate pulmonary hypertension, myocardial infarction, or mitral valve disease (Liu et al., 2010; Vranka, Penz, and Dukat, 2007).

As the action potential moves from atria to ventricles, shortened or prolonged PR intervals can reveal a lack of coordination between the atria and ventricles and possible dysrhythmias. Increased ventricular amplitudes and duration of the QRS complex provide early indications of conduction abnormalities and ventricular hypertrophy (Grigioni et al., 2002; Iler et al., 2008; Bacharova, Szathmary, and Mateasik, 2010). After the systole, the ventricle's repolarization is reported by QT intervals. The widening of the QT intervals can indicate an increased risk of ventricular arrhythmias, syncope, and sudden death (Johnson et al., 2011; Liu et al., 2011). The measurement serves as a prognostic index for hypertrophic cardiomyopathy and heart failure (Cygankiewicz et al., 2008; Bayrak et al., 2007). Non-invasive, surface ECG has proven a valuable tool in identifying disruptions in the cardiac conduction system and one's risk for cardiac events and chronic conditions.

1.3.3 Diabetes related ECG abnormalities

ECG changes have been observed in both human and murine diabetic heart disease studies (Okin et al., 2004; Stern and Sclarowsky, 2009; Howarth et al., 2009). In human, QT_c prolongation was presented as a common finding associated with diabetes (Matel, Chiochina, and Stratone, 2010; Nagaya et al., 2010a; Laitinen et al., 2008). ECG assessments in diabetic animal models have supported the abnormalities seen in human studies. Bradycardia, decline in HRV, and QT_c prolongation were noted in STZ rat model (Annapurna et al., 2009; Squadrito et al., 1986; Howarth et al., 2005). Reductions in HRV and heart rate were also observed in Goto-Kakizaki and Kob rats (Howarth et al., 2008; Sanyal, Arita, and Ono, 2002). **ECG investigations of the ZDF model were absent in the current literature** until our published comprehensive study (VanHoose, 2010). In this dissertation work, we have characterized ECG abnormalities in the ZDF rat and the recovery of parameters after exercise training as reported in Chapter 2.

1.4 Hemodynamic Assessment of Diabetic Heart Disease

1.4.1 Normal left ventricular hemodynamics

The ventricular cycle was first described a century ago by Otto Frank (Frank, 1895). Starling expanded the concepts in 1914 and developed the current terminology, including Starling's Law of the Heart (Burkhoff, Mirsky, and Suga, 2005; Konhilas, Irving, and de Tombe, 2002). The Law states that the amount of blood ejected from the ventricles (stroke volume) is dependent on the volume in the chambers (end diastolic volumes) (Solaro, 2007). The theory indicates the interplay between the ventricular hemodynamic pump and the viscoelastic properties of the cardiac muscle. The

interaction is captured by the assessment of the relationship between instantaneous ventricular pressures and volumes during the cardiac cycle. Plotting of the relationship is commonly described as pressure volume loops. The pressure volume loop provides a visual description of the intricate balance between the phases of systole and diastole as influenced by intramyocardial and extramyocardial forces and is shown in Table 1.

Three Phases of Systole	Four Phases of Diastole
Isovolumic contraction	Isovolumic relaxation
Ejection	Atrial systole
Rapid inflow	Rapid inflow
	Diastasis

Table 1 Phases of systole and diastole. The three phases of systole describe the initial development of pressure within the ventricle, followed by the ejection into the aorta, and the rapid inflow into the aorta. Systole is trailed by diastole, which accounts for the majority of the cardiac cycle duration. During isovolumic relaxation, pressure decreases in the ventricle. The contraction of the atrium causes a rapid inflow of blood into the ventricle. Diastasis is the final filling phase of the ventricle.

At point A in Figure 4, the mitral valve opens and the atrium fills quickly (rapid inflow). Diastasis, middle third to the end of diastole, contributes a small volume to the ventricle during interval AB. Atrial contraction is the culmination of interval AB. When pressure in the left ventricle is higher than in the left atrium, the mitral valve closes as indicated by point B, at which time end diastolic volume is measured. The diastolic load at the end of ventricular relaxation is defined as preload. The compliance of the ventricle will determine the ability of the ventricle to stretch and accommodate variable preloads. Interval BC reflects the initial contraction of the ventricles known as the isovolumic contraction. During this interval, the ventricular volume remains constant, but pressure

increases substantially. At point C, the LV pressure exceeds the aortic root pressure and the aortic valve opens.

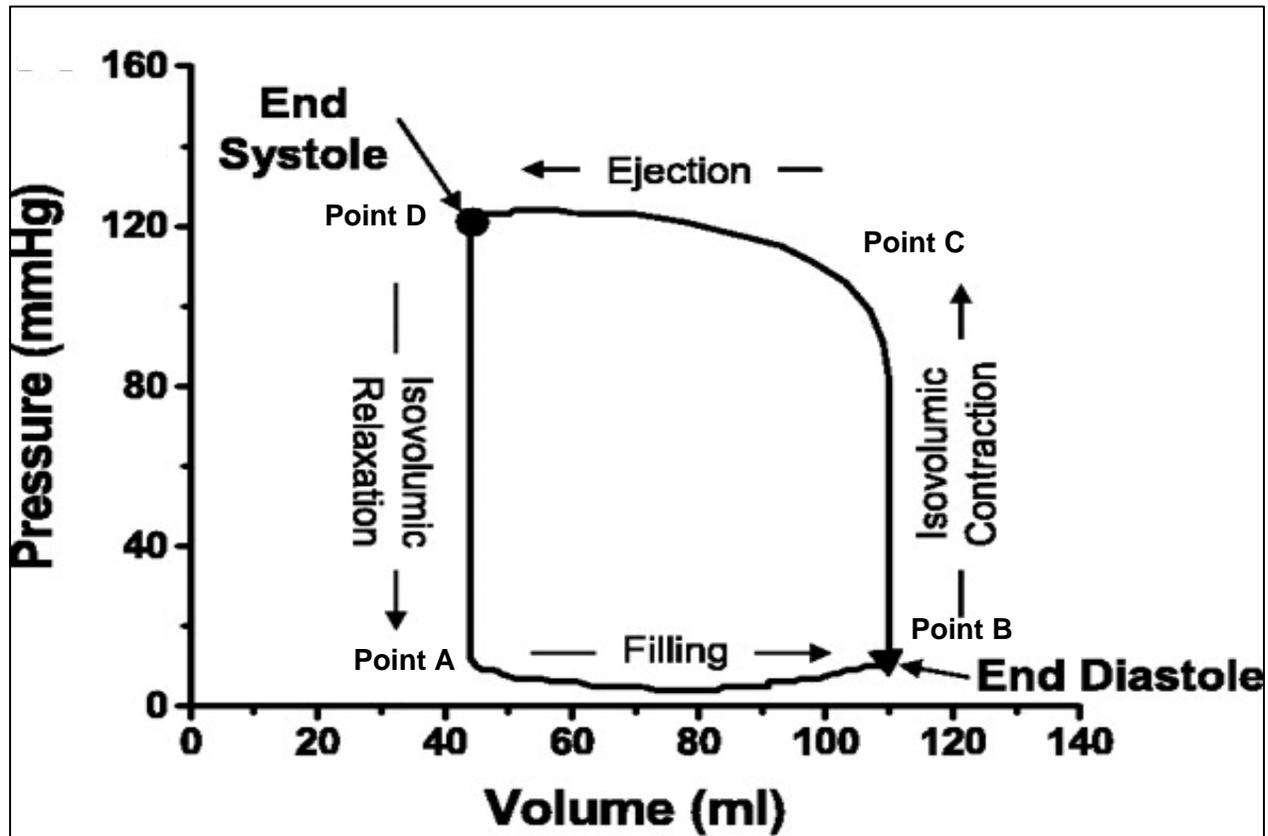


Figure 4 Pressure volume loop Modified from (Burkhoff, Mirsky, and Suga, 2005). The diagram depicts the phases and distinct points of the cardiac cycle. Point A is the end of isovolumic relaxation. Point B is commonly identified as the end diastolic volume. Point C indicates the end of isovolumic contraction and the initiation of ejection. Point D is the end systolic volume and initiation of isovolumic relaxation.

Interval CD represents ventricular systole, which includes the ejection of blood and rapid inflow into the aorta. The amount of pressure that the ventricle must develop for ejection is directly related to peripheral resistance or afterload. Afterload is represented by the factors that oppose contraction and ejection fraction, such as systemic hypertension. End systolic volume is measured at point D and signifies the closure of the aortic valve due to a lower LV pressure than aortic root pressure. Interval

DA indicates another time in the cardiac cycle where volume is constant, but pressure changes. During isovolumic relaxation, pressure decreases in the left ventricle as reported by the tau value. The elastance of the ventricle will recoil the ventricle to its resting state in preparation for diastole. During ventricular relaxation, the cardiac cycle recommences with filling of the chamber. Table 2 outlines the hemodynamic parameters attained from the pressure volume loop analysis.

Cardiac Cycle Points	Hemodynamic Parameters	Cardiac Cycle Intervals	Hemodynamic Parameters
Point A Start of ventricular filling	Minimum pressure (P_{min}) Minimum volume		
		Interval AB Ventricular filling	dV/dt_{max} $P@dV/dt_{max}$
Point B Start of isovolumic contraction	End diastolic volume End diastolic pressure		
		Interval BC Isovolumic contraction	dP/dt_{max} $V@dP/dt_{max}$ dV/dt_{min}
Point C Start of ejection			
		Interval CD Ejection	Maximum pressure (P_{max}) Ejection fraction
Point D Start of isovolumic relaxation	End systolic volume End systolic pressure		
		Interval DA Isovolumic relaxation	dP/dt_{min} $V@dP/dt_{min}$ Tau

Table 2 Hemodynamic parameters obtained from the pressure volume loop analysis.

The hemodynamic parameters directly derived from the pressure volume loops allow for the calculation of additional measures. Heart rate is based on the number of contractions per minute. Stroke volume (SV) is calculated as the difference between end diastolic and systolic volumes. Cardiac output (CO) is the product of heart rate and

SV. The area within the loop is defined as the stroke work (SW). SV, CO and SW are often adjusted for body weight and described as indices (Jin et al., 2000). Maximal power and arterial elastance are determined from standard hemodynamic software.

1.4.2 Common hemodynamic abnormalities

The ventricle can respond to chronic volume and pressure overloading with physiological and structural changes. Persistent volume increases result in eccentric hypertrophy. The heart will respond with slight increases in end diastolic pressure and significant expansion of the end diastolic volume. This rightward shift of the pressure volume loop will deliver a larger stroke volume to maintain function. Wall thickness also increases under chronic pressure conditions and is labeled concentric hypertrophy, where the loops will shift upward with increases in end diastolic pressures (Burkhoff, Mirsky, and Suga, 2005). The compensated cardiac function results from cardiomyocyte hypertrophy in the enlarged ventricles, not direct increases in contractility and stroke work (Heineke and Molkenin, 2006). Unfortunately, the heart cannot maintain compensation long-term due to afterload mismatches and decline in preload reserve.

1.4.3 Diabetes related hemodynamic abnormalities

Diabetes can cause afterload mismatches due to impaired contractility and declines in preload related to diastolic dysfunction. Inotropy or contractility is lessened under diabetic conditions due to geometrical and hypertrophic alterations (Lee et al., 1997; Baynes and Murray, 2009). The CHS study reported increased septal and left posterior ventricular wall thickness in men and women with T2D without CAD (Lee et al., 1997). These alterations impaired cardiac function, which presented as reductions in stroke volume and cardiac output. The ZDF rat has shown a decrease in heart rate and

CO (Radovits et al., 2009), which supported the loss of cardiac function under diabetic condition. In another report on the ZDF rat, systolic pressures, dP/dt_{max} and dP/dt_{min} decreased with pressure volume analysis (Yue et al., 2005). The diabetic heart could not maintain function via the Frank-Starling mechanism, because the heart was incapable of producing the necessary pressures for ejection or other compensatory responses. An assessment of 16 and 36 week old ZDF rats showed an adaptive response in stroke volume in 16 week old animals. This compensatory mechanism was lost in the older animals. The progressive loss of ejection fraction was suggested as evidence of LV dilatation (Baynes and Murray, 2009).

Diastolic dysfunction in the diabetic heart may result from myocardial wall thickening and fibrosis (Jellis et al., 2011; Ihm et al., 2010). These changes can attenuate the lusitropic or filling ability of the diseased ventricle. The upward and leftward shift in pressure volume loops confirmed the increased afterload and poor ventricular filling reported in biomechanical investigations (Villars et al., 2004). Echocardiographic evaluations of asymptomatic subjects with T2D revealed impaired diastolic function in 70% of the sample population (Shrestha et al., 2009). **To explore the mechanisms responsible for diabetic hemodynamic changes, an animal model is crucial.** The ZDF rat has been characterized with relaxation and contractility deficiencies due to increases in LV stiffness (Radovits et al., 2009) and thus is suitable for fully exploring cardiac function with and without therapeutic interventions. In Chapter 3, we will report our successful characterization of the hemodynamic changes of the ZDF rat and the beneficial effects of exercise training.

1.5 Mechanisms of Diabetic Heart Disease

Diabetic heart disease has multiple etiologies that occur across organismal to cellular levels. **Metabolic disturbances** (Ku et al., 2011), **myocardial fibrosis** (Mohamad, Askar, and Hafez, 2011), **small vessel disease** (Mourot et al., 2009), and **DAN** (Spallone et al., 2011) are reported to play a complex and concomitant role in the development and progression of the disease (Boudina and Abel, 2007).

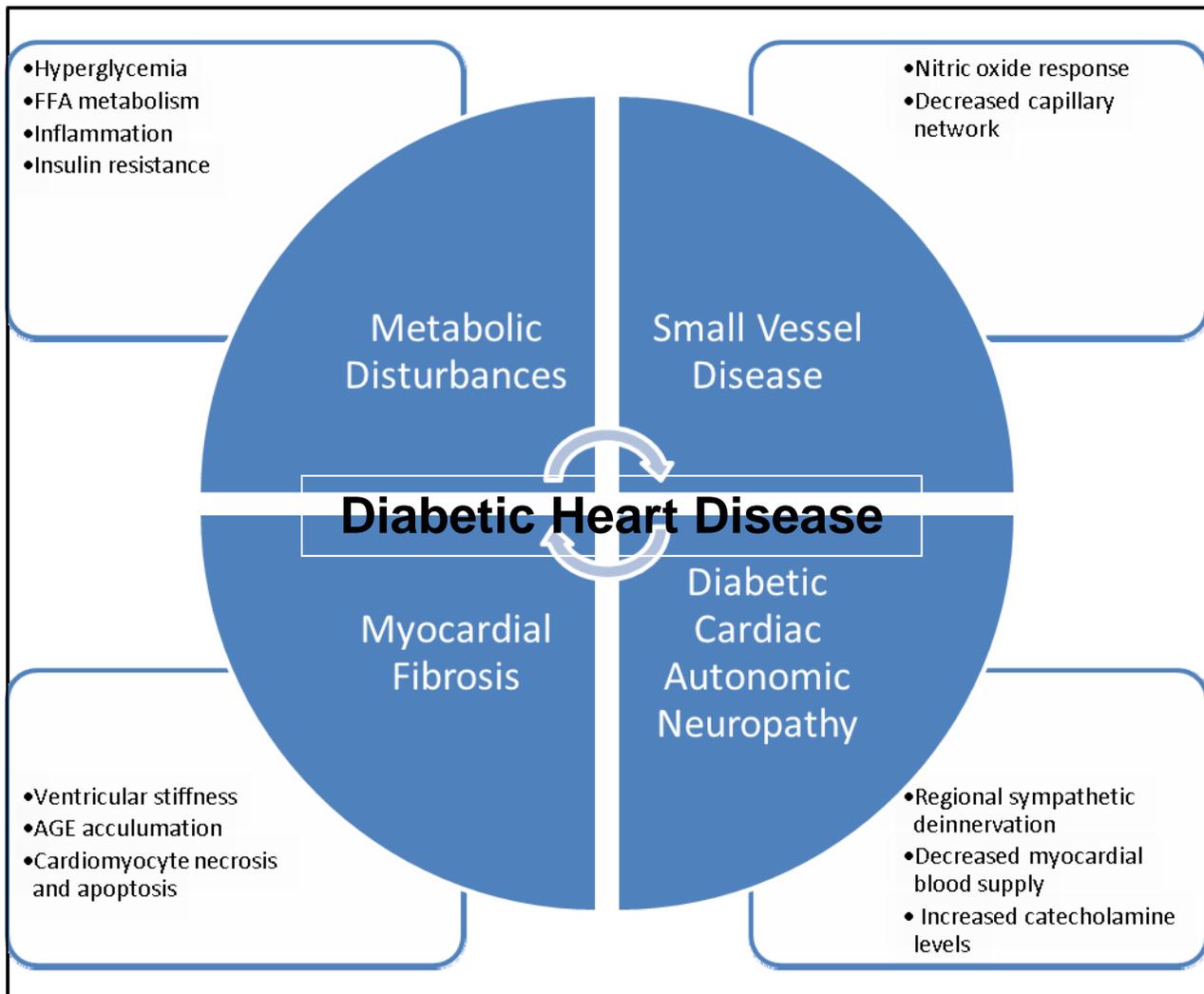


Figure 5 Mechanisms involved in diabetic heart disease. The figure outlines the four major contributors to the development and progression of diabetic heart disease and the reported mechanisms from the current literature.

1.5.1 Metabolic disturbances

A loss in glucose supply and utilization by the cardiomyocyte has been suggested as the initial injury in diabetic heart disease (Rodrigues, Cam, and McNeill, 1998). Under normal conditions, cardiac energy demands are met primarily with FFAs and secondarily with glucose metabolism (Taegtmeyer, 1994). Substrate utilization switches predominately to glucose under diabetic conditions, but a reduction in glucose transporters 1 and 4 has been observed in rat diabetic hearts (Garvey et al., 1993). Glucose transporters are necessary for the passage of glucose into the cardiomyocyte for energy. Overexpression of glucose transporter 4 restored cardiac function in db/db mice (Semeniuk, Kryski, and Severson, 2002). Utilization of glucose is required to protect the heart against the deleterious effects of hyperglycemia. The accumulation of FFAs, secondary to the metabolic shift, can inhibit glucose utilization leading to or exacerbating hyperglycemia (Eckel and Reinauer, 1990).

Fatty acids are carboxylic acids with long aliphatic tails and they are derived from triglycerides. When the fatty acids are unbound, they are called “free”. FFA levels are elevated systemically under diabetic conditions (Fang, Prins, and Marwick, 2004). A relationship has been reported between diabetic heart disease and carnitine deficiency in rats (Malone et al., 1999). Carnitine is a substance required for myocardial FFA metabolism. A decline in carnitine availability increases FFA levels. The level of circulating FFAs has a direct correlation with the severity of diabetic heart disease (Yazaki et al., 1999). The diminished FFA metabolism also has a direct effect on glucose utilization. The high circulating FFA levels in the diabetic heart reduces glucose oxidation secondary to decreases in glucose transporters (Eckel and Reinauer, 1990);

(Garvey et al., 1993) through activation of protein kinase C (Griffin et al., 1999). These imbalances in FFA metabolism promote hyperglycemia, morphological changes of the ventricle (Nakayama et al., 2001), and cellular insulin resistance (Boden et al., 2002; Jiao et al., 2011).

Insulin resistance can induce DAN and alterations in cardiac structure and function (Meyer et al., 2004). Insulin resistance is a metabolic dysfunction in which the body has a lowered response to insulin and raises insulin production to counter glucose levels. The low grade inflammation commonly associated with T2D and obesity, primarily through tumor necrosis factor-alpha (TNF- α) activity, plays a role in the development of insulin resistance (Sun et al., 2011; Moon et al., 2010). TNF- α suppressed insulin signaling in rodent models of diabetes (Hotamisligil, Shargill, and Spiegelman, 1993). Other inflammatory cytokines, such as interleukin (IL)-6, activated pathways in cardiomyocytes that induced insulin resistance through oxidative stress and inflammation (Gwechenberger et al., 1999). The literature, however, is inconclusive on the independent influence of insulin resistance on functional and structural changes in diabetic heart disease (Poornima, Parikh, and Shannon, 2006; de Kreutzenberg et al., 2000; Galvan et al., 2000; Rutter et al., 2003).

1.5.2 Myocardial fibrosis

The replacement of cardiomyocytes by fibrotic tissue is an initial element of diabetic heart disease (Mizushige et al., 2000), resulting from the interstitial accumulation of glycoproteins, collagen, and hyaluronan. Hyperglycemic conditions can increase intracellular and extracellular advanced glycation end (AGE) products (Degenhardt, Thorpe, and Baynes, 1998). As early as one week of exposure to high

glucose, intracellular AGE levels are increased and ventricular remodeling begins (Brownlee, 2001). Collagen levels are upregulated and their function is compromised due to AGE formation on various collagens altering extracellular matrix interactions and composition (Brownlee, 2001). Cardiac AGE accumulation is related to increases of collagen I (Tanaka et al., 1988). Increased collagen I levels have been suggested to lead to ventricular dysfunction in rats (Wei 1999, 117). Collagen I has different physical characteristics than collagen III. The impact of diabetes on collagen I/III ratios in animal and human eye and cardiac samples is currently under debate (Kern, Sebert, and Robert, 1986; Kita et al., 1996; Shimizu et al., 1993). AGE crosslinking of these collagens decreases the compliance of the ventricle, resulting in declines in cardiac function and permanent changes in the ventricle under prolonged hyperglycemic conditions (Willemsen et al., 2011).

Fibrotic alterations can occur in the interstitium (spaces between the cells) or around the blood vessels (perivascular). LV mass has been shown to increase significantly as a result of fibrosis and increased diameters of ventricular cardiomyocytes (Wang et al., 2006). End systolic LV wall diameters were increased with echocardiographic evaluation of STZ diabetic rats, likely due to increases in interstitial and perivascular fibrosis (Castoldi et al., 2010). Hyperglycemia and increased oxidative stress can also contribute to LV hypertrophy and loss of cardiac function through cardiomyocyte necrosis and fibrosis (Chen et al., 2000).

Hyperglycemia can induce cell death through necrotic and apoptotic pathways. Apoptosis is controlled programmed cell death (Toldo et al., 2011). Necrosis is cell death, which is spontaneous in nature and related to increases in oxidative stress

(Frustaci et al., 2000). Both processes occur in the diabetic heart, but necrosis transpires more often under diabetic conditions (Frustaci et al., 2000) compared to normal cellular environments. Cardiomyocyte cell death, through either necrosis or apoptosis, makes way for increased interstitial collagen accumulation (Li et al., 1997, Anversa et al., 1998) contributing to fibrosis. An examination of the left ventricles of ZDF animals revealed increased collagen deposits consisting of collagen I and III (Huang et al., 2005). These morphological changes result in hypertrophy and loss of cardiac function under diabetic conditions.

1.5.3 Small vessel disease

Debate has existed regarding whether myocardial fibrosis and small vessel disease are independent factors of diabetic heart disease (Poornima, Parikh, and Shannon, 2006). Physical changes to the microvascular wall can impair the response to nitric oxide (Mayhan, Simmons, and Sharpe, 1991). Nitric oxide controls the vasodilatation of the vessels, which provides the nutritional support for the myocardium and its vessels. The losses in endothelial function are coupled with structural changes in the vascular bed as seen in investigations using the STZ diabetic rat model (Messaoudi et al., 2009). Histological examinations showed a reduction in surface density and total surface area of blood capillaries in the left ventricles of diabetic rats (Warley, Powell, and Skepper, 1995). Declines in the number of capillaries and losses in function reduce the vascular supply to the myocardium, increase necrosis, and affect neuronal input to the myocardium (Yarom, 1994).

1.5.4 Diabetic cardiac autonomic neuropathy

Sympathetic denervation is a common characteristic of diabetic heart disease (Nagamachi et al., 1996; Kahn et al., 1986). Regional sympathetic damage can result in conduction irregularities and increased mortality risk (Valensi et al., 2002), partially due to the impact of DAN on cardiac function. Studies have indicated that DAN limited the cardiovascular compensatory responses in diabetes to external stress (Scognamiglio et al., 1995; Erbas et al., 1992). The loss of neural response also has a direct relationship to declines in myocardial blood flow (Stevens et al., 1998a) causing myocardial ischemia that can result in ventricular remodeling, which is triggered by cell death and fibrosis (Backlund et al., 2004). Myocardial catecholamine levels increased during the early months of diabetes in subjects with T2D (Kondo et al., 2002) and can induce prolonged overstimulation of the heart resulting in alterations in ventricular geometry (Samuels, 2007). The upregulation of β -adrenergic receptors, in response to myocardial catecholamines, can cause increases in fibrosis, cardiomyocyte hypertrophy, and loss of cardiac function as shown in transgenic mouse studies (Bisognano et al., 2000).

1.6 Interventions for Diabetic Heart Disease

The complexity and coexistence of the above mentioned mechanisms has required the investigation of singular and combined therapeutic approaches to address the impairments associated with diabetic heart disease. Interventions have focused on delaying the onset and progression of the disease. Glycemic control has altered many of the mechanisms responsible for cardiac dysfunction (Hayat et al., 2004). Intensive glucose management reduced all diabetic complications by 25% in humans (UKPDS, 1998). Every 1% reduction in hemoglobin A1C (HbA1C) has been shown to equate to

significant declines in mortality, myocardial infarction risk, and microvascular complications (Stratton et al., 2000).

Clinical research focused on pharmaceuticals and diet modification has proven successful in normalizing glucose levels (Sprafka et al., 1992; Davis and Edelman, 2004; Umpierre et al., 2011). Insulin has been shown to reverse hemodynamic abnormalities and structural changes in cardiomyocytes (Fang, Prins, and Marwick, 2004). Other medications have been identified to address biochemical and morphological cardiac alterations related to diabetes (Lowes et al., 1999; Afzal et al., 1988; Cohn and Tognoni, 2001; Hanif, Bid, and Konwar, 2010; Horwich, MacLellan, and Fonarow, 2004; Minchenko et al., 2003; Nesto et al., 2004; Packer et al., 1996). Numerous factors, including cultural beliefs and finances, may affect adherence to diet and pharmaceutical recommendations for long term diabetic care (Schechtman, Nadkarni, and Voss, 2002; McQuaid et al., 2009). However, aerobic exercise can provide a low-cost, non-invasive, and culturally neutral treatment for diabetic heart disease.

1.7 Cardioprotective Effects of Aerobic Exercise for Diabetic Heart Disease

The American Heart Association has encouraged regular physical activity to combat diabetes for decades (Marwick et al., 2009). The current recommendations from a collaborative taskforce with the American College of Sports Medicine suggest 150 minutes per week (Haskell et al., 2007). Medical clearance is necessary, because exercise can unmask early, subclinical signs of DAN and other symptoms of diabetic heart disease (Jellis et al., 2011; Palmieri et al., 2008). However the benefits of exercise

outweigh the risk. **Exercise has a direct impact on the factors related to the development and progression of diabetic heart disease.**

1.7.1 Exercise training can normalize metabolic disturbances

Exercise can improve HbA1C (Umpierre et al., 2011) and blood glucose levels (Nyenwe et al., 2011), recover insulin sensitivity (Cederberg et al., 2011), modulate inflammation (Colbert et al., 2004; Batista et al., 2009), and improve weight management with appropriate dosing (Greaves et al., 2011). Low intensity aerobic activity of less than 150 minutes per week has failed to attenuate hyperglycemia (Wing et al., 1988; Khan and Rupp, 1995), but moderate level aerobic exercise for 135 to 270 minutes per week has been shown most effective in lowering HbA1C in adults with T2D (Sigal et al., 2007; Snowling and Hopkins, 2006). Resistance exercise has been reported as exhibiting greater control of blood glucose than aerobic exercise for humans (Bweir et al., 2009). However, a recent publication suggested that exercise of any mode must be combined with dietary advice to maximally impact HbA1C levels in humans (Umpierre et al., 2011). Thirteen weeks of swimming improved glucoregulation through increased beta cell size and metabolism in ZDF rats (Kiraly et al., 2008). The impact of exercise on glucose homeostasis is also attributed to its effect on glucose transporters. Glucose transporters are necessary for the passage of glucose into the cardiomyocyte for energy. Increased activity of the glucose transporters has been reported with muscle contractions and accounts for the improved utilization and clearance of glucose in diabetic rats (Ploug, Galbo, and Richter, 1984; Lehnen et al., 2010). FFAs, which inhibit glucose metabolism, were decreased with treadmill training in ZDF rats (Colombo et al., 2005). Insulin sensitivity also improved with regular exercise in humans (Borghouts et

al., 1999). Glycemic control through exercise has an ancillary advantage of depressing oxidative stress and inflammation associated with diabetic heart disease.

The delicate balance between pro- and anti-inflammatory cytokines can be maintained through exercise (Martin-Cordero et al., 2011; Cesar et al., 2011). Controversy about the rise in inflammatory cytokines exists in the literature regarding acute exercise (Ostrowski et al., 1998), because the amount of exercise can regulate the expression of inflammatory cytokines. Pro-inflammatory cytokines such as interferon gamma (IFN- γ), tumor necrosis factor (TNF)- α , IL-6, IL-4 and IL-1 β are increased in plasma and serum under diabetic and/or sedentary conditions (Jankord and Jemiolo, 2004; Pischon et al., 2003; Colbert et al., 2004; Smith et al., 1999). Exercise training has been beneficial in restoring the equilibrium between pro- and anti-inflammatory regulation. Fourteen weeks of swimming reduced circulating TNF- α and IL-6 levels in ZDF animals (Teixeira de Lemos et al., 2009). Research has suggested that IL-10 and IL-1 α counter the chronic inflammation observed with diabetes. The cardioprotective effects of exercise may be mediated primarily by IL-10 (Batista et al., 2009).

Obesity is an independent risk factor for heart disease, but obesity and T2D typically coexist (Yeung et al., 2011). The increased fat mass can have negative influences on oxidative stress, glucose metabolism, and inflammation (Ong et al., 2011; Goossens et al., 2011). Regular exercise is a proven intervention for weight management (Wong et al., 2011) and obesity related complications, including diabetes (Balducci et al., 2006; Bidasee et al., 2008; Bweir et al., 2009). A moderate amount of exercise can lessen one's risk of developing T2D and control the progression of the disease (Church, 2011; Psaltopoulou, Ilias, and Alevizaki, 2010). The Look Ahead trial

reported improvements in glycemic control and an 8.8% weight loss with an average of 175 minutes per week of aerobic exercise over 1 year.(Belalcazar et al., 2010; Solomon et al., 2010). Even exercise without weight loss has proven beneficial because the increase in muscle mass and decrease in fat mass can be cardioprotective (Lee et al., 2005). Exercise has a positive impact on weight management and other metabolic disturbances related to diabetes.

1.7.2 Exercise training can reduce myocardial fibrosis

Exercise can reduce the accumulation of collagen and fibrotic appearance of the myocardial interstitium. In an alloxan model of T1D in Wistar rats, 8 weeks of swimming one hour per day decreased the deposition of collagen I and III (Castellar et al., 2011). Nine weeks of treadmill training reduced the cross-sectional surface area of collagen fibers in a STZ animal study (Searls et al., 2004). However, habitual exercise of STZ rats had no impact on myocardial collagen fluorescence, an indication of the amount of collagen in the tissues, but did attenuate myocardial stiffness (Woodiwiss, Kalk, and Norton, 1996). These investigations indicate that exercise can reduce collagen deposition, myocardial fibrosis, and related cardiac dysfunction.

1.7.3 Exercise can impact small vessel disease

Exercise can improve vascular function for those with diabetes. Increased exercise tolerance decreased myocardial infarction risk by 48% for person with diabetes (Pierre-Louis et al., 2010). The decline in ischemia may be due to improvements in the regulation of nitric oxide. Moderate levels of exercise in db/db mice enhanced nitric oxide bioavailability and vascular function (Moien-Afshari et al., 2008). Similar advances in vascular function have been observed in humans after 6 weeks of multi-modal

exercise training. Arterial compliance improved in both small and large arteries, and the authors speculated it was secondary to nitric oxide production (Mouroto et al., 2009). A cross sectional study of persons with T1D revealed that increased physical activity levels have a positive correlation with small artery compliance (Mason et al., 2006). Exercise training can improve vascular function in persons with diabetes.

1.7.4 Exercise training can reverse diabetic cardiac autonomic neuropathy

Although exercise is commonly recommended as an intervention for diabetic complications, studies regarding the effect of exercise on DAN are limited due to concerns about abnormal responses and safety. Much of the literature has focused on the use of exercise as a screening tool for DAN (Bottini et al., 1995; Endo et al., 2000). However, a study evaluating 6 months of aerobic exercise training showed an improvement of RR intervals (heart rate) and HRV in T2D subjects (Pagkalos et al., 2008). The benefits of exercise are duration dependent. Longer durations of training, such as 12 months of cycling and treadmill tasks, reversed autonomic dysregulation with improvements in HRV of T2D subjects (Sridhar et al., 2010). Although reports are minimal in number, early investigations have suggested that exercise has the potential to reverse or attenuate DAN.

1.8 Myocardial Edema as a Possible Target of Exercise

The benefits of exercise are attributed to changes in the circulatory or endocrine systems, such as normalization of blood glucose and insulin levels and improved circulation. Also, exercise can address obesity and the chronic inflammation commonly observed in persons with T2D. However, **researchers must thoroughly identify and stratify all possible mechanism(s) of action** for the treatment of diabetic heart

disease. **One potential mechanism that has been completely overlooked is myocardial edema.** Edema formation in the heart is a delicate balance between cardiac function, endothelial permeability, and the cardiac lymphatic system.

1.8.1 Anatomy and physiology of the lymphatic system

The body's lymphatic system is a network of organs and vessels that parallel the venous system. Lymphatic organs include the thymus, bone marrow, spleen, lymph nodes, tonsils, and lymphoid follicles (von der Weid and Rainey, 2010). The conducting portion of the lymphatic system consists of capillaries, collectors, and large ducts that drain tissues regionally, ultimately leading to the thoracic or right lymphatic ducts. This network is responsible for transporting waste and immune cells along with removing interstitial fluid and macromolecules. As plasma escapes the vascular capillary into the interstitium, approximately 90% of it is reabsorbed by the vascular capillary and the remaining 10% is collected by the lymphatic capillary (Greitz, 2002). When the residual interstitial fluid enters the lymphatic capillary, it becomes lymph, which is a clear fluid, except in the digestive system where the addition of fats gives lymph a milky, white appearance.

Lymphatic capillaries are open-ended, irregular, shaped vessels with a single layer of endothelium, anchoring filaments, and an incomplete basement membrane as shown in Figure 6. Anchoring filaments allow the gap junctions in the lymphatic capillaries to remain open under pressure for fluid and macromolecule removal (Witte et al., 2001). The lymphatic vessels are larger than vascular capillaries to allow for macromolecule filtration. The lymphatic capillaries join with larger vessels, the lymphatic collectors, which have smooth muscle and adventitia. Lymphatic pre-collectors appear

between superficial and deep layers of tissues and provide perpendicular access. As the lymphatic vessels become larger and form lymphatic ducts, an increase in the layers of smooth muscle and adventitia is observed.

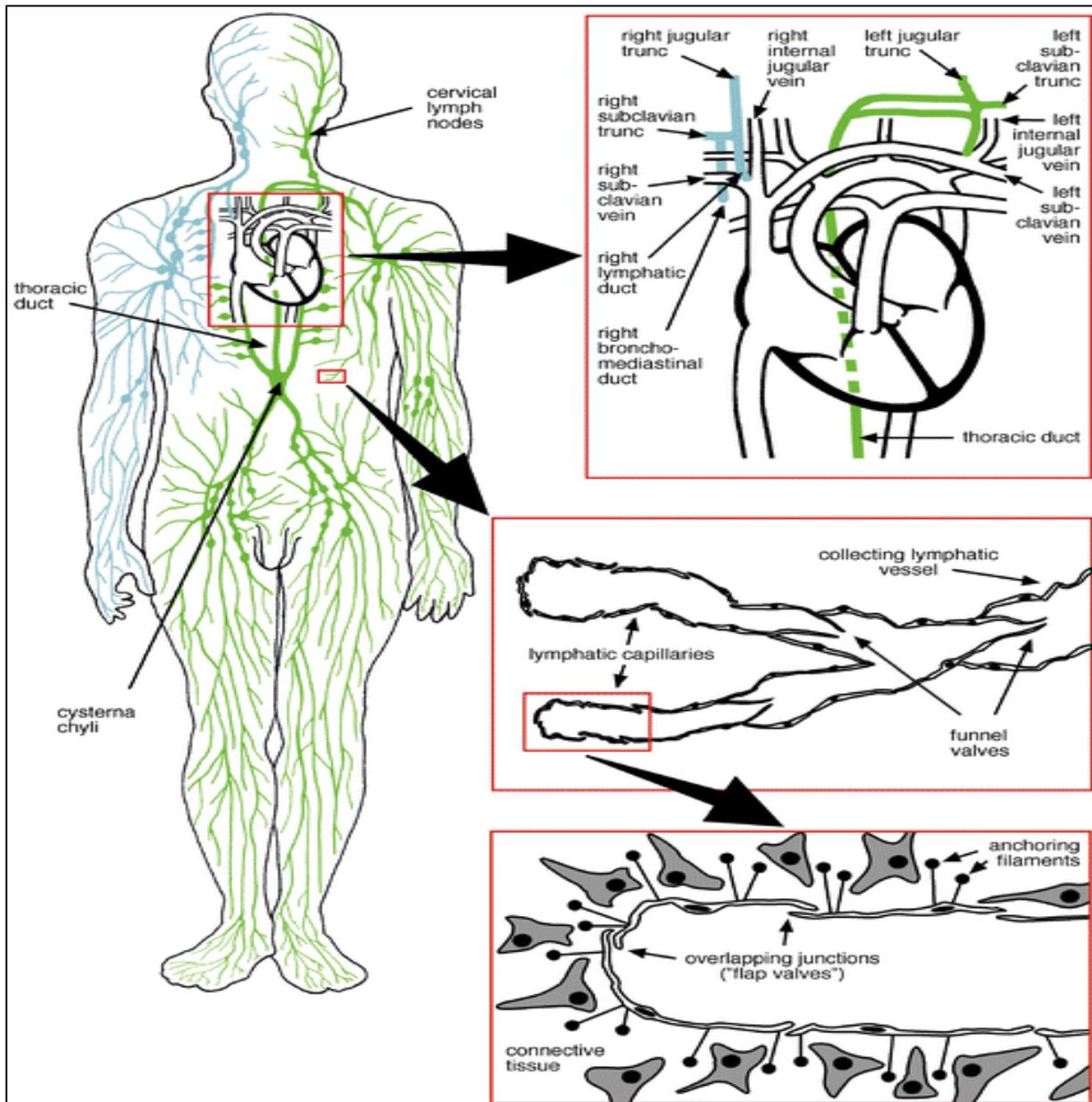


Figure 6 Lymphatic capillaries and lymph drainage (Jeltsch et al., 2003)

The lymphatic network is divided into sections drained by regional lymph nodes for filtering. Efferent lymphatic vessels transport the lymph to the node regions, depending on anatomical locations, through lymphatic collectors and/or ducts (Jussila and Alitalo, 2002). Within the lymphatic collectors and ducts are valves with each section identified as lymphangions (Mislin, 1971), shown in Figure 7.

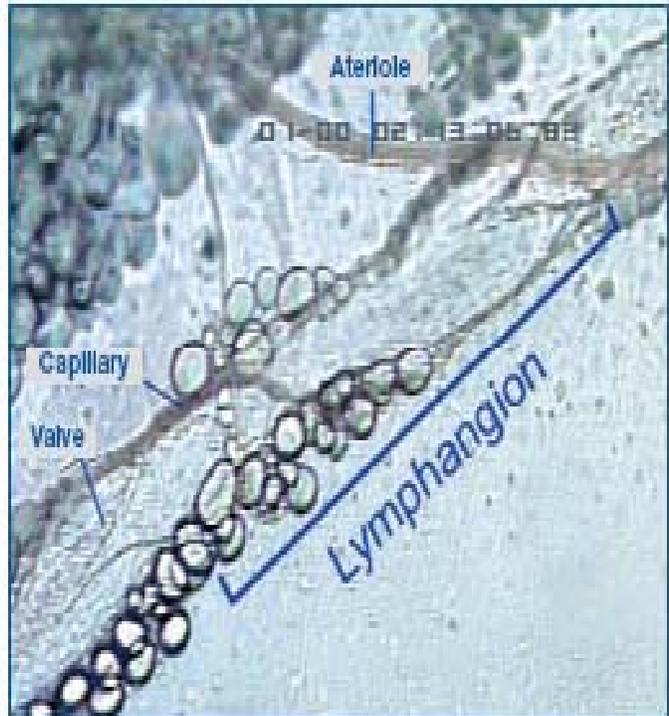


Figure 7 The lymphangion (Stucker, 2009)

Lymphangions are the “hearts” of the vessels, because the lymphatic system is not directly connected to the heart, but can respond to muscle contractions and arterial pulsations. Alpha-adrenergic sympathetic nerves mediate the lymphangion response. Regional vessel differences present in the response to interstitial pressure and flow demands. Flow mediated inhibition has been noted in the thoracic duct under chronic high lymph conditions including infections, inflammation, and diabetes (Miller, Ellis, and Hirsch, 1964; von der Weid and Muthuchamy, 2010). The thoracic and right lymphatic ducts empty into the subclavian veins; thereby making the lymphatic system an open system as observed in Figure 6. Obstructions, inflammation, and elevated venous pressure result in edema due to disruptions in lymphatic flow (Kline, Miller, and Katz, 1963; von der Weid and Muthuchamy, 2010). The lymphatic system responds to

inflammation with dilation of vessels, expansion of lymph nodes, and compensatory lymphangiogenesis (Flister et al., 2010).

1.8.2 The anatomy and physiology of the cardiac lymphatic system

The cardiac lymphatic system imitates the network seen in the extremities and abdominal areas; however its system is more extensive due to the importance of proper cardiac function and flow. Early investigations of the cardiac lymphatic system indicated a possible connection with conduction due to its close proximity with the conduction apparatus (Miller, 1963). Cardiac lymphatic vessels drain the SA and atrioventricular (AV) nodes and the bundle of HIS. However, **researchers have agreed that the primary role of the cardiac lymphatic system is fluid balance** (Konuralp, Idiz, and Unal, 2001; Shimada et al., 1989). Unlike the vessels in the extremities, the majority of lymphatic vessels in the heart lack lymphangions and drainage occurs from the endocardium to the epicardium through the contractions (Shimada et al., 1989).

At rest, the heart has a myocardial interstitial fluid pressure of approximately 15 mmHg. With each contraction, myocardial pressures increase to approximately 120 mmHg (Laine and Granger, 1985). The large pressure gradient allows each contraction to wring fluid from the base of the heart toward the apex. The endocardium has primarily lymphatic capillaries (Johnson and Blake, 1966). These vessels empty into the lymphatic pre-collectors located in the myocardium. The self-squeezing motion of the heart propels fluid to the surface of the heart, where lymphatic vessels are most abundant. Once lymph moves to the epicardium, it is transported in lymphatic vessels feeding into progressively larger vessels (Johnson and Blake, 1966). As shown in Figure 8, these larger vessels drain into posterior and anterior interventricular trunks

(PVT and AVT) and then into the left and right coronary channels (LCC and RCC). The coronary channels travel with the vascular system, the coronary veins. The lymphatic collectors merge into the primary cardiac lymphatic vessel, main supracardiac channel (MSC), which takes fluid to the pretracheal and/or cardiac nodes (CLN) located above the aortic arch. Nodes empty into the right lymphatic duct (RLD) draining into the venous angle formed by the subclavian vein (Sc V) and the internal jugular vein (IJV) (Miller, 1963; Nakamura and Rockson, 2008).

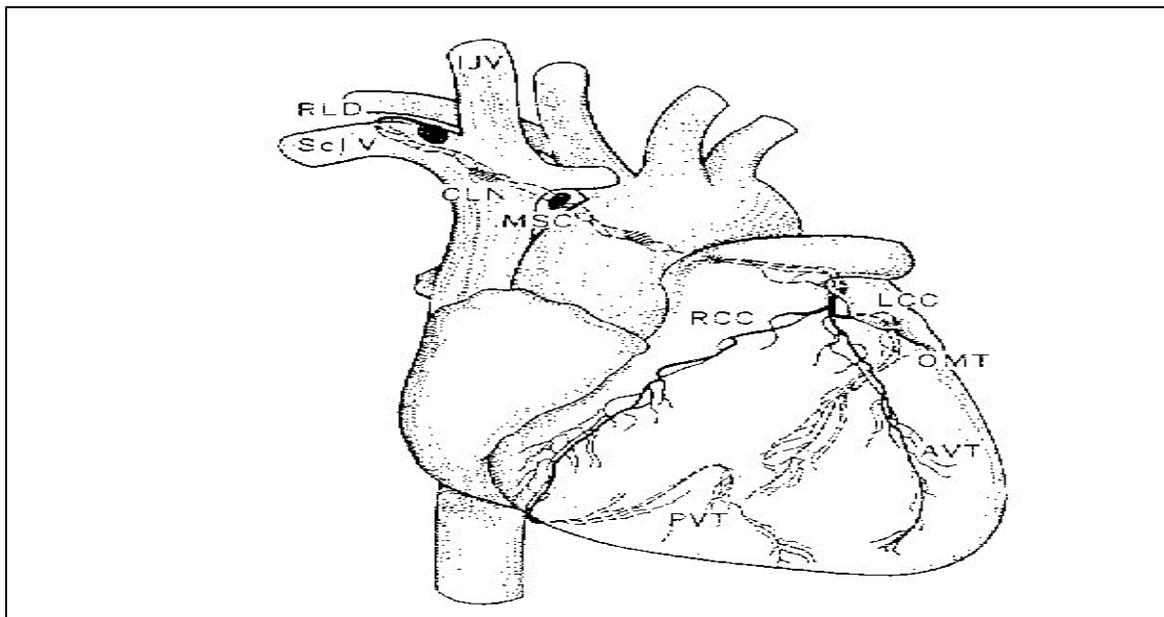


Figure 8 The epicardial lymphatic system (Miller, 1981). OMT is an obtuse marginal trunk, an accessory lymphatic vessel. Posterior and anterior interventricular trunks (PVT and AVT), left and right coronary channels (LCC and RCC), main supracardiac channel (MSC), cardiac lymph nodes (CLN), right lymphatic duct (RLD), subclavian vein (ScV) and the internal jugular vein (IJV) are outlined in the figure.

1.8.3 Pathophysiology of myocardial edema

Myocardial edema is an imbalance between vascular hyperpermeability, the quantity and function of lymphatic vessels, lymph flow, and cardiac function (Dongaonkar et al., 2010). Endothelial hyperpermeability of the vascular or lymphatic vessels (Huxley and Scallan, 2011) can increase the fluid and protein load in the

cardiac interstitium resulting in a significant protein gradient between interstitial and lymph fluid. Under normal conditions, the body would respond with a “washdown” to decrease interstitial protein concentrations. The washdown is an increase in fluid filtration into the interstitium causing enhanced protein uptake into the lymphatic vessels (Stewart et al., 2000). This protective mechanism can only occur when the myocardium is relaxed, as in diastole, so fluid can flow across the myocardium. Under diseased conditions, the myocardial lymphatic vessels cannot adequately absorb interstitial fluid. This inefficiency may result from decreased vessel density, hypertension, or diastolic dysfunction (Stewart et al., 2000). Edema is further exacerbated when the heart cannot adequately pump fluid toward the venous angle. As myocardial edema increases, myocardial pressures elevate and chronic dysfunction can result in concentric remodeling of the ventricle (Stewart et al., 1997).

1.8.4 Myocardial edema and heart disease

Although cardiac dysfunction can induce myocardial edema (Stewart et al., 2000), **myocardial edema can independently diminish diastolic and systolic function** (Laine and Allen, 1991). Without proper flow, interstitial fluid can accumulate and decrease left ventricular compliance. A cycle of edema formation begins with the attraction of macrophages, free radicals, and cytokines. Chronic edema can promote interstitial fibrosis and permanent losses in ventricular compliance (Rubboli, Sobotka, and Euler, 1994; Kong, Kong, and Wang, 2006). Such remodeling results in decreased ventricular filling as seen with common LV diseases, diabetes and heart failure. Diastolic dysfunction can limit lymphangion contractions and the propulsion of lymph (Nakamura and Rockson, 2008), which can result in additional edema formation.

Changes in myocardial water content greater than 2% are associated with systolic dysfunction (Fischer et al., 2006). Edema resolution is dependent on systolic function because strong contractions are needed for lymph flow. The rate and force of contractions maintain fluid balance in the heart (Dongaonkar et al., 2010). Inefficient contractions cause lymphostasis (stagnation of lymph) and edema (Barsotti et al., 1993). The edematous myocardium presents with decreased contractility and subsequent reductions in cardiac output. Although diastolic and systolic dysfunctions are common characteristics of diabetic heart disease, **the identification of myocardial edema under the backdrop of diabetes is lacking from the current literature.**

1.8.5 Possible relationship between myocardial edema and diabetic heart disease

Myocardial edema can be caused by underlying, fundamental, and precipitating factors related to diabetes that span different biological levels. These three categories of factors are commonly used to describe the causes of heart failure (Dumitru, 2011) and provide a structure to discuss the possible relationship between myocardial edema and diabetic heart disease. Underlying factors are structural abnormalities in the vascular or lymphatic vessel that promote fluid imbalance. Biochemical or physiological changes in the myocardial interstitium or lymphatic system are the fundamental factors involved in myocardial edema formation. Precipitating factors are external factors, such as obesity and inflammation that could impede lymph flow and clearance. By investigating the three categories of factors, **we chose to identify if diabetes creates an edematous state in the heart, which could account for the related ECG and hemodynamic changes** observed in the diabetic heart disease of the ZDF rat. The results are described in Chapter 4.

1.8.5.1 Underlying factors

Structural changes that can promote myocardial edema include endothelial permeability and vessel density. Vascular endothelial growth factor C (VEGF-C) is a disulfide-linked prepropeptide dimer that regulates endothelial permeability as a full length protein, but following proteolytic cleavage the ligand binds with VEGFR-3, its receptor, for lymphangiogenic signaling (Partanen and Paavonen, 2001). Diabetes has been shown to increase vascular permeability (Kivela et al., 2007) and decrease reabsorption through the vascular capillaries (Kumar et al., 2009). However, comparison of VEGF-C mRNA expression levels indicated no differences between diabetic and control skeletal muscle samples. (Kivela et al., 2007). VEGFR-3 is the receptor for VEGF-C. At embryonic day 10, VEGFR-3 activity switches from angiogenesis to lymphangiogenesis development and maintenance in normal mice. VEGFR-3 expression is restricted to lymphatic vessels in the mature mouse and protein levels decrease with age (Laakkonen et al., 2007). However, the receptor can be reactivated with upregulation of its ligand, VEGF-C (Partanen and Paavonen, 2001) or as a response to inflammation. The mature VEGF-C binds to VEGFR-3 to promote lymphangiogenesis. In human skeletal muscle, VEGFR-3 mRNA levels mimicked VEGF-C levels (Kivela et al., 2007). No change in the ligand or receptor levels was observed under diabetic conditions. An examination of lymphatic vessels in skeletal muscle revealed no differences in the density between control and diabetic subjects (Kivela et al., 2007). The lack of change may have resulted from an insufficient diabetic lymphatic load, which did not trigger a response, or from a possible loss of lymphangiogenesis in the diabetic skeletal muscle. Although unknown for cardiac

tissue, the quantity of lymphatic vessels can be decreased under diabetic conditions as reported in evaluations of corneal wound healing in mice (Maruyama et al., 2007). The cornea is normally void of lymphatic vessels, which develop during wound healing under non-diabetic conditions. Lymphangiogenesis follows angiogenesis during wound healing (Cursiefen et al., 2006). The decline in lymphatic vessels in diabetic mice may be evidence of an impaired inflammatory response. Diabetes, causing vascular hyperpermeability, could increase the risk for myocardial edema. However, **the evidence regarding the density of both mature and new lymphatic vessels is lacking.**

1.8.5.2 Fundamental factors

Diabetes related alterations in the physiology and biochemistry of the lymphatic system have been discussed in the literature. Vascular permeability has been shown to increase systemically due to hyperglycemia in a T1D animal model (Moriguchi et al., 2005). The study also reported that lymph node activity was decreased in the diabetic rats, but the node uptake and lymph flow was improved with glycemic control (Moriguchi et al., 2005). In a study of 163 patients with T2D, lymphatic dysfunction or the decreased clearance of albumin was present in 72% of patients and the dysfunction was present before increased capillary permeability (Valensi et al., 1997). **It is unknown whether lymphatic dysfunction occurs in the cardiac lymphatic system due to changes in the lymphangiogenic signaling pathway under diabetic conditions, which would maintain the structure and function of the system.**

PROX-1 is a homologue of the *Drosophila* homeobox gene prospero and its product, a transcription factor, has been reported as the master regulator of

lymphangiogenesis and development of the lymphatic system (Al-Rawi, Mansel, and Jiang, 2005). This transcription factor can regulate the expression of lymphatic vessel endothelial receptor 1 (LYVE-1, see below in Section 1.8.5.3) and VEGFR-3 through PROX-1 nuclear translocation (Flister, Volk, and Ran, 2011) or possibly through NOTCH signaling (Shawber et al., 2007). The influence of diabetes on PROX-1 expression and function has yet to be investigated. However, inflammation has been reported to have a direct effect on PROX-1, by limiting its nuclear translocation (Oka et al., 2008). PROX-1 activity is needed for lymphatic vessel development and stability (Wigle et al., 2002). The loss of PROX-1 control can result in declines of the LYVE-1 and VEGFR-3 protein levels, which have direct implications for the structure and function of the lymphatic system (Wigle and Oliver, 1999).

1.8.5.3 Precipitating factors

External factors associated with diabetes also increase the risk of myocardial edema. Obesity, inflammation, extracellular matrix quality, and cardiac functional changes interplay with structural and fundamental factors. Circulating AGE levels were elevated in obese db/db mice, contributing to endothelial dysfunction (Gao et al., 2008). The binding of AGE to the endothelium increased the microvascular hyperpermeability of endothelial cells in diabetic rats (Bonnardel-Phu et al., 1999). Endothelial function is further impaired by inflammation induced by hyaluronan fragments (Nanji et al., 1996). Hyaluronan is a primary component of the extracellular matrix. This glycosaminoglycan is removed from the interstitium by LYVE-1 and it is systematically elevated with diabetes (Lewis et al., 2008). Hyaluronidases are responsible for degrading hyaluronan into fragments with various molecular weights. Each fragment has a different function,

but most are pro-inflammatory and induce oxidative stress and/or endothelial dysfunction (Day and de la Motte, 2005). The fragments are removed by the lymphatic vessels and transported to the lymph nodes for degradation (Smedsrod, 1991). While the relationship between hyaluronan and diabetes has been investigated, studies regarding the role of LYVE-1 in diabetic heart disease have yet to be described in the literature.

Finally, weak ventricular contractions limit the efficiency of edema resolution through the lymphatic system. Chronic edema is further exacerbated by diastolic and systolic dysfunction commonly seen with diabetes (Nakamura and Rockson, 2008). Currently, pharmaceutical treatments are limited to address risk factors for myocardial edema, but exercise appears promising in addressing myocardial edema and lymphatic dysfunction.

1.8.6 Exercise and the lymphatic system

Dogma has supported the influence of exercise on circulation, but knowledge regarding its impact on vascular permeability and lymph transport is evolving. Exercise has been reported to increase microvascular filtration in normal populations (Charles et al., 2006). Exercise appears to also have a positive relationship to lymph flow (Knott et al., 2005). The lymphatic system responds to various modes and dosages of exercise. Upper extremity exercise increased lymphatic clearance in a dose dependent response in healthy humans (Lane et al., 2006). In canine models, increases in treadmill speeds resulted in significant rises in lymph flow rates (Downey et al., 2008; Knott et al., 2005) up to 419% higher than resting flow (Desai et al., 2010). Seated cycle ergometer exercise decreased transit time of lymph from the feet to the groin compared to

stationary standing in humans (Unno et al., 2008). A study of hyaluronan clearance, which is removed from the interstitium by the lymphatic system, showed elevated clearance with submaximal and moderate intensity bicycle ergometrical tasks (Hinghofer-Szalkay et al., 2002). Each of these studies suggested that exercise does improve lymph flow and nodal clearance. Biomechanical experiments revealed that when a muscle can reach its shortest length and maximum force production then lymph propulsion is most efficient. The lymphatic vessels dilate to accommodate the increased lymphatic flow induced by exercise (Kivela et al., 2007). Therefore, aerobic exercise may provide a non-invasive, simple intervention for increasing cardiac lymph flow and clearance, which could enhance cardiac function. **Knowledge about the influence of exercise on molecular and structural components of the cardiac lymphatic system in general and in diabetes specifically has yet to be obtained and disseminated in the literature.**

1.9 Significance of the Proposed Work

Aerobic exercise has been shown to delay or attenuate cardiac dysfunction in other animal models of T2D. An understanding of the exercise response of the ZDF rat is limited. Intervention discussions favor glucoregulation as the primary benefit of exercise. However, exercise has an effect on several mechanisms and the cardioprotective benefits of exercise are the result of its ability to address the multi-faceted nature of diabetic heart disease. We chose to investigate the impact of exercise on diabetic heart disease in the ZDF rat, and explore a novel mechanism. **The objectives of the dissertation were to investigate the cardiac dysfunction in the ZDF model, report if aerobic exercise training can reverse the ECG and**

hemodynamic changes induced by diabetes, and identify whether cardiac edema may be one of the contributing factors of diabetic heart disease. We hypothesized that ZDF rats develop cardiac dysfunction; aerobic exercise would attenuate the cardiac dysfunction; and myocardial edema would be present in this model as a possible contributor to diabetic heart disease. The subsequent chapters will report the findings of the following specific aims:

Aim 1: To identify electrocardiographic changes induced by diabetes and determine the impact of exercise on these changes.

Aim 2: To identify hemodynamic changes in the left ventricle induced by diabetes and determine the impact of exercise on these changes.

Aim 3: To identify clinical and subclinical signs of myocardial edema in left ventricular tissue induced by diabetes.

Chapter 2

Electrocardiographic changes with the onset of diabetes and the impact of aerobic exercise training in the Zucker Diabetic Fatty (ZDF) rat

2.1 Abstract

Background: Early markers of diabetic autonomic neuropathy (DAN) in an electrocardiogram (ECG) include elevated R wave amplitudes, widening of QT_c intervals and decreased heart rate variability (HRV). The severity of DAN has a direct relationship with mortality risk. Aerobic exercise training is a common recommendation for the delay and possible reversal of cardiac dysfunction. Limited research exists on ECG measurements for the evaluation of aerobic exercise training in the Zucker Diabetic Fatty (ZDF) rat, a model of type 2 diabetes. The objective of this study was to assess whether aerobic exercise training may attenuate diabetes induced ECG changes.

Methods: Male ZDF (obese fa/fa) and control Zucker (lean fa/+) rats were assigned to 4 groups: sedentary control (SC), sedentary diabetic (SD), exercised control (EC) and exercised diabetic (ED). The exercised groups began 7 weeks of treadmill training after the development of diabetes in the ED group. Baseline (prior to the training) and termination measurements included body weight, heart weight, blood glucose and glycated hemoglobin levels, and ECG parameters. One way repeated measures ANOVA (group) was used to analyze within and between subject differences and interactions. Pearson coefficients and descriptive statistics described variable relationships and animal characteristics.

Results and conclusion: Diabetes caused crucial changes in R wave amplitudes ($p < 0.001$), HRV ($p < 0.01$), QT intervals ($p < 0.001$) and QT_c intervals ($p < 0.001$). R wave amplitude augmentation in SD rats from baseline to termination was ameliorated by exercise, resulting in R wave amplitude changes in ED animals similar to control rats.

Aerobic exercise training neither attenuated QT or QT_c interval prolongation nor restored decreases in HRV in diabetic rats. This study revealed alterations in R wave amplitudes, HRV, QT and QT_c intervals in ZDF rats. Of these changes, aerobic exercise training was able to correct R wave amplitude changes. In addition, exercise has beneficial effect in this diabetic rat model in regards to ECG correlates of left ventricular mass.

2.2 Introduction

Cardiovascular disease (CVD) accounts for the majority of deaths for people with type 2 diabetes mellitus. CVD is a broad term which includes any condition causing pathological changes in blood vessels, cardiac muscle or valves, and cardiac rhythm. The electrocardiogram (ECG) offers a quick, non-invasive clinical and research screening tool for the early detection of CVD.

Electrocardiographic changes in raw and corrected QT intervals and R wave amplitudes **are early indicators of evolving CVD and increased risk of cardiovascular events.** Prolonged QT and QT_c intervals are considered reliable predictors of heart disease and fatal ventricular arrhythmias (Cardoso, Salles, and Deccache, 2003; Christensen et al., 2000; Gorodeski et al., 2009). A positive linear relationship exists between QT_c interval prolongation and diabetic cardiac autonomic neuropathy (DAN) severity in the diabetic population (Mathur, 2006). Heart rate variability (HRV), one indicator of DAN, decreases with diabetes, which indicates increased mortality risk (Schroeder et al., 2005). QT and QT_c interval abnormalities reflect changes in cardiac architecture. A positive correlation between QT or QT_c interval prolongation and left ventricular (LV) mass has been reported (Davey, Barlow, and Hart, 2000; Oikarinen et al., 2001; Pshenichnikov et al., 2003). LV hypertrophy presents as exaggerated R wave amplitudes on ECG recordings. Elevated R wave amplitudes are an independent risk factor for cardiovascular events (Nakamura et al., 2006). LV hypertrophy and QT interval alterations coupled with decreased cardiac function are commonly observed with diabetes related CVD (Zhang et al., 1999).

Non pharmacological interventions for CVD focus primarily on lifestyle changes with physical activity as the primary focus and as a risk reduction strategy. Physical activity reduces QT_c interval prolongation and cardiac dysfunction in healthy subjects (Genovesi et al., 2007; Perhonen et al., 2006). Exercise lowers heart rate and increases HRV in healthy and diseased populations (Sandercock, Bromley, and Brodie, 2005; Tuomainen et al., 2005). **Physical activity can serve as a potent prescription in the delay and attenuation of the CVD complications for persons with type 2 diabetes (T2D)**, but additional comparative studies are needed regarding the cardiac response to exercise under diabetic conditions at various time points of the disease.

The Zucker Diabetic Fatty (ZDF) rat is a model of T2D. The ZDF rat develops hyperglycemia and hyperlipidemia by week 8 and overt diabetes by week 12. The progression mimics the obesity-related insulin resistance and inflammation seen in humans (Leonard et al., 2005; Schmidt et al., 2003). The ZDF rat is commonly used to investigate the prevention of diabetes; however, research characterizing diabetic heart disease in the model including ECG studies is limited. **We hypothesized that ECG changes occur in ZDF rats early in the disease process and that aerobic exercise training will alleviate these changes.** We detected changes in ECG parameters that were partially corrected by exercise training. Our findings add to the characterization of the ZDF model for studying T2D effects on the heart and explore the benefits of an early exercise intervention in the presentation and progression of diabetes related CVD.

2.3 Methods

2.3.1 Animals

Male Zucker Diabetic Fatty (fa/fa) rats of 11 weeks of age were utilized for this study. Male lean, age-matched Zucker (fa/+) rats (both from Charles River Laboratory, Saint Louis, MO) served as non-diabetic controls. The animals were allowed food and water ad libitum and were placed on a 12:12 light-dark cycle. As per vendor's recommendations, the animals were fed Purina 5008 diet during the entire study for the development of a disease process resembling T2D and its complications. All animal procedures were performed according to the University of Kansas Medical Center Institutional Animal Care and Use Committee guidelines and an approved Animal Care and Use Protocol.

2.3.2 Measurements

Body weights and blood glucose levels were measured weekly on all animals. Blood glucose levels were determined from rat tail blood samples using the Accu-Check Active meter (Roche Diagnostics, Indianapolis, IN). Glycated hemoglobin (HbA1c) levels were measured at the end of the experiment using an antibody-based A1cNow meter (Metrika, Sunnyvale, CA). When rats had blood glucose or HbA1c levels higher above the range of detection for the method utilized, the highest detectable value (600 mmol/L or 13%, respectively) was used in the statistical analyses. Animals were killed within 36 hours of the last exercise training episode.

2.3.3 Aerobic exercise training

The rats started a treadmill exercise program at 12 weeks of age, immediately after the onset of diabetes, and continued exercising for 7 weeks. Four groups of rats

were used: sedentary control (SC, n=12), sedentary diabetic (SD, n=10), exercised control (EC, n=10), and exercised diabetic (ED, n=12). The exercise training protocol has been used by our group previously for a rat model of type 1 diabetes (T1D) (Searls et al., 2004) and was adapted for the obese diabetic rats as they were unable to perform at that intensity level. During the first week of exercise training, the animals ran at 10 m/min initially at 10 min per day and increasing to 40 min per day by the end of the week. The progression allowed the rats to acclimate to the treadmill during the first five training days. Starting at week two and until the completion of the exercise training session the rats ran at 15 m/min, for 40 minutes, 5 days per week. To accommodate for the disease progression in the diabetic rats, any animals showing signs of fatigue were allowed breaks of a few minutes until they were able to continue, for a total run time of 40 min per day. All rats assigned to the exercise groups completed the exercise training protocol.

2.3.4 Electrocardiogram (ECG) assessment

Animals received ketamine (60 mg/kg) and xylazine (7 mg/kg) prior to the resting ECG recording. ECG leads I, II, III, aVR, aVL, aVF were recorded with surface electrodes (ADInstruments, Colorado Springs, CO). Measurements were collected at baseline, prior to training, and after 7 weeks of exercise training. The mean value for each rat was obtained from four measurements consisting of four consecutive cardiac cycles using LabChart software (ADInstruments, Colorado Springs, CO). Corrected QT (QT_c) was calculated with mean values and the Bazett's formula, $QT_c = QT \text{ Interval} / \sqrt{RR \text{ interval}}$ (Heffernan, Jae, and Fernhall, 2007). The heart rate (bpm) for each animal

was calculated by dividing 60 by the mean RR interval. HRV was calculated as the standard deviation of the RR intervals.

2.3.5 Statistical analysis

Descriptive statistical analyses were performed on animals' means for each group. One way repeated measures ANOVA (group) was used to analyze within and between subject differences and interactions. Single time point measurements or change scores were completed with one way ANOVA (group) with Least Significant Difference (LSD) post-hoc analysis. Pearson correlations were utilized to assess relationships between variables. Partial eta squared values are reported for the proportion of total variability attributed to a factor. Statistics were conducted with PASW Version 17 software (SPSS Inc, Chicago, IL, USA). Significance was defined at $p < 0.05$. Results are presented as means \pm standard errors (SEs). The effect size of baseline body weights was large with Cohen's $d = 2.8$ and power was greater than 90% with sample sizes of 10-12 per group.

2.4 Results

2.4.1 Animal characteristics

A summary of animal characteristics is reported in Table 1 and weekly body weights are plotted in Figure 1. A significant difference in body weight between diabetic and control animals was observed at both baseline and termination time points, $F(3,40)=19.37$; $p < 0.001$. The mean difference between baseline and termination body weights indicated that control animals gained approximately 14% of their baseline body weights compared to a 5% gain for diabetic animals ($p < 0.001$). Diabetic animals outweighed their control counterparts by 9-12% at termination even with the

discrepancy in weight gain. Although ED rats weighed less than SD animals at the termination, the ED rats were significantly heavier than SC and EC animals ($p=0.016$ and $p<0.001$, respectively). However, aging accounted for 64% of the variance in body weights as evidenced by average weight gains of 16-57 grams in all animal groups, $F(1,39)=71.29$; $p<0.001$.

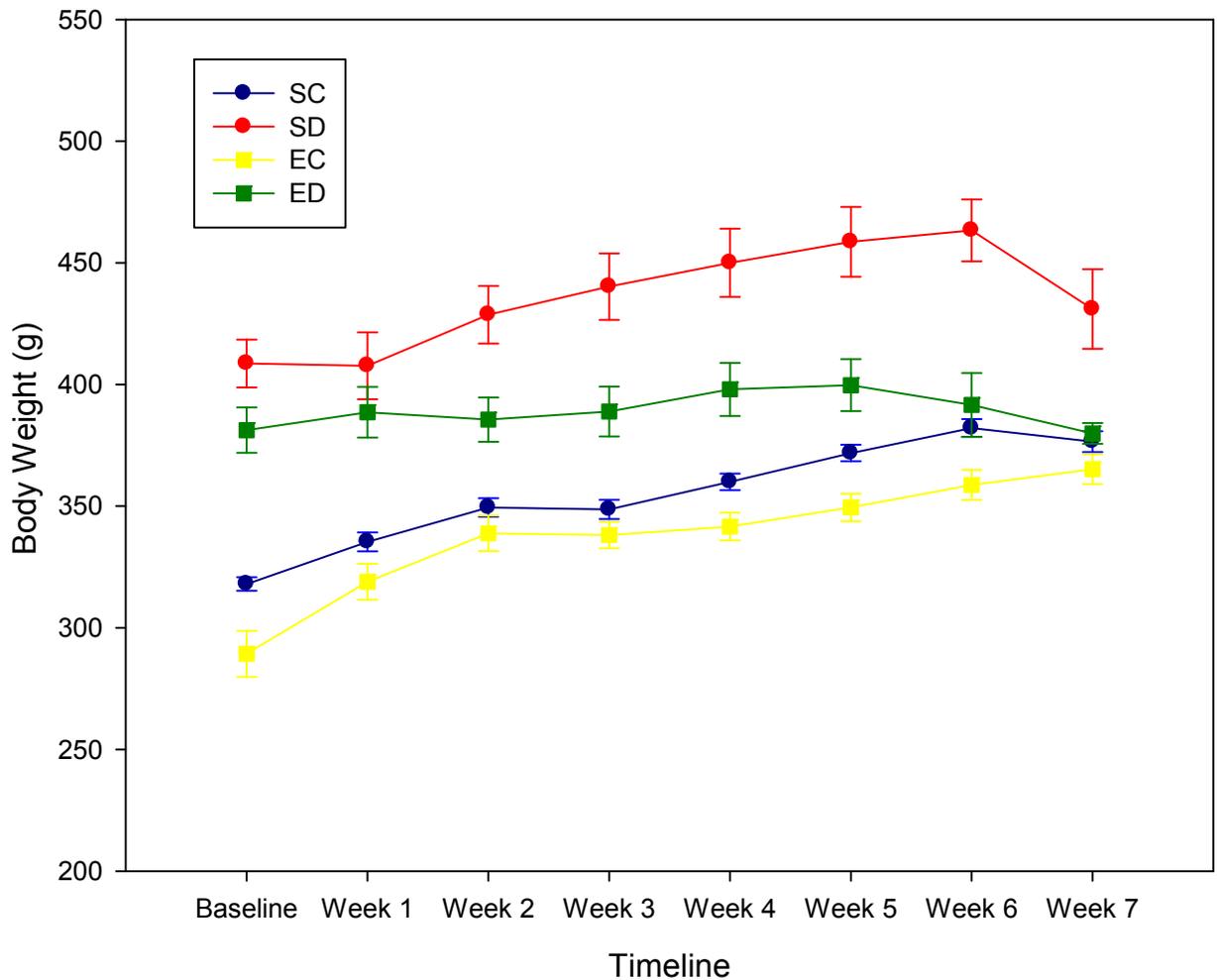


Figure 1 Weekly mean body weight measurements. Data are means \pm SEs for each group with $n=12$ for sedentary diabetic (SD) and exercised control (EC); and $n=10$ for sedentary control (SC) and exercise diabetic (ED) rats.

A moderate relationship between body weight and blood glucose levels existed at both baseline, $r(44)=0.56$; $p<0.001$ and termination, $r(44)=0.41$; $p<0.01$. The main effect of the group on blood glucose levels was modified by aging, $F(1,39)=3.02$; $p<0.04$. Although all animals had an increase in blood glucose levels, ED animals had only a 10% change compared to the 21% change of SD rats. HbA1C levels were elevated in diabetic groups compared to control groups, $F(3,40)=99.27$; $p<0.001$. Exercise did not attenuate HbA1C levels in ED animals ($p=0.92$). Hyperglycemia is a factor in LV hypertrophy development and progression, but a correlation between HbA1C levels and heart weight/body weight ratios was not observed, $r(44)=-0.17$ $p=0.27$. Heart weight/body weight ratios were similar for the animal groups, $F(3,40)=1.39$; $p=0.26$.

	Sedentary Control (SC) n=10		Sedentary Diabetic (SD) n=12		Exercised Control (EC) n=12		Exercised Diabetic (ED) n=10	
	Baseline	Term	Baseline	Term	Baseline	Term	Baseline	Term
Body weight (g)	326±13	381±13	409±34 ^{a,b,f}	457±41 ^{a,b,f}	309±30 ^{c,f}	366±22 ^{c,f}	374±24 ^{b,c,e}	390±23 ^{b,c}
Blood glucose (mmol/L)	115±9	145±9	433±144 ^{a,f}	579±35 ^{a,f}	111±11 ^{c,f}	156±12 ^{c,f}	491±136 ^c	584±22 ^{c,e}
HbA1c (%)	ND	4.2±0.3	ND	13.0±0.0 ^{a,f}	ND	4.7±0.3 ^{c,d,f}	ND	13.0±0.0 ^{c,e}
Heart rate (bpm)	355±148	416±190	393±68 ^{a,f}	423±84	358±139 ^{c,f}	408±100	397±73 ^{c,e}	413±81
Heart weight (g)	1.24±0.12		1.29±0.09 ^f		1.15±0.08 ^{d,f}		1.22±0.10	
Heart weight/ Body weight (mg/g)	3.27±0.04		3.02±0.03		3.16±0.02		3.09±0.03	

Table 1 Animal characteristics at baseline and termination. Data are means ± SEs for each group with n as indicated. Statistical significance: Between group differences at baseline or termination (term) of $p\leq 0.05$; ND – not determined; ^aSC vs SD, ^bSD vs ED, ^cEC vs ED, ^dSC vs EC, ^eSC vs ED and ^fSD vs EC.

2.4.2 ECG wave amplitudes

2.4.2.1 R wave amplitude

R wave amplitudes were similar for groups at baseline except for the SD groups, $F(3,40)=15.16$, $p<0.001$. Therefore, statistical analyses used the change value for R wave amplitude to account for the difference at baseline. Gains in R wave amplitudes from baseline to termination of the experiment were only observed in the SD animals as reported in Figure 2. The SD rats had a 17% increase in R wave amplitude, suggesting left ventricular hypertrophy. A reduction in R wave amplitude was found in ED animals at termination. ED rats had change values similar to SC and EC animals, $F(3,40)=4.13$, $p=0.84$ and $p=0.87$.

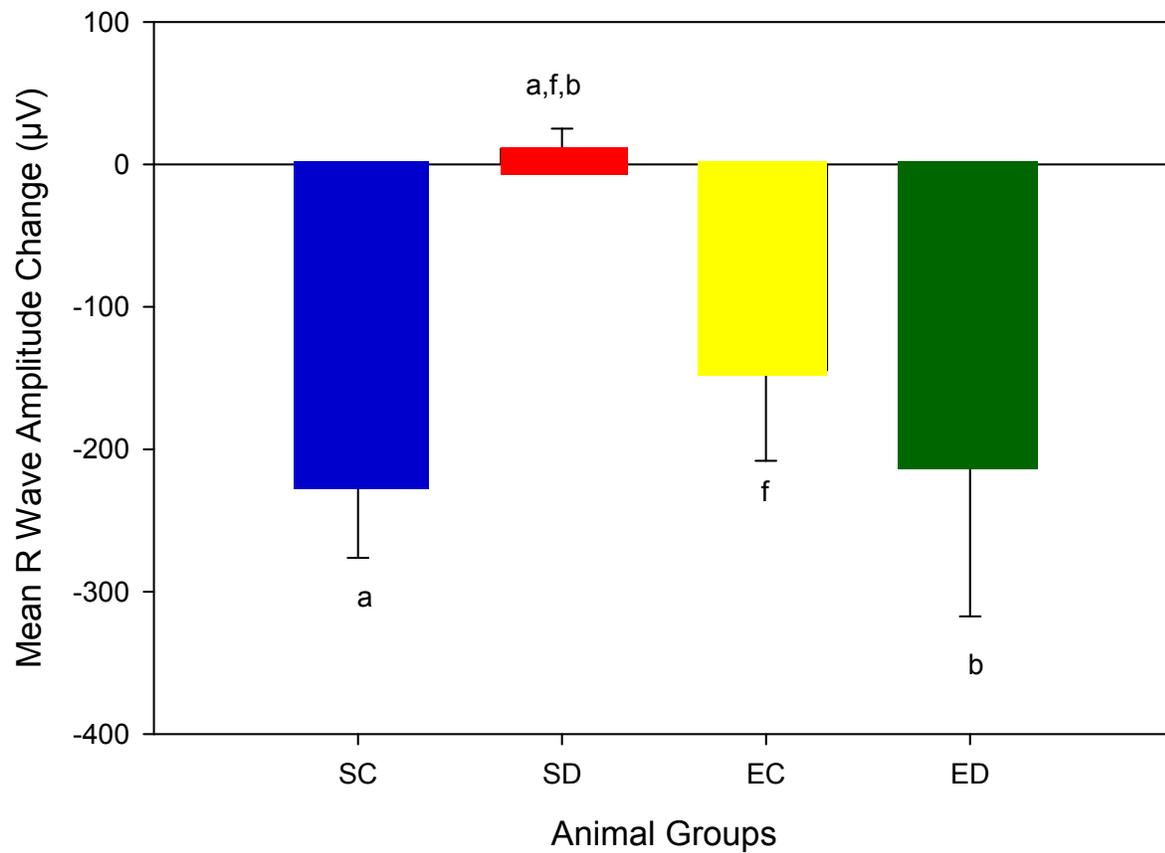


Figure 2 Mean R wave amplitude changes. Data are means \pm SEs for each group with n=12 for sedentary diabetic (SD) and exercised control (EC); and n=10 for sedentary control (SC) and exercise diabetic (ED) rats. ^aSC vs SD, ^bSD vs ED and ^fSD vs EC.

2.4.2.2 T wave amplitude

Due to the significant differences between diabetic groups at baseline (Figure 3), we analyzed the change of T wave amplitudes from baseline to termination. T wave amplitudes changes were similar in the four groups, $F(3,40)=1.81$, $p=0.16$.

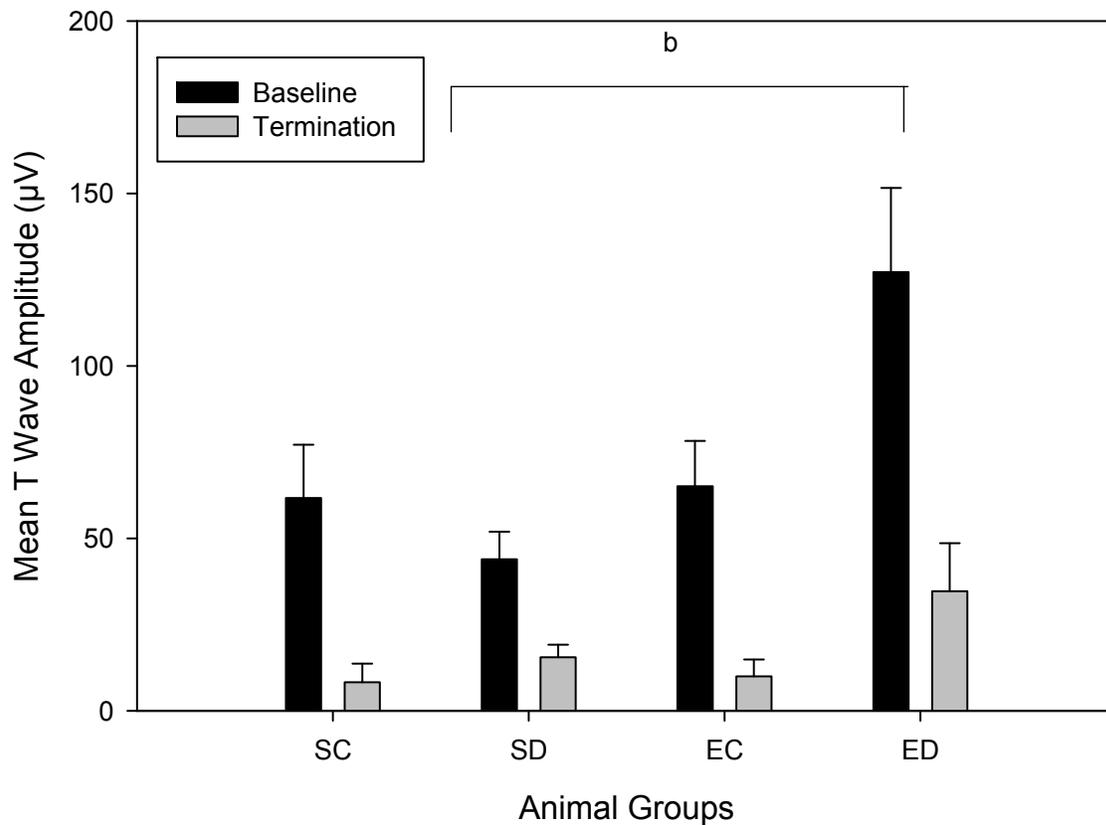


Figure 3 Mean T wave amplitudes. Data are means \pm SEs for each group with $n=12$ for sedentary diabetic (SD) and exercised control (EC); and $n=10$ for sedentary control (SC) and exercise diabetic (ED) rat. ^bSD vs ED.

2.4.2.3 P wave amplitude

P wave amplitudes were similar between control and diabetic animals at baseline and termination, $F(3,40)=0.40$, $p=0.99$ (Figure 4). The amplitudes were decreased

between baseline and termination in all four animal groups, as evident by time modifying the group effect, $F(1,40)=25.05$, $p<0.001$.

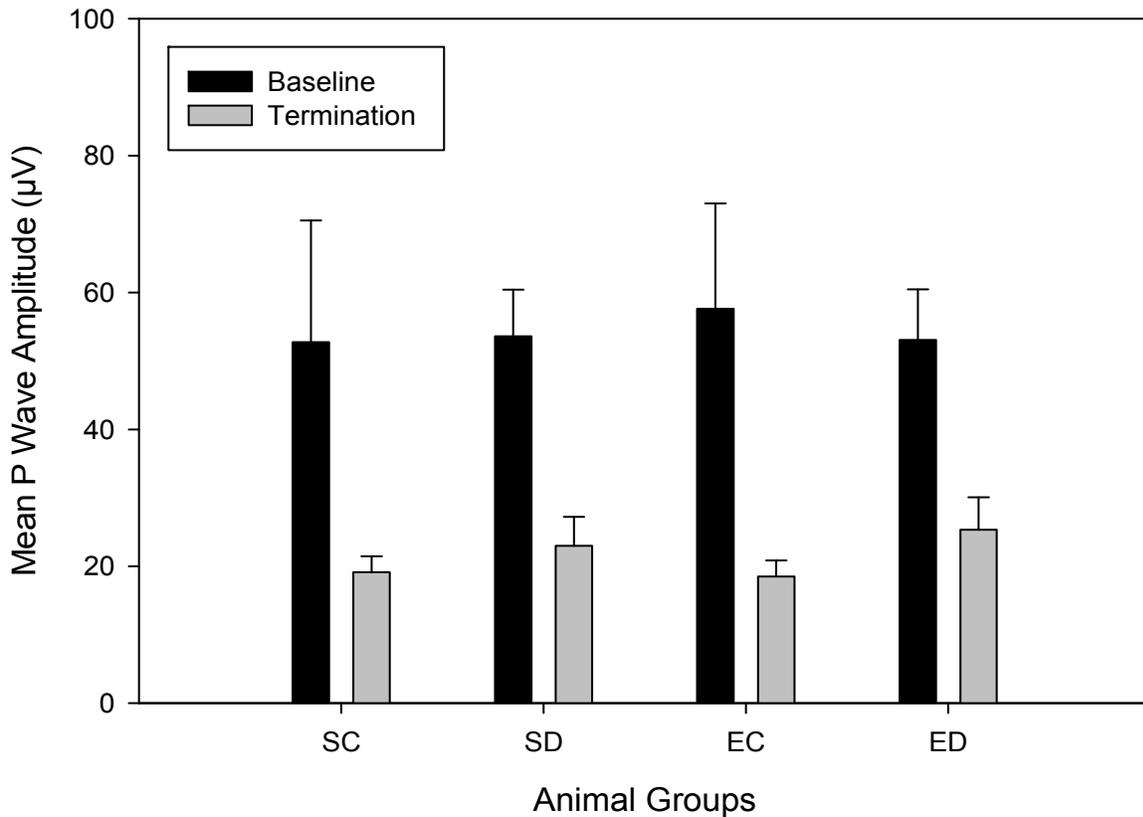


Figure 4 Mean P wave amplitudes. Data are means \pm SEs for each group with $n=12$ for sedentary diabetic (SD) and exercised control (EC); and $n=10$ for sedentary control (SC) and exercise diabetic (ED) rats. Statistical significance ($p<0.001$) between baseline and termination values was found in all four animal groups, but no difference observed between groups.

2.4.3 ECG intervals

2.4.3.1 RR intervals

A significant shortening of RR intervals and increased heart rates was observed in SD group at baseline when compared to the SC group, $F(1,38)=8.83$, $p<0.01$ (Table 2). At termination, only a trend toward tachycardia was observed SD animals compared to SC animals, $F(3,36)=2.79$, $p=0.06$. Exercise did not reverse the heart rate pattern for

diabetic animals, $p=0.92$. Table 2 shows that all animal groups had various levels of RR interval shortening at termination, age was not a statistically significant effect, $F(1,38)=0.34$, $p=0.56$. Decreased HRV was observed in all diabetic animals, sedentary and exercised, compared to control animals $F(1,38)=1662.23$, $p<0.01$. HRV was not improved with exercise, $p=0.91$ as calculated with the termination RR intervals of the ED animals.

	Sedentary Control (SC) n=10		Sedentary Diabetic (SD) n=12		Exercised Control (EC) n=12		Exercised Diabetic (ED) n=10	
	Baseline	Term	Baseline	Term	Baseline	Term	Baseline	Term
RR interval (sec)	0.195 ±0.077	0.152 ±0.041	0.158 ±0.033	0.132 ±0.012	0.191 ±0.073	0.158 ±0.048	0.157 ±0.036	0.135 ±0.012
QRS interval (sec)	0.016 ±0.005	0.022 ±0.002	0.020 ±0.002 ^{a,f}	0.022 ±0.004	0.016 ±0.004 ^{c,f}	0.023 ±0.003	0.020 ±0.002 ^{c,e}	0.021 ±0.004
QT interval (sec)	0.043 ±0.012	0.040 ±0.005	0.064 ±0.013 ^{a,f}	0.051 ±0.012 ^{a,f}	0.045 ±0.012 ^{c,f}	0.041 ±0.006 ^{c,f}	0.062 ±0.011 ^{c,e}	0.052 ±0.013 ^{c,e}
QT_c interval (sec)	0.105 ±0.043	0.105 ±0.020	0.163 ±0.033 ^{a,f}	0.134 ±0.034 ^{a,f}	0.111 ±0.043 ^{c,f}	0.106 ±0.018 ^{c,f}	0.159 ±0.026 ^{c,e}	0.134 ±0.037 ^{c,e}
PR interval (sec)	0.032 ±0.010	0.030 ±0.006	0.045 ±0.011 ^{a,f}	0.034 ±0.008	0.036 ±0.012 ^{c,f}	0.030 ±0.005	0.046 ±0.011 ^{c,e}	0.034 ±0.009

Table 2 ECG interval measurements. Data are means ± SEs for each group with n as indicated. Statistical significance: Between group differences at baseline or termination (term) of $p\leq 0.05$; ^aSC vs SD, ^cEC vs ED, ^eSC vs ED and ^fSD vs EC.

2.4.3.2 QRS intervals

Widening of QRS intervals, a sign of abnormal intraventricular conduction, was found at baseline in diabetic animals, $F(3,40)=3.72$, $p<0.01$ (Table 2). An 18% difference existed in the duration of QRS intervals between the SC and SD animals. At termination, QRS intervals increased 6-9% for SD animals in comparison to 27-29% increases in SC animals. These changes imply that aging interacted with the main effect of the group, $F(1,41)=16.78$, $p<0.001$ and accounted for 29% of the variability of QRS intervals at termination. Therefore, the difference between SC and SD animals at

baseline was lost at termination, $F(3,40)=0.57$, $p=0.64$. An impact of exercise on QRS intervals was not observed in the EC and ED animals.

2.4.3.3 QT and QT_c intervals

SD animals presented with QT intervals (a measure of ventricular repolarization) 31% wider than SC animals at baseline, $F(3,40)=9.37$, $p<0.001$ (Table 2). After 7 weeks of exercise, QT intervals in ED animals remained widened, $F(3,40)=14.13$, $p=0.85$. At termination, the difference between SC and SD animals decreased to 21%. Aging accounted for 20% of the variability in termination QT intervals, but the group effect of diabetes accounted for 51% of the variability. An interaction between aging and group factors was not significant, and QT intervals were not affected by exercise in control or diabetic animals, $p=0.91$ and $p=0.61$.

QT_c intervals (QT intervals corrected with RR intervals) were analyzed as a measure independent of heart rate. SD animals displayed significant widening of QT_c intervals compared to SC animals, $F(1,42)=10.58$, $p<0.001$ (Table 2). Exercise did not attenuate the widening of QT_c intervals in ED animals, $p=0.66$. Compared to QT intervals, aging accounted for 85% of the variability in QT_c intervals and 43% was due to group effects at termination. The interaction between time and group was significant, $F(3,40)=4.54$, $p<0.01$.

2.4.3.4 PR intervals

Baseline measurements revealed significant prolongation of PR intervals in SD animals compared to SC animals, $F(1,39)=5.40$, $p<0.01$ (Table 2). At termination, the difference between groups was not observed, $p=0.34$. Exercise did not impact PR intervals in EC and ED animals.

2.5 Discussion

CVD risk is increased up to four-fold in people with diabetes compared with their nondiabetic counterparts (Preis et al., 2009). Researchers are aggressively trying to identify early detection methods and explore the factors contributing to diabetes related heart disease. The ZDF rat model is routinely used to investigate physiological and molecular hypotheses regarding diabetes and its related complications. Reports are available indicating cardiac dysfunction in the ZDF rat established primarily with hemodynamic or echocardiographic measurements or through experiments on the isolated heart (Baynes and Murray, 2009; Boudina and Abel, 2007; Radovits et al., 2009; van den Brom et al., 2010). However, limited information exists about the ECG changes that occur in the ZDF rat and its response to physical activity, specifically to aerobic exercise that is commonly recommended for those at risk or diagnosed with diabetes related heart disease (Chipkin, Klugh, and Chasan-Taber, 2001; Mizuno et al., 2010). Our project aimed to address this gap in the current literature.

2.5.1 Diminished heart rate variability in the ZDF rat

Although we did not measure autonomic function directly, ECG indicators of autonomic dysfunction were observed in the ZDF rats at baseline with HRV alterations, tachycardia, and QT interval prolongation. Autonomic innervations control HRV and cardiac function through a delicate balance of sympathetic and parasympathetic responses. Diabetes stimulates the sympathetic nervous system initially, but prolonged exposure to hyperglycemia and elevated catecholamine levels cause a decrease in adrenergic receptors (Scott and Kench, 2004). As diabetes creates a sympathetic predominance, it also produces a corresponding parasympathetic imbalance by

denervation of the vagus nerve. Subclinical symptoms of DAN, primarily decreased HRV, are believed to appear in humans within one year of T2D diagnosis and clinical presentations may not emerge until years into the diagnosis (Vinik et al., 2003). Bergstrom et al identified symptoms of DAN in T1D patients with durations of diabetes as short as two months (Bergstrom, 2009). In our animal study, diminished **HRV was noted in ECG recordings as early as within one week of diabetes onset** for the ZDF rat.

2.5.2 Tachycardia in the ZDF rat

Clinical DAN symptoms include resting tachycardia, exercise intolerance, and orthostatic hypotension and heart rate syndromes (Vinik and Ziegler, 2007).

Tachycardia was observed in diabetic animals at baseline, within a week of diabetes onset. Tachycardia is also a common finding in humans with uncontrolled diabetes (Kitabchi et al., 2006). Similarly, our animals were not treated for their hyperglycemia as evident by elevated blood glucose and HbA1C levels. The role of tachycardia is controversial because research indicates that it may be a diabetic complication or a causative factor of diabetes. Nagaya et al argue that elevated resting heart rates and systolic blood pressure increase the risk for T2D (Nagaya et al., 2010b).

2.5.3 Prolonged QT_c intervals in the ZDF rat

Another study by the same group indicated that prolonged QT_c intervals were also an independent risk factor for the development of diabetes (Nagaya et al., 2010a). Our analysis indicates that **widening of QT_c intervals was present with the onset of diabetes at twelve weeks of age** in ZDF rats. Thus abnormalities of ventricular repolarization are present at an early stage of diabetes in this model. An earlier time

study would be useful to verify the presence of tachycardia and QT_c interval prolongation during a pre-diabetic state. Of interest, several ECG parameters in our study showed an impact of aging in agreement with findings by others (Baynes and Murray, 2009) indicating cardiac and renal changes in the ZDF rat model with aging up to 36 months.

2.5.4 Lack of P and T wave amplitude changes in the ZDF rat

P wave amplitudes were unaffected with the onset of diabetes. Future studies need to investigate the relationship between the dispersion of the P wave and the onset of diabetes. Obesity is commonly linked to diabetes and is reported to increase the dispersion of the P wave (Seyfeli et al., 2006). PR intervals were widened in diabetic animals at baseline, but normalized at termination. PR intervals are commonly associated with atrial fibrillation (Homoud, 2009; Lorscheid et al., 2005); however our ECG assessments of diabetic animals did not indicate atrial dysfunction. Diabetes and exercise appeared to have no effect on T wave amplitudes. T wave amplitudes were skewed at baseline and therefore change scores were used for analysis. However, the morphology of the T wave has been shown to have prognostic value for CVD, but not so for amplitude changes (Huang et al., 2009; Nair et al., 2008).

2.5.5 The impact of exercise on electrocardiographic changes

2.5.5.1 Exercise influences R wave amplitudes

After seven weeks of aerobic exercise, **ED animals showed R wave amplitudes comparable to control animals.** Only SD animals had an increase in R wave amplitude. Hyperglycemia is associated with LV hypertrophy in type 2 diabetes (Goraksha-Hicks and Rathmell, 2009). The increase in R wave amplitude may indicate

a change in left ventricular mass. With diabetes, pathological hypertrophy results from myocardial damage and fibrosis (Fukui et al., 2009; Kannel, 1983; Seferovic et al., 2007). Fibrosis affects the filling and contractility of the ventricles. Subsequently, cardiac dysfunction presents as decreased activity tolerance, ejection fraction, and cardiac output resulting in heart failure (Kannel, Levy, and Cupples, 1987).

However, other factors can alter R wave amplitudes, including electrical axis deviations, altered electrodes position, and differences in chest wall thickness. Ideally, the ECG should be correlated with an echocardiography. Unfortunately, we were unable to use echocardiography, thus we recognize this as a limitation of our study. In analyzing other indicators of heart hypertrophy, we found no difference in the heart weight/body weight ratio in our study. Alternatively, the LV weight/tibial length ratio has been validated as an index of cardiac hypertrophy in mature rats (Yin et al., 1982). Due to the fact that the onset of diabetes in the ZDF rat occurs early in their age, we were restricted to using relatively young animals that were still in the latter stages of their growth phase. Thus, normalizing heart size to the changing tibial bone length during growth would not have provided an accurate index in our study. Darmellah et al reported that normalization of the heart weight per body weight or tibial length resulted in similar measurements of cardiac hypertrophy in Goto-Kakizaki animals, another rat model of T2D (Darmellah et al., 2007).

Aerobic exercise did not impact the hyperglycemia in the ZDF rat. Exercise is postulated to improve glucose uptake and decrease lipid accumulation in persons with controlled diabetes, but the protective mechanism of exercise is lost if hyperglycemia persists (Sato et al., 2000). A comparative study of fenobirate and metformin validated

the role of lipid oxidation in the development and progression of diabetes related heart disease, with fenofibrate decreasing triglycerides content and fibrosis in diabetic myocardium (Forcheron et al., 2009). The switch in myocardial substrate from glucose to fatty acids has been shown to result in systolic and diastolic dysfunction in the ZDF model (van den Brom et al., 2009). Exercise training has also been suggested to improve microcirculation through enhanced endothelial function (Erbs et al., 2010) by normalizing glycemic levels. However, the severity of diabetes will determine if the body can adapt to the demands of exercise or whether regional flow has already been compromised beyond recovery (Joshi et al., 2010). Microcirculatory disturbances or small vessel disease may lead to declines in myocardial blood flow which could influence ECG parameters (Cosyns et al., 2008).

2.5.5.2 Exercise does not recover QTc interval prolongation

A 31% difference in QT intervals was reported between SC and SD animals at baseline. We hypothesize that cardiac remodeling was already in the process in the SD rats when baseline measurements were taken, as evidenced by the presence of hyperglycemia and obesity in the ZDF cohorts. **QTc intervals did not respond to exercise**, but the chronic tachycardia shortened the intervals as a compensatory effect. Commonly, exercise may cause a decrease in heart rate and increased ventricular relaxation which presents as longer QT intervals. A study investigating the effects of a seven month endurance training program in dogs revealed an increase in QT intervals (Constable et al., 2000). Acute resistance exercise resulted in a similar effect on QT_c intervals in humans. (Heffernan et al., 2008). Since our obese, diabetic animals were showing early signs of autonomic disturbance, exercise tolerance was lowered and

animals required frequent rest breaks during our training program. The mode and duration of exercise might not have been sufficient for QT_c interval adaptation. In the future, we will investigate whether longer durations of exercise training can return heart rates to normal or restore HRV in diabetic animals. Pagkalos et al reported improvements in cardiac autonomic function with six months of aerobic exercise training (Pagkalos et al., 2008). Another alternative is to evaluate HRV during post-exercise recovery. Training may not affect resting HRV, but its benefits may be evident during the post-exercise recovery. This conclusion is supported by a study investigating cardiac autonomic function in women with and without diabetes (Figuroa et al., 2007).

2.6 Conclusions

In summary, our investigation showed that ECG alterations do occur with diabetes in the ZDF rat. These alterations include prolongation of the QT_c interval and tachycardia which constitute important electrophysiological alterations in this animal model of diabetes. These modifications coupled with the high R wave amplitude illustrate the early cardiac anatomic and electrophysiological alterations in this diabetic model. After seven weeks of exercise training, R wave amplitude changes from baseline to termination were similar in both diabetic and control animals from baseline to termination. However, aging may have an impact on several ECG parameters and the ZDF model showed changes in atrial and ventricular conduction possibly due to an interaction of aging and group effects. Future studies are needed to investigate ECG changes in the ZDF model before the onset of diabetes which will provide additional information about the use of QT_c intervals and HRV in the early detection of DAN.

Chapter 3

Effects of aerobic exercise on left ventricular hemodynamics in Zucker diabetic fatty (ZDF) rats

3.1 Abstract

Introduction: Diabetic heart disease has been described as a hybrid of diastolic and systolic dysfunction. Diastolic dysfunction is defined as decreased relaxation and filling of the ventricles. Reduced contractility and circulation are recognized as clinical manifestations of systolic dysfunction. Aerobic exercise has been suggested to have cardioprotective benefits, which delay or lessen the effects of diabetic heart disease. We aimed to characterize left ventricular hemodynamics in the Zucker Diabetic Fatty (ZDF) rat, a model of type 2 diabetes that resembles the human disease, and to evaluate changes induced by exercise training.

Methods: Male ZDF and Zucker lean (control) rats were assigned to four groups: sedentary control (SC, n=16), sedentary diabetic (SD, n=18), exercised control (EC, n=12), and exercised diabetic (ED, n=10). Exercise training consisted of seven weeks of progressive running on a treadmill. Hemodynamic alterations were determined using left ventricular catheterization and analysis of pressure volume loop relationships.

Results and conclusion: Of the 24 hemodynamic parameters tested, 15 were negatively affected by diabetes. The debility of diabetic heart disease was evident in the diastolic filling, isovolumic contraction, ejection, and isovolumic relaxation phases. Specifically, ventricular filling was impaired in SD rats with a 23% loss in end diastolic volume. dP/dt_{max} , an indicator of contractility, showed a 40% reduction while the ejection fraction was 8% lower in SD rats. In addition to compromised ventricular contraction, impaired relaxation was present in SD rats with dP/dt_{min} levels at 59% of those seen in controls. Importantly, exercise training restored 13 of the 15 hemodynamic parameters affected by diabetes. Specifically, training was beneficial for restoring end diastolic

volumes in ED rats. Exercise improved the velocity of the ventricular contraction, dP/dt_{max} , in the ED rats. Volumes at dP/dt_{max} were restored for ED rats to SC and EC levels. Exercise returned maximum pressure, end systolic volume, and end systolic pressure to levels of SC rats. Exercise recovered dP/dt_{min} for ED rats and related volumes to levels similar to control animals. We concluded that diastolic and systolic dysfunction was present in the ZDF rat model and that exercise had a definite cardioprotective effect on left ventricular hemodynamics in these diabetic rats.

3.2 Introduction

Over 100 million people suffered from diabetes mellitus or prediabetes in the United States in the last year (ADA, 2011). Almost 95% of the diabetic cases were identified as type 2 diabetes (T2D). Stroke and heart disease were reported as the first and third leading causes, respectively, of mortality in the United States, for persons with T2D, and accounted for the majority of diabetes-related deaths (CDC, 2011a). Heart disease in persons with T2D has many concomitant illnesses (Kelly et al., 2009), which have normally eluded a single clinical diagnosis. Therefore, diabetic heart research has utilized animal models that have a similar fusion of cardiovascular diseases.

The Zucker diabetic fatty (ZDF) rat has a phenotype that resembles the human progression of T2D and its complications. Diabetes in the ZDF rat correlates well with the obesity-related insulin resistance and inflammation seen in humans (Leonard et al., 2005; Schmidt et al., 2003). The rats develop hyperglycemia and hyperlipidemia by 8 weeks and overt diabetes by 12 weeks of age (Phillips et al., 1996). These metabolic disturbances become disastrous for the diabetic heart. Previous hemodynamic studies have shown decreases in heart rate and cardiac output along with signs of ventricular stiffness and diastolic dysfunction in the ZDF rat (Lavanchy, 2003; Radovits et al., 2009). In addition, investigations have documented atherosclerosis (Blankenberg et al., 2001; Vaskonen et al., 2002) and hypertension (Osmond et al., 2009) in the rodents. The existence of Impaired systolic function in ZDF rat remains debatable with reports of compensation in the early stages of the disease (Baynes and Murray, 2009). This complex interaction of cardiovascular diseases is similar to diabetic heart disease observed in humans. Consequently, the model has been established as ideal to

investigate diabetic heart disease and possible interventions (Clark, Palmer, and Shaw, 1983; Golfman et al., 2005; Wang et al., 2005). However, **the cardioprotective influence of exercise on hemodynamic parameters affected by diabetes in the ZDF rat model has not been reported.**

Exercise has been designated as one of the major tenets of the T2D treatment regimen (Creviston and Quinn, 2001; Schafer et al., 2007). An inverse relationship has existed between exercise and diabetic heart disease in humans with higher physical activity levels associated with lower cardiovascular risk (Haapanen et al., 1997). Metabolic disturbances, oxidative stress, and inflammation are reported to play a complex and coexistent role in the development and progression of the disease. Therefore, exercise has the potential to address many of the mechanisms responsible for diabetic heart disease. Exercise has been suggested to improve glucoregulation in humans (Kiraly et al., 2008). Animal studies indicate that exercise reduced inflammation (Teixeira de Lemos et al., 2009) and had a dose dependent impact on reducing oxidative stress (Fukai et al., 2000; Rush, Turk, and Laughlin, 2003). Our study was designed to test the hypothesis that the cardioprotective effects of physical exercise would attenuate left ventricular (LV) pressure volume changes seen in ZDF rats.

3.3 Methods

3.3.1 Animals

Male ZDF (fa/fa) rats of 11 weeks were used in the study. Age-matched male Zucker lean (fa/+) rats served as non-diabetic controls. Rats were purchased from Charles River Laboratory (Saint Louis, MO) and allowed one week for acclimation. The rats received husbandry services at the Laboratory Animal Resources facility at the

University of Kansas Medical Center. The rats were allowed food and water ad libitum and were maintained on a 12:12 light-dark cycle. As per vendor's recommendations, the rats were fed Purina 5008 during the entire study, for the development of a disease process similar to T2D in humans. All animal procedures were performed according to the IACUC guidelines of the University of Kansas Medical Center.

3.3.2 Aerobic exercise training

Rats were divided into four groups: sedentary control (SC, n=16), sedentary diabetic (SD, n=18), exercised control (EC, n=12), and exercised diabetic (ED, n=10). Rats in the exercised groups were trained by running on a treadmill starting at 12 weeks of age, which is the beginning of diabetes progression. An endurance training protocol as described previously was used (VanHoose et al., 2010). Briefly, rats ran at 15 m/min, for 40 minutes, 5 days per week. In order to accommodate for diabetic rats showing signs of fatigue, breaks were given until the rested rats were able to continue for a total run time of 40 minutes per day. All rats assigned to the exercised groups completed the exercise training.

3.3.3 Animal measurements and sample collection

Body weights were recorded weekly on all rats. Blood samples were collected from the tail vein before the hemodynamic assessment. Blood glucose levels were assessed by a digital blood glucose meter (Accu-Chek Active, Roche Diagnostics, Indianapolis, IN). Hemoglobin A1C (HbA1C) levels were determined by a home evaluation unit (A1C Now At Home System, Bayers, Pittsburgh, PA). When rats had blood glucose or HbA1c levels higher than the range of detection by the method used, we performed the statistical analysis as described previously (Loganathan et al., 2007;

VanHoose et al., 2010). Briefly, we used the highest detectable value for blood glucose (600 mmol/L) and HbA1C (13%) levels for statistical analyses. Blood ketone levels were assessed using test strips and the Precision Xtra meter (Roche Diagnostics, Indianapolis, IN). Urine ketones were measured with test strips (KetoDiasix, Bayers, Pittsburgh, PA) as the animal voluntarily voided. Rats were sacrificed within 36 hours of the last exercise training episode.

3.3.4 Left ventricular hemodynamic measurements

Functional evaluation was performed using LV catheterization through the right carotid artery with a 2 French microtip pressure volume catheter (Millar Instruments, Houston, TX) under ketamine and xylazine anesthesia (80 mg/Kg and 10 mg/Kg, respectively). Rat core temperatures were maintained at 37°C with a heating lamp. Alligator clip electrodes were placed on the upper extremities and left lower extremity to monitor heart rate through electrocardiography during the procedure. A 4 cm long incision was made from the lower mandible extending to the sternum. The adipose tissue and salivary glands were secured to the sides with retractors. The muscles of the anterior neck region were cleared by blunt dissection to expose the right carotid artery. The vessel was ligated superior to the site of catheter entry. Subsequently, a loose inferior suture was applied, in addition to a metal clip close to the inferior suture. A fine incision was made close to the superior suture and the catheter was introduced gently through that incision. The metal clip was removed as the catheter slid past the inferior suture to enter the aorta. Then, the inferior suture was tightened around the catheter. The catheter was gently pushed to reach the chamber of the left ventricle, which was monitored by the appearance of the pressure volume loops on the computer monitor.

After allowing the pressure volume loops to stabilize for 3-5 minutes, steady state pressure volume loops were recorded at a sampling rate of 1000 samples per second using Millar Pressure Volume System (MPVS-400, ADInstruments, Colorado Springs, CO). After recording the measurements for approximately 1 minute, the catheter was gently removed and the inferior suture was tightened.

Cardiac Cycle Phase	Hemodynamic Parameters		
	Measured at the initiation	Measured throughout the phase	Measured at the termination
Diastolic filling	Minimum pressure (P_{min}) Minimum volume	dV/dt_{max} $P@dV/dt_{max}$	End diastolic volume End diastolic pressure
Isovolumic contraction		dP/dt_{max} $P@dP/dt_{max}$ $V@dP/dt_{max}$ dV/dt_{min}	
Ejection		Maximum pressure (P_{max})	End systolic volume End systolic pressure
Isovolumic relaxation		dP/dt_{min} $V@dP/dt_{min}$	

Table 1 Hemodynamic parameters obtained from the left ventricular pressure volume relationship analysis. The parameters are categorized based on the phases of the cardiac cycle.

Table 1 specifies the hemodynamic parameters collected as they correspond to the cardiac cycle phases. These parameters along with 9 additional parameters (below) were extracted and analyzed using the Chart 5.0 software (ADInstruments, Colorado Springs, CO). On average, 10-20 loops per animal were used for analysis. The additional parameters included heart rate, ejection fraction, arterial elastance, tau (based on the Weiss method), maximal power, and preload adjusted maximal power. The Weiss method calculated tau based on the regression of log of pressure versus time (Frais et al., 1990). To control for body weight differences, cardiac output, stroke volume and stroke work were normalized to body weight and reported as indices:

Cardiac Output Index (COI), Stroke Volume Index (SVI) and Stroke Work Index (SWI) (Jin et al., 2000). Calibration of the pressure volume catheter was performed per manufacturer's instructions with hypertonic saline and fresh heparinized rat blood. A blood sample was taken from each animal group to calculate the formula for volume calibration.

3.3.5 Statistical analysis

One way ANOVAs (group) with Least Significant Difference (LSD) post-hoc analysis detected group differences in hemodynamic parameters. Statistics were conducted with PASW Version 18 software (IBM, Somers, NY). Significance was determined at $p < 0.05$. Results are described as means \pm standard errors (SEs). Pressure volume loops were not recorded for all rats due to death during the procedures or unstable waveforms. Only those rats with recordable pressure volume loops were included in the data analysis (SC, $n=13$; SD, $n=14$; EC, $n=11$; ED, $n=8$).

3.4 Results

3.4.1 Animal characteristics

Diabetic rats, SD and ED, had higher body weights when compared to the control rats from the time the rodents developed diabetes at the age of 12 weeks (baseline) and throughout the seven weeks of diabetes duration. In Figure 1, SD rats weighed 22% more than SC rats, $F(3,52)=40.31$, $p \leq 0.001$, and 7% more than ED rats ($p \leq 0.02$) at baseline. SD rats were heavier than other groups throughout the experiment. At termination, at week 7, SD rats weighed 13% more than ED rats, $F(3,52)=22.75$, $p \leq 0.001$, reflecting the benefits of exercise on weight maintenance. However, increases

in body weight were observed in all rodents from baseline to termination. An interaction between age and animal group was recognized ($p < 0.001$).

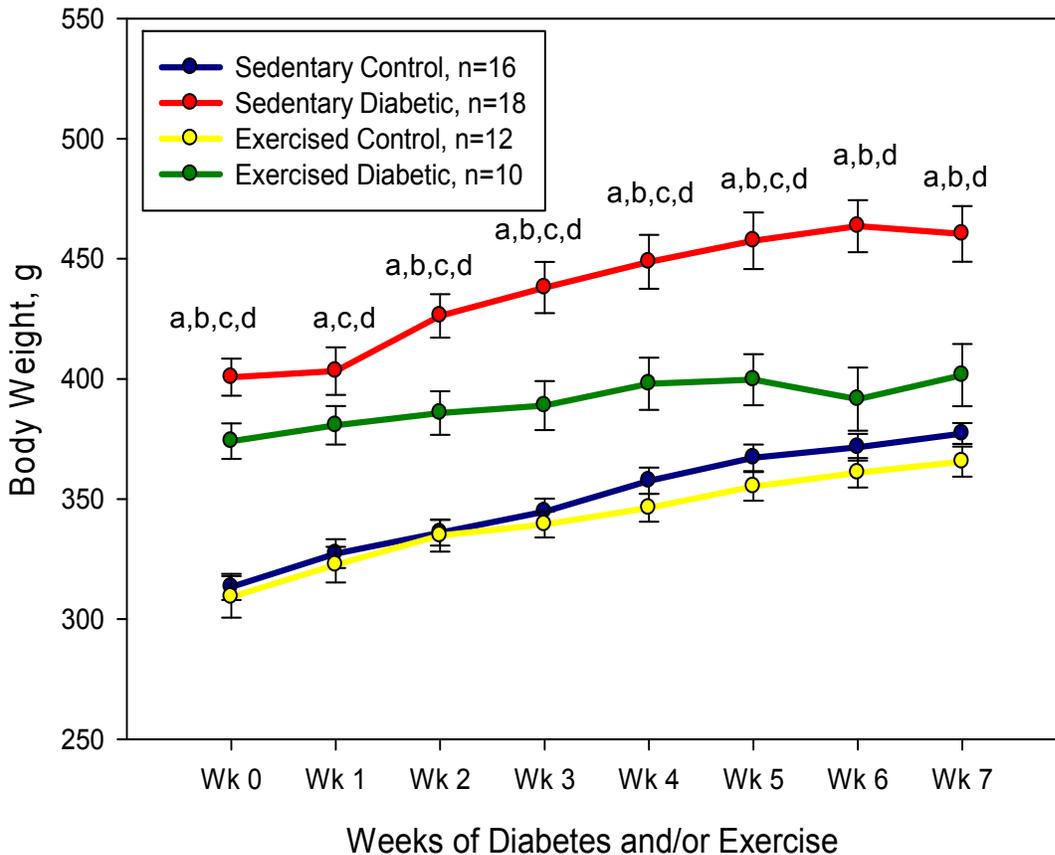


Figure 1 Weekly rat body weights. Rats in the sedentary diabetic group had greater weekly body weights than the rats in the sedentary control and the exercised groups throughout the experiment. Sedentary control (SC, $n=16$), sedentary diabetes (SD, $n=18$), exercised control (EC, $n=10$), and exercised diabetes (ED, $n=12$). ^aSC vs SD, ^bSD vs ED, ^cSC vs ED, ^dEC vs ED.

As expected, blood glucose and HbA1C levels were elevated in SD rats compared to the SC group, $F(3,51)=154.02$, $p \leq 0.001$ and $F(3,47)=90.07$, $p \leq 0.001$ (Table 2). Blood ketones were not impacted by diabetes, $F(3,41)=4.71$, $p=0.09$. A descriptive analysis revealed no dissimilarity in urine ketones between SC and SD rats. Interestingly, the exercise regimen did not lower blood glucose or HbA1C levels ($p=0.62$ and $p=0.57$, SD vs ED, respectively). No differences were noted in blood ($p=0.76$) or

urine ketones levels between SD and ED rats. These results indicate that while our exercise regime was beneficial in terms of body weight reduction, it did not impact metabolic status.

	<i>Sedentary Control (SC)</i>	<i>Sedentary Diabetic (SD)</i>	<i>Exercised Control (EC)</i>	<i>Exercised Diabetic (ED)</i>
Number of rats	16	17	12	10
Blood Glucose, mg/dL	180±17	565±15 ^a	156±3 ^d	551±33 ^{c,d}
Hemoglobin A1C, %	4.3±0.1	11.8±0.5 ^a	4.7±0.1 ^d	12.2±0.8 ^{c,d}
Blood Ketones, mmol/L	0.41±0.05	0.63±0.11	0.28±0.02 ^d	0.67±0.11 ^d
Urine Ketones, qualitative	Negative	Negative	Negative	Negative

Table 2 Rat characteristics. Rats in the SD and ED groups had indications of persistent hyperglycemia when compared to control rats. Exercise had no impact on hyperglycemia. ^aSC vs SD, ^cSC vs ED, ^dEC vs ED.

Since a few reports have indicated that Zucker lean and ZDF rats may develop hydronephrosis (Baynes and Murray, 2009; Marsh et al., 2007; Vora et al., 1996) that affects cardiovascular function, we performed gross analysis of the kidneys of all rats in this study. We found evidence of hydronephrosis in only one ZDF rat from the SD group. Data obtained on this rat were subsequently removed from the analysis.

3.4.2 Left ventricular hemodynamics

3.4.2.1 Pressure volume loop diagram analysis

Pressure volume loops were graphed in Figure 2 with the mean values for each animal group that corresponded to the 4 major relationships within the cardiac cycle. The representative pressure volume loops show a leftward and downward shift for the SD rats. A decrease in ventricular volumes and pressures was observed in SD rats compared to SC rats. Stroke work is defined as the area inside the loop and

qualitatively SD rats had the lowest stroke work compared to the other groups. Aerobic exercise increased volumes and pressures for control and diabetic rodents, which was evident as the rightward and/or upward shift in the pressure volume loop. A comparison of the pressure volume loops from ED and SC rats showed striking similarities in pressures, volumes, and stroke work. EC rats had the greatest volume and stroke work of the four animal groups.

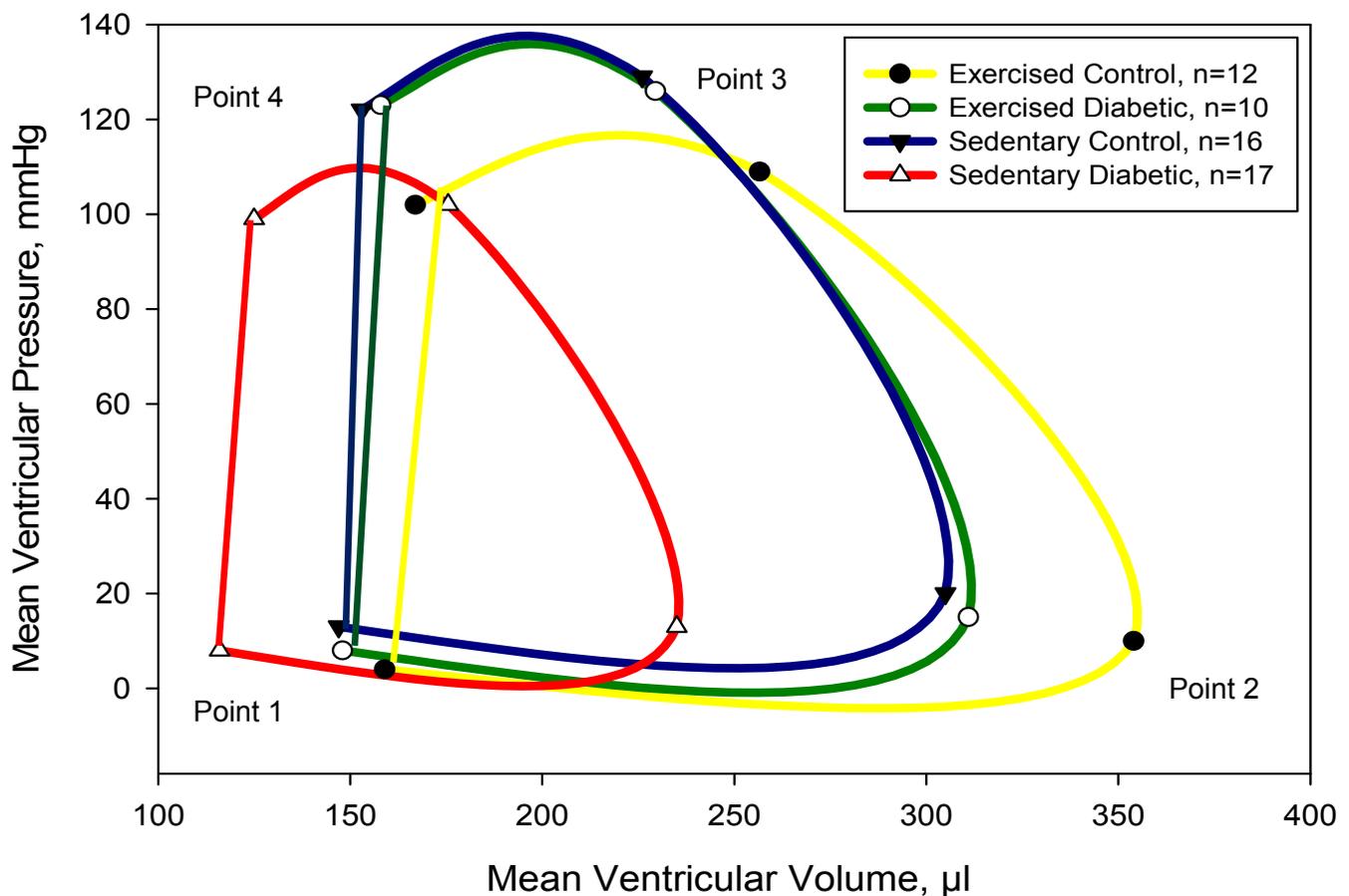


Figure 2 Representative pressure volume loops collected from rats in sedentary and exercised control and sedentary and exercised diabetic groups. Sedentary diabetic rats had the lowest ventricular volumes and pressures compared to the other groups. Pressure volume loops were based on four relationship points (mean values): Point 1: P_{min} and minimum volume; Point 2: End diastolic volume and pressure; Point 3: P_{max} and the midpoint of end diastolic volume-minimum volume; Point 4: End systolic pressure and volume. Sedentary control (n=16), sedentary diabetes (n=17), exercised control (n=12), and exercised diabetes (n=10).

3.4.2.2 Selected measures of cardiac performance

As depicted in Figure 3, bradycardia was observed in the SD rats, $F(3,42)=4.78$, $p\leq 0.01$, compared to SC rats, but heart rate was normalized with exercise training in diabetes (SD vs ED, $p\leq 0.01$). SVI, COI and SWI were lower for SD rats ($p\leq 0.001$) when compared to SC rats, $F(3,42)=25.44$, $F(3,42)=15.03$, $F(3,42)=23.11$, respectively in Figures 4-6. After seven weeks of training, the ED group differed from the SD animals ($p\leq 0.001$), and had similar indices compared to the SC group ($p=0.63$, 0.98 and 0.99). In Table 3, no difference was observed in maximal power between SC and SD rats, $F(3,42)=5.40$, $p=0.15$. However, maximal power was two times higher in the ED rats (SD vs ED, $p\geq 0.001$). Preload adjusted maximal power was not affected by diabetes or exercise, $F(3,42)=2.18$, $p=0.10$. Peripheral arterial resistance was similar for the SC, SD and ED rats as evident by arterial elastance measurements, $F(3,42)=3.77$, $p\geq 0.05$.

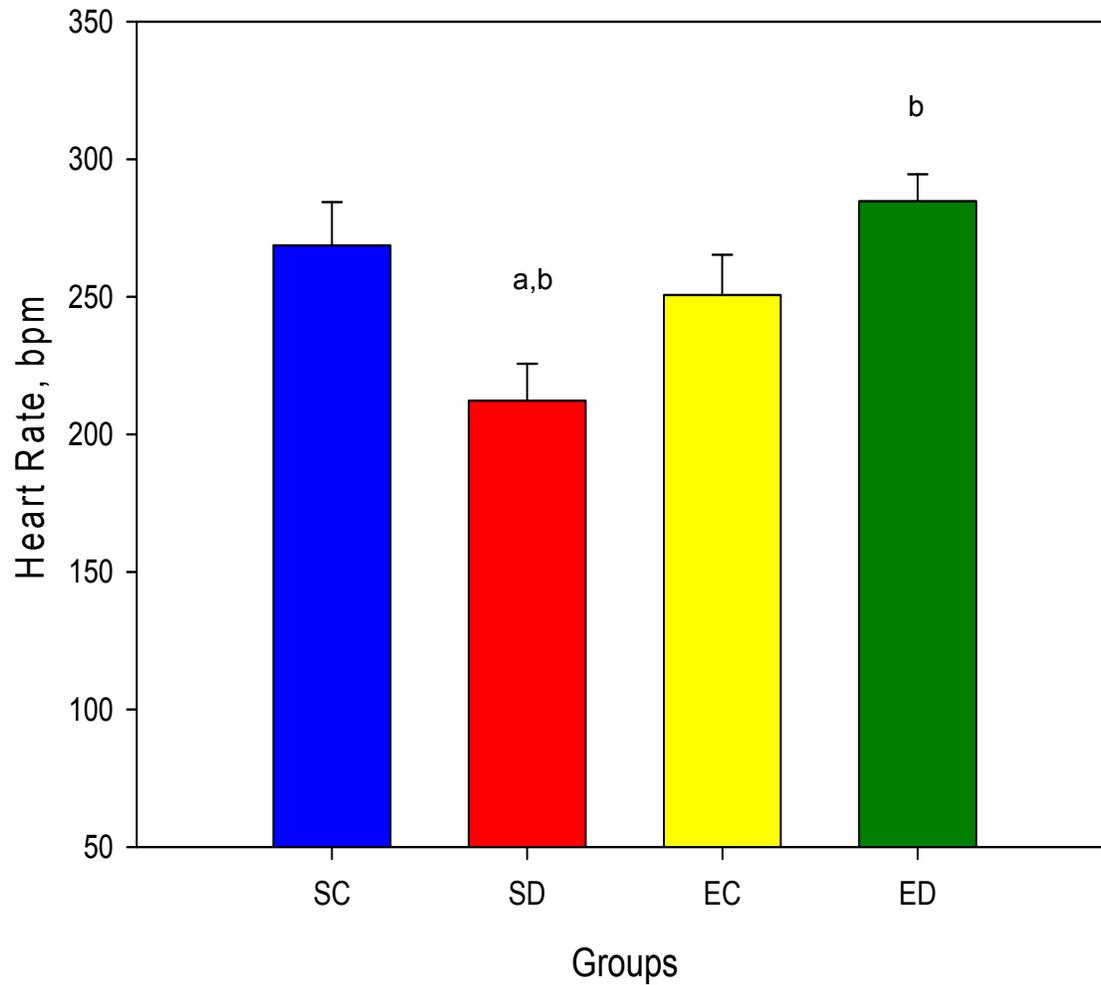


Figure 3 Heart rate of rats in sedentary (SC, n=13) and exercised (EC, n=11) controls and sedentary (SD, n=14) and exercised diabetic (ED, n=8) groups collected at termination. Rats in the SD group had lower heart rates than the rats in the SC and the exercised groups. Exercise increased heart rate in diabetic rats. ^aSC vs SD, ^bSD vs ED.

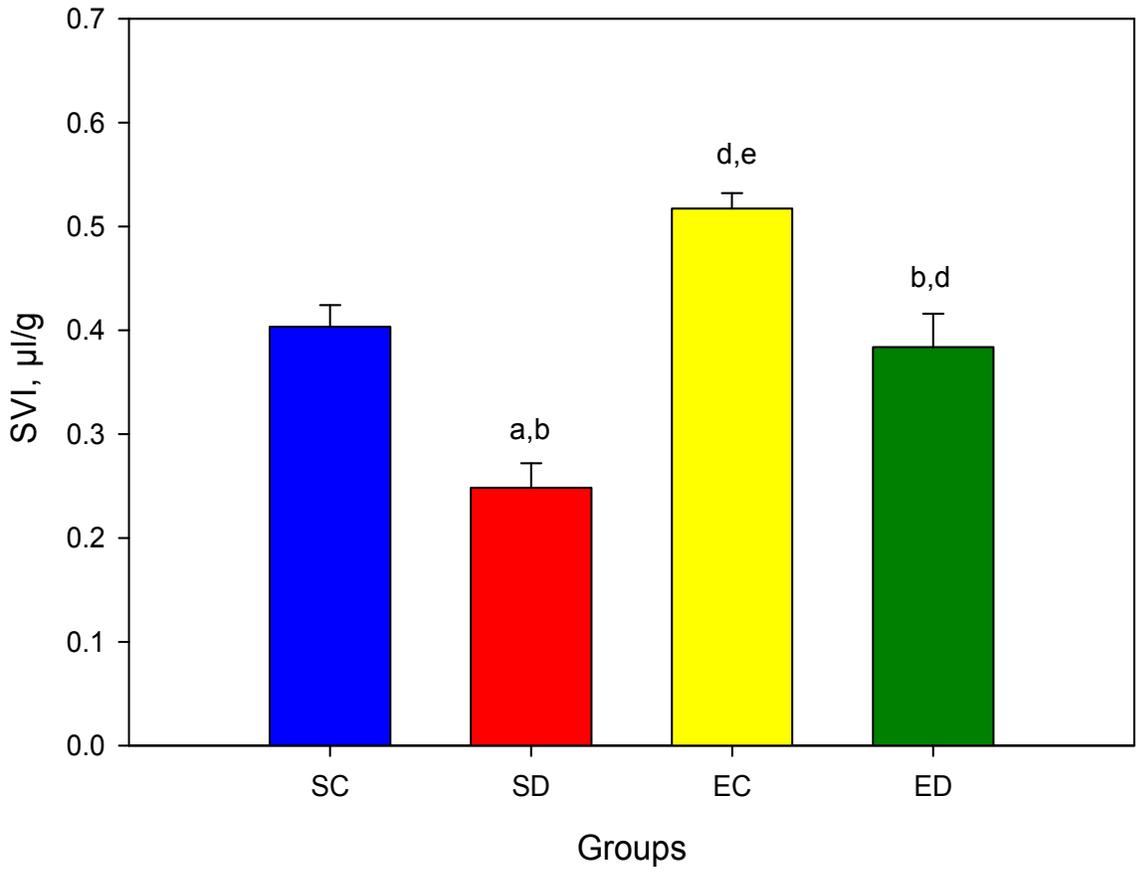


Figure 4 SVI calculated from SV adjusted by rat body weight. Rats in the sedentary diabetic (SD, n=14) group had lower SVI than the rats in the sedentary control (SC, n=13) and the exercised control (EC, n=11) and diabetic (ED, n=8) groups. Exercise improved SVI in diabetic rats. ^aSC vs SD, ^bSD vs ED, ^dEC vs ED, ^eSC vs EC.

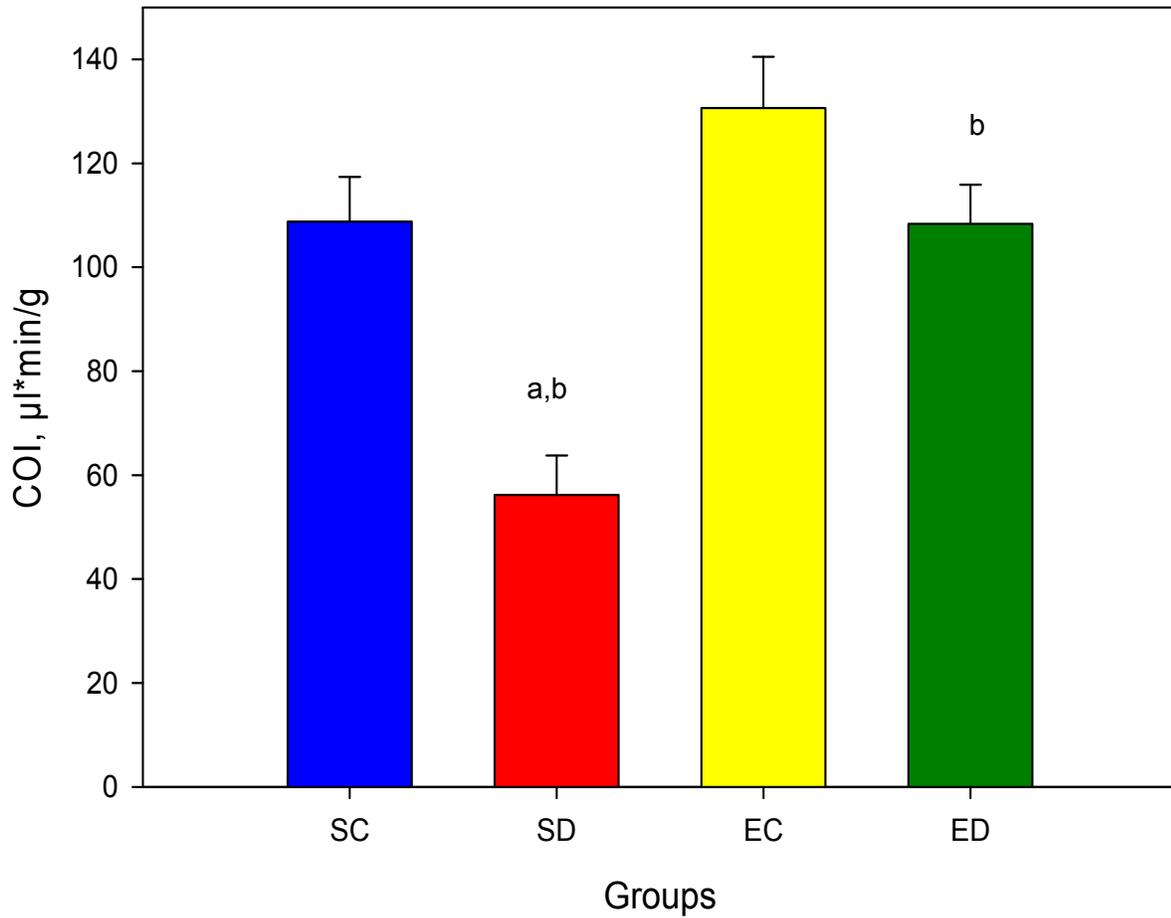


Figure 5 COI calculated from CO adjusted by rat body weight. Rats in the sedentary diabetic (SD, n=14) group had lower COI than the rats in the sedentary control (SC, n=13) and the exercised control (EC, n=11) and diabetic (ED, n=8) groups. Exercise had a positive effect on COI in diabetic rats. ^aSC vs SD, ^bSD vs ED.

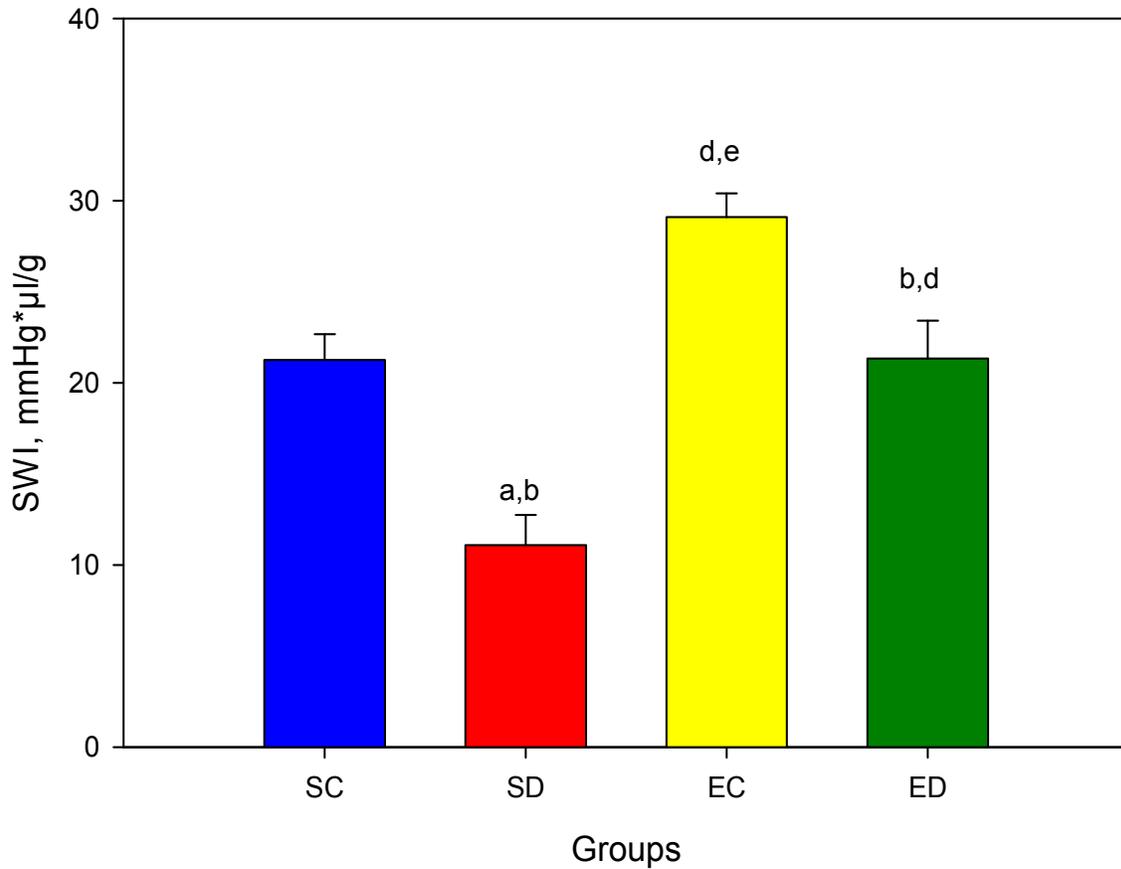


Figure 6 SWI calculated from SW adjusted by rat body weight. Rats in the sedentary diabetic group (SD, n=14) had lower SWI than the rats in the sedentary control (SC, n=13) and the exercised control (EC, n=11) and diabetic (ED, n=8) groups. Exercise increased SWI in diabetic rats. ^aSC vs SD, ^bSD vs ED, ^dEC vs ED, ^eSC vs EC.

	<i>Sedentary Control</i> (SC, n=13)	<i>Sedentary Diabetic</i> (SD, n=14)	<i>Exercised Control</i> (EC, n=11)	<i>Exercised Diabetic</i> (ED, n=8)
Maximal power, watts	42±4	30±5 ^b	53±6	65±11 ^{b,c}
Preload adjusted maximal power, watts	10±0.8	13±2	9±0.8	16±8
Arterial elastance, mmHg/ul	2.1±0.2	2.1±0.2	1.2±0.1 ^{d,e}	2.0±0.3 ^d

Table 3 Selected LV hemodynamic parameters of rats in sedentary (SC) and exercised (EC) control and sedentary (SD) and exercised diabetic (ED) groups. Maximal power, preload adjusted maximal power, and arterial elastance were not affected by diabetes. Maximal power was two times higher with exercise training. ^bSD vs ED, ^cSC vs ED, ^dEC vs ED, ^eSC vs EC.

3.4.2.3 Diastolic filling

After isovolumic relaxation, the P_{\min} of the left ventricle was similar for SD and SC rats as noted in Table 4, $F(3,42)=1.50$, $p=0.23$. P_{\min} indicates the relaxation state of the ventricle and a lower resting value would have allowed for greater diastolic filling. SD rats had a 21% decrease in resting volume (minimum volume, Figure 7) compared to the SC group, $F(3,42)=6.38$, $p\leq 0.001$. A loss of compliance in the diseased ventricle may have accounted for the decreased minimum volume. The rate of volume change, dV/dt_{\max} , signifies the filling of ventricle over time and was comparable between SC and SD rats, $F(3,42)=5.16$, $p=0.16$, (Table 4). Changes in pressure during the interval were not different between SD and SC rats, $F(3,42)=3.91$, $p=0.14$. At the completion of filling, end diastolic pressures were unchanged by diabetes, $F(3,42)=1.50$; $p=0.23$. Diastolic dysfunction was observed in the SD rats with end diastolic volumes decreased by 23% as compared to SC rats, $F(3,42)=11.26$; $p\leq 0.001$, (Figure 8).

	<i>Sedentary Control (SC, n=13)</i>	<i>Sedentary Diabetic (SD, n=14)</i>	<i>Exercised Control (EC, n=11)</i>	<i>Exercised Diabetic (ED, n=8)</i>
P _{min} , mmHg	13±5	8±2	4±0.42	8±1
dV/dt _{max} , ul/sec	4322±744	3347±348	6018±437 ^{d,e}	3570±292 ^d
P@dV/dt _{max} , mmHg	55±9	41±6	21±3 ^e	37±10
End diastolic pressure, mmHg	20±5	13±2	10±0.61	15±2

Table 4 Hemodynamic parameters related to rat LV diastolic filling. No difference was observed between SC and SD rats for either parameter. ^dEC vs ED, ^eSC vs EC.

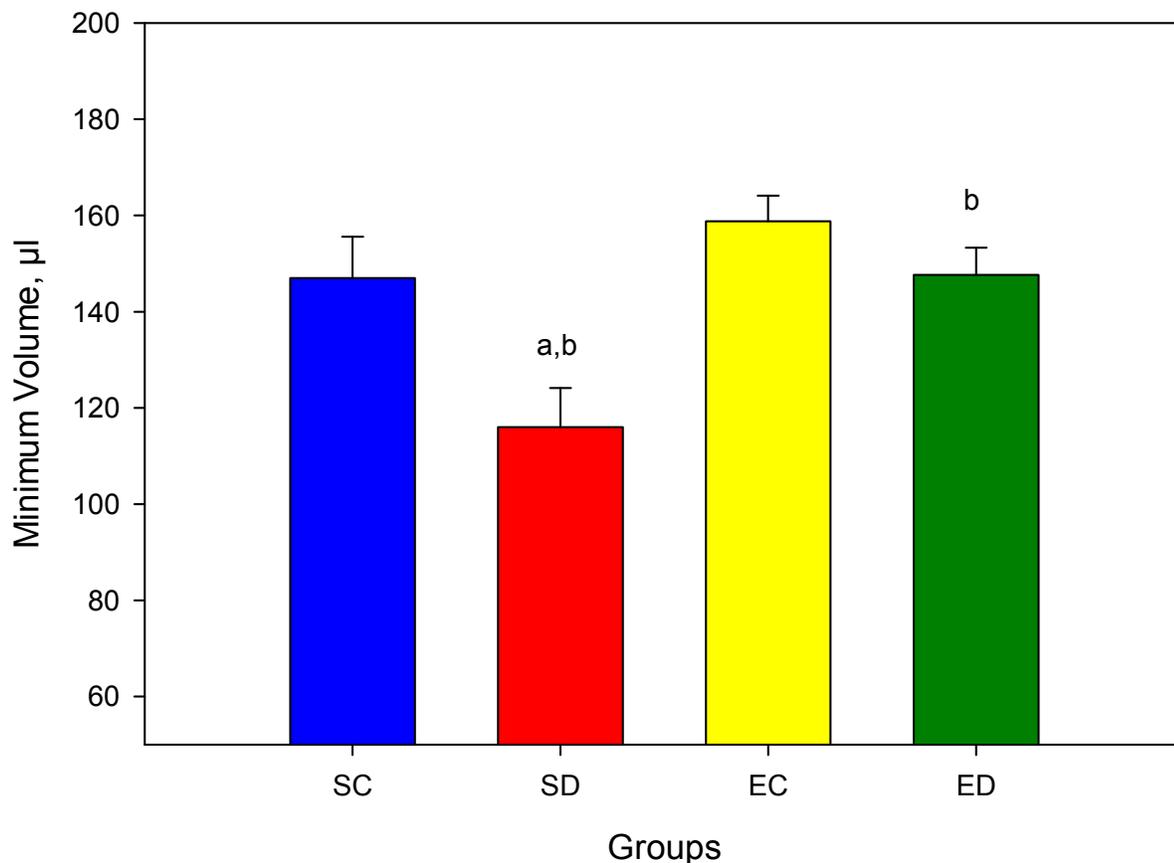


Figure 7 Rat LV minimum volumes. A difference was observed in minimum volumes between sedentary control (SC, n=13) and sedentary diabetic (SD, n=14) rats. The exercised diabetic (ED, n=8) group had larger volumes than the SD group. ED rats had levels comparable to SC and exercised control (EC, n=11) rats. ^aSC vs SD, ^bSD vs ED.

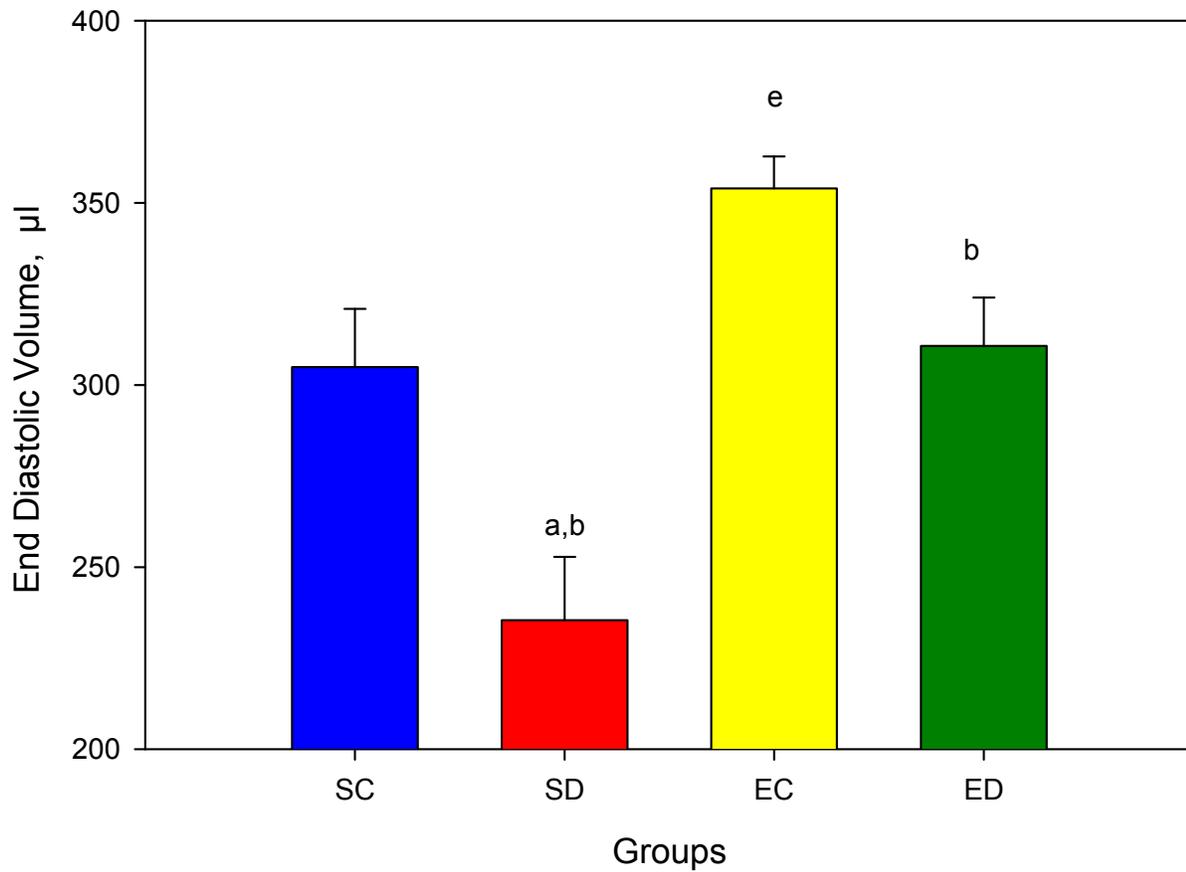


Figure 8 Rat LV end diastolic pressures volumes. A difference was observed in end diastolic pressures between sedentary control (SC, n=13) and sedentary diabetic (SD, n=14) rats. The exercised diabetic (ED, n=8) group had larger volumes than the SD group. ED rats had levels comparable to SC and exercised control (EC, n=11) rats ^aSC vs SD, ^bSD vs ED, ^eSC vs EC.

After 7 weeks of exercise training, no difference was observed in P_{\min} between SD and ED rats, ($p=0.23$, Table 4). A significant difference ($p\leq 0.01$) was observed between the minimum volumes of SD and ED rats (Figure 7). Table 4 shows that exercise influenced dV/dt_{\max} values in EC rats ($p<0.05$), but no change was observed in ED rats ($p=0.78$). SC, SD and ED rats had similar dV/dt_{\max} values (SC vs SD, $p=0.16$ and SC vs ED, $p=0.35$). $P@dV/dt_{\max}$ was also comparable for SC, SD and ED rats (SC vs SD, $p=0.14$ and SC vs ED, $p=0.11$). Likewise, end diastolic pressures were unaffected by exercise ($p=0.23$) in diabetic rats. Figure 8 showed that exercise training had a restorative effect on end diastolic volumes, with ED ventricles having higher filling capacities compared to SD rats ($p\leq 0.001$) and similar values to SC rats ($p=0.81$). Our results indicate that diastolic dysfunction is present at 19 weeks of age in the ZDF rats and 7 weeks of exercise training improved LV compliance as evident by increased end diastolic volumes.

3.4.2.4 Isovolumic contraction

The initial velocity of the ventricular contraction, dP/dt_{\max} , an indicator of contractility is reported in Table 5. A 40% reduction in dP/dt_{\max} was observed in SD rats as compared to SC rats, $F(3,42)=12.12$ $p\leq 0.001$, with lower volumes and pressures also at this measurement (Figures 9-10). Calculated from the negative slope of the QRS wave, dV/dt_{\min} values indicated no difference between SC and SD rats, $F(3,42)=2.06$, $p=0.12$.

Exercise showed a trend toward providing compensation for decreased diabetic end diastolic volumes with increases in dP/dt_{\max} , but lacked significance ($p=0.07$, Table 5). In Figure 9, a 24% difference was observed in volumes at dP/dt_{\max} between SD and

ED groups ($p \leq 0.01$). Exercise produced 21% increase in $P@dP/dt_{max}$ in EC rats when compared to SD rats in Figure 10 ($p \leq 0.05$). In Table 5, exercise had no effect on the decay of the ventricular contraction (dV/dt_{min} , $p=0.12$) in control or diabetic rats.

	<i>Sedentary Control</i> (SC, n=13)	<i>Sedentary Diabetic</i> (SD, n=14)	<i>Exercised Control</i> (EC, n=11)	<i>Exercised Diabetic</i> (ED, n=8)
dP/dt_{max} , mmHg/sec	9219±536	5591±456 ^a	8022±248	6919±600 ^c
dV/dt_{min} , ul/sec	-4367±765	-3899±504	-5601±565	-5787±625

Table 5 Rat LV hemodynamic measures related to isovolumic contraction. No difference was observed between SC and SD rats for either parameter. ^aSC vs SD, ^cSC vs ED.

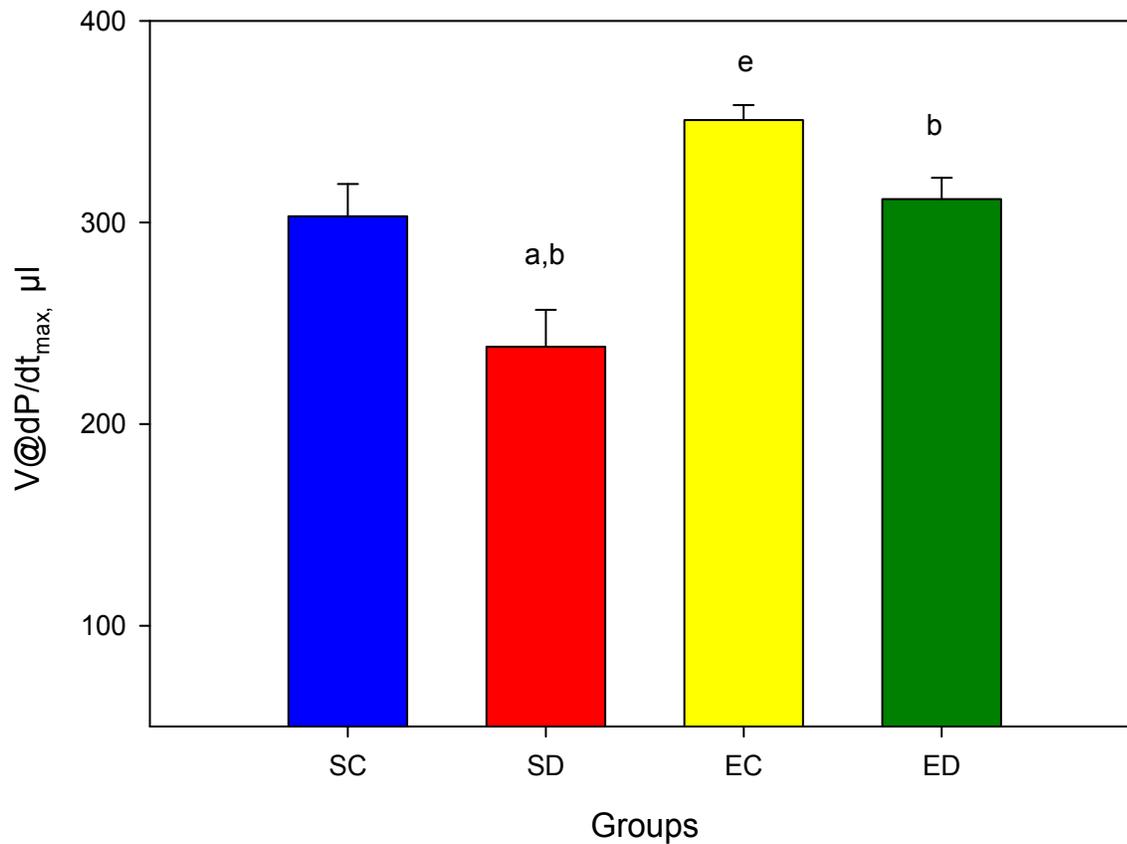


Figure 9 Rat LV $V@dP/dt_{max}$. A difference was observed between sedentary control (SC, n=13) and sedentary diabetic (SD, n=14) rats. The exercised diabetic (ED, n=8) group had larger volumes than the SD group. ED rats had levels comparable to SC and exercised control (EC, n=11) rats. ^aSC vs SD, ^bSD vs ED, ^eSC vs EC.

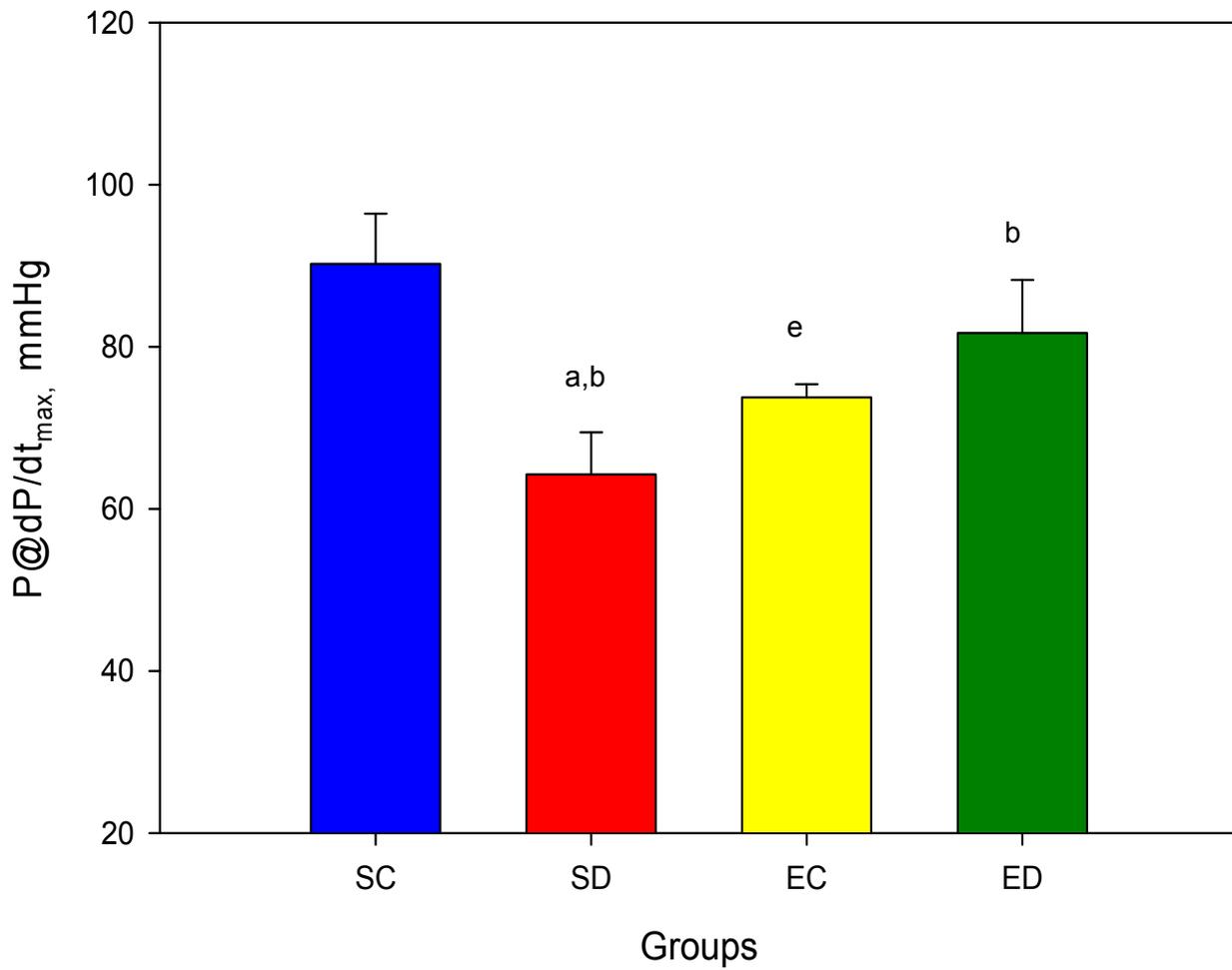


Figure 10 Rat LV P@dP/dt_{max}. A difference was observed in between sedentary control (SC, n=13) and sedentary diabetic (SD, n=14) rats. Exercised diabetic (ED, n=8) rats had higher pressure levels than the SD group. EC - exercised control (n=11). ^aSC vs SD, ^bSD vs ED, ^eSC vs EC.

3.4.2.5 Ejection

After the isovolumic contraction, ventricular pressure quickly rises to push blood into the aorta. Maximum pressures in the SD left ventricles were significantly lower than SC rats, $F(3,42)=5.45$, $p\leq 0.001$, as measured by the highest point of the pressure volume loop (Figure 2) and presented as P_{\max} values (Figure 11). As the ventricle completed the contraction phase, end systolic pressures and volumes were decreased 18-19% in SD rats compared to control rats, $F(3,42)=5.70$, $p\leq 0.01$ and $F(3,42)=4.85$, $p\leq 0.01$ (Figures 12a-b). SD rats demonstrated lower ejection fractions ($46\pm 1\%$) compared to SC rats ($50\pm 0.9\%$), $F(3,42)=5.32$, $p\leq 0.05$.

Exercise improved cardiac function in diabetic rats through increases in P_{\max} . A 17% increase was observed in ED rats compared to the SD group ($p\leq 0.001$, Figure 11). Exercise enhanced end systolic pressures and volumes (Figures 12a-b) compared to SD rats. Levels were similar to SC values ($p= 0.69$ and 0.87 , respectively). Exercise did not improve ejection fraction in ED rats ($49\pm 6\%$, $p=0.14$). It appeared that exercise increased ventricular pressures and end systolic volume. However, these changes did not equate to improvements in ejection fractions.

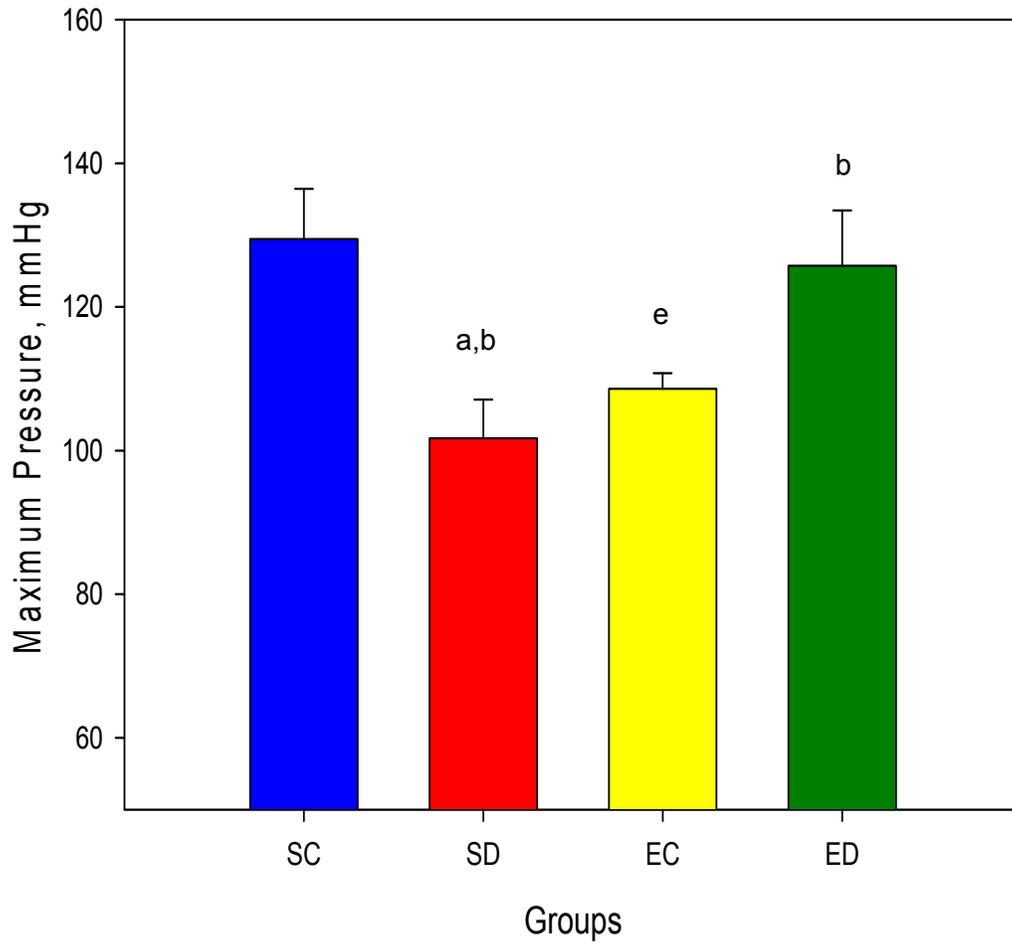
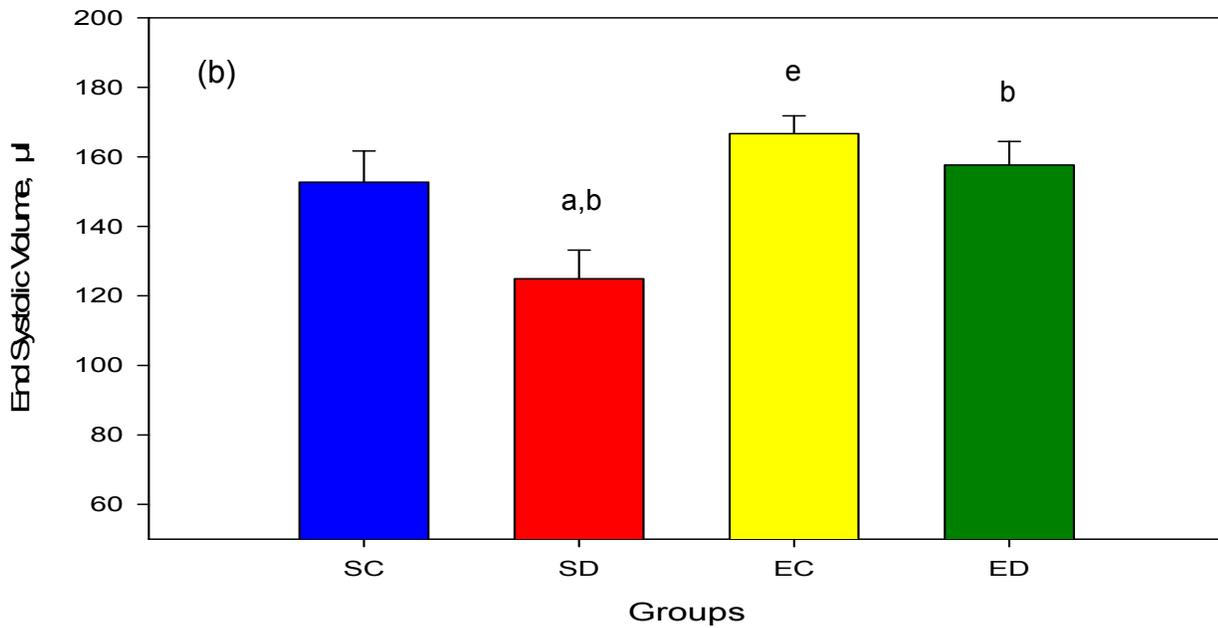
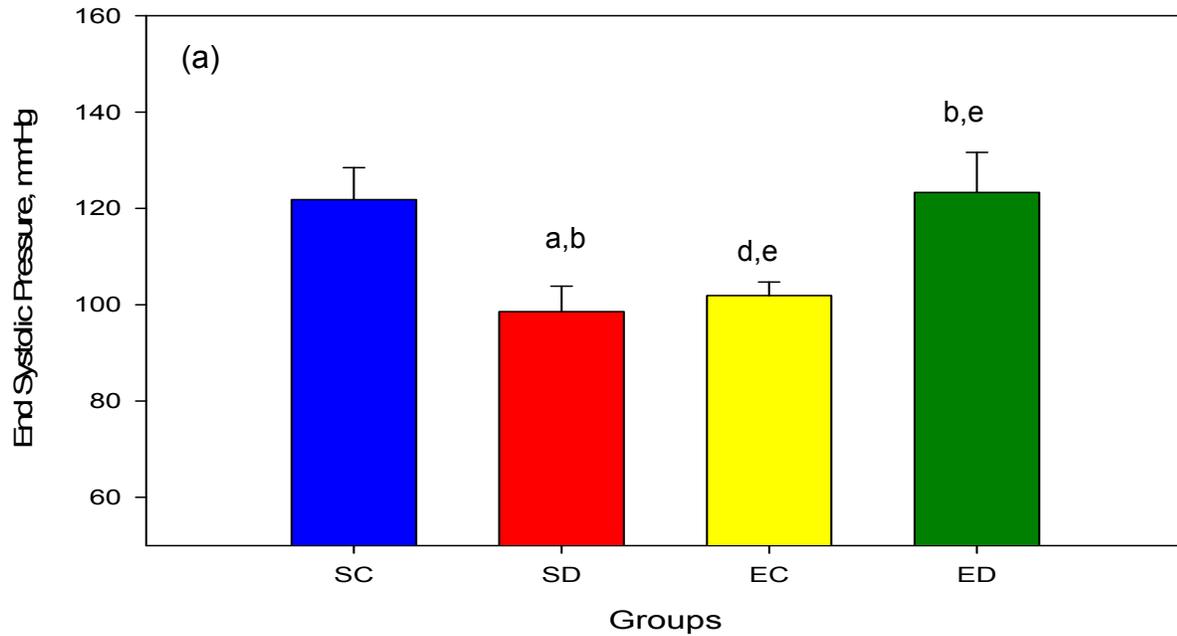


Figure 11 Rat LV maximum pressure. Sedentary diabetic (SD, n=14) rats had the lowest maximum pressure values. Maximum pressures increased significantly for exercised diabetic (ED, n=8) rats. SC - sedentary control (n=13), EC - exercised control (n=11). ^aSC vs SD, ^bSD vs ED, ^eSC vs EC.



Figures 12 Rat LV end systolic pressures (a) and volumes (b). Sedentary diabetic (SD, n=14) rats showed reduced end systolic pressures and volumes. Exercise improved both measures for exercised diabetic (ED, n=8) rats. SC - sedentary control (n=13), EC - exercised control (n=11). ^aSC vs SD, ^bSD vs ED, ^dEC vs ED; ^eSC vs EC.

3.4.2.6 Isovolumic relaxation

Impaired relaxation was present in SD rats with dP/dt_{\min} levels 59% of those seen in the SC group, $F(3,42)=4.45$, $p\leq 0.01$ (Figure 13). Volumes at dP/dt_{\min} were also reduced in the SD rats, (Figure 14), $F(3,42)=5.44$, $p\leq 0.01$. However, the isovolumic relaxation constant, tau, was similar across SC (24 ± 5 msec) and SD (28 ± 3 msec) groups, $F(3,42)=0.25$, $p=0.86$.

A significant difference was observed in dP/dt_{\min} ($p\leq 0.05$) and $V@dP/dt_{\min}$ ($p\leq 0.001$) between SD and ED groups. Tau values were unchanged with exercise in the ED cohort (26 ± 6 msec, $p=0.71$).

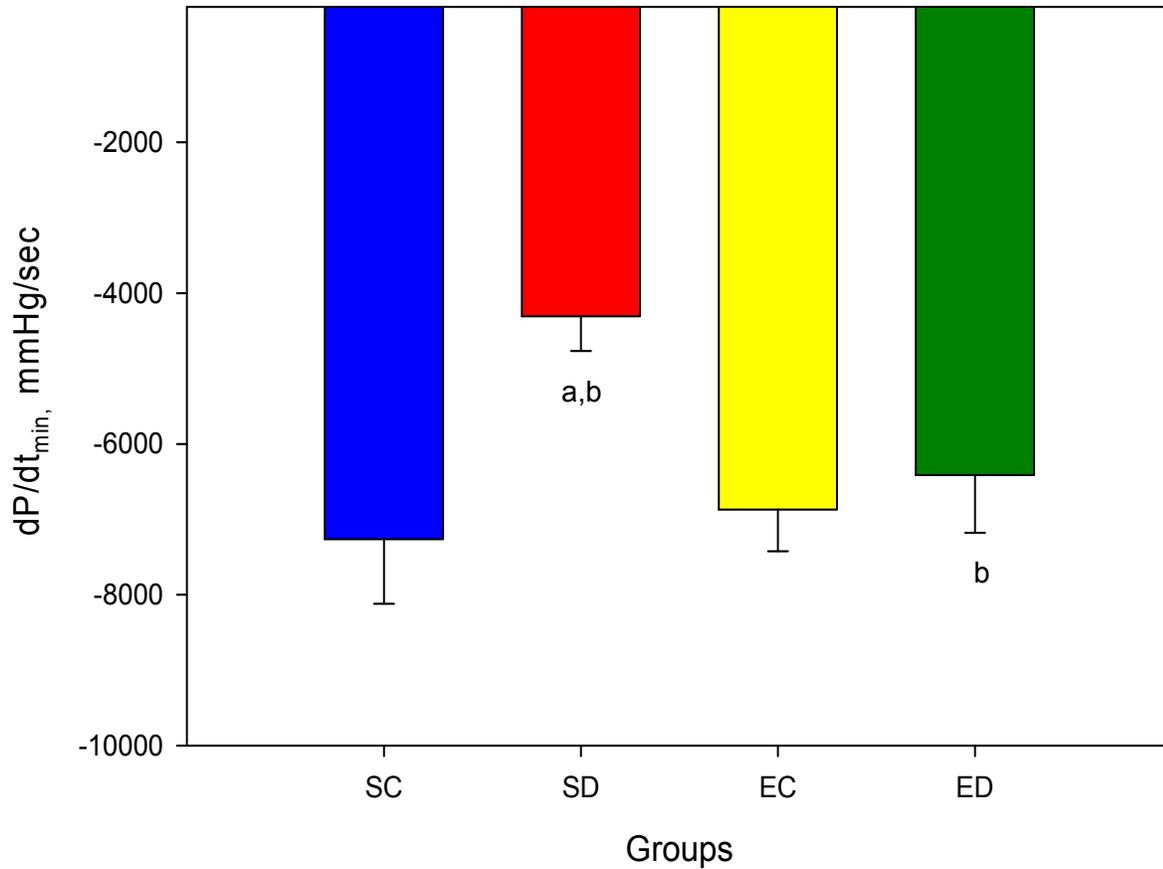


Figure 13 Rat LV dP/dt_{min}. Sedentary diabetic (SD, n=14) rats had lower values than sedentary control (SC, n=13) rats. Exercise restored levels in diabetes (ED, n=8) to that of SC and exercised control (EC, n=11) rats. ^aSC vs SD, ^bSD vs ED.

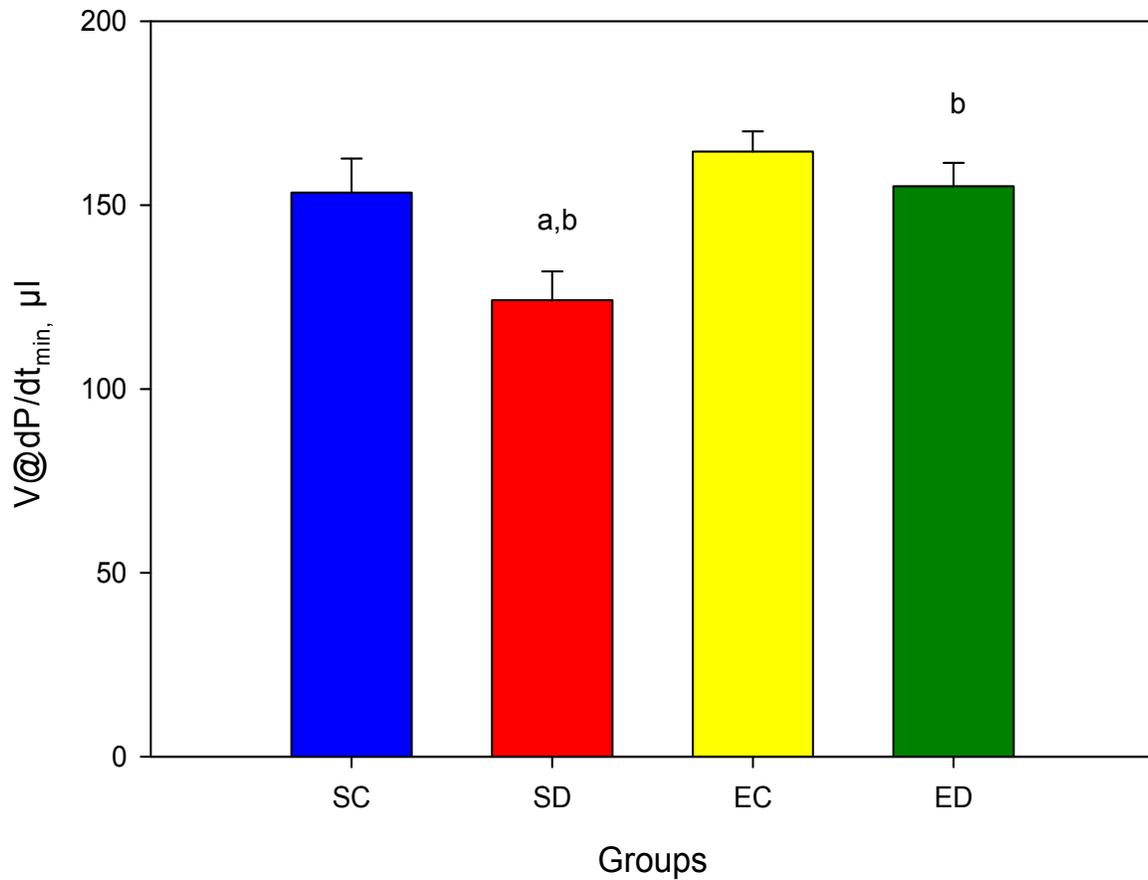


Figure 14 Rat LV $V@dP/dt_{min}$. Volume was decreased for sedentary diabetic (SD, n=14) rats compared to the sedentary control (SC, n=13) group. Exercise significantly improved the volume levels in diabetic (ED, n=8) rats. EC - exercised control (n=11). ^aSC vs SD, ^bSD vs ED.

3.4.2.7 dP/dt_{max} and end diastolic volume

Due to the loading dependency of dP/dt_{max} , an assessment of the relationship between dP/dt_{max} and EDV was used to nullify the preload effect. Figure 15 summarizes the cardiac function of the four rat groups. The flat fit line of the SC rats indicates that the relationship between dP/dt_{max} and end diastolic volume was unpredictable and unrelated. The mean dP/dt_{max} value was 9219 ± 526 mmHg/sec with a mean end diastolic volume of 305 ± 14 μ l. The SC rats demonstrated increased contractility to maintain cardiac function. SD rats were unable to compensate as shown by decreases in end diastolic volume (235 ± 14 μ l) and declines in dP/dt_{max} (5591 ± 456 mmHg/sec). These findings suggested that SD rats had losses in systolic and diastolic function. The fit line indicated that 75% of variability in dP/dt_{max} was the result of the end diastolic volume. EC rats displayed the highest levels of cardiac efficiency with the highest mean end diastolic volume (354 ± 9 μ l) and lower contractility measures than other groups. Exercise in the diabetic group also improved diastolic and systolic function through the Frank-Starling mechanism with a rightward shift in volume measurements (311 ± 13 μ l). Contractility also improved in ED rats with mean dP/dt_{max} values of 6919 ± 600 mmHg/sec, which was higher than in SD rats. Our results supported the presence of systolic and diastolic dysfunction in the ZDF rat model and the protective benefits of exercise training on both components.

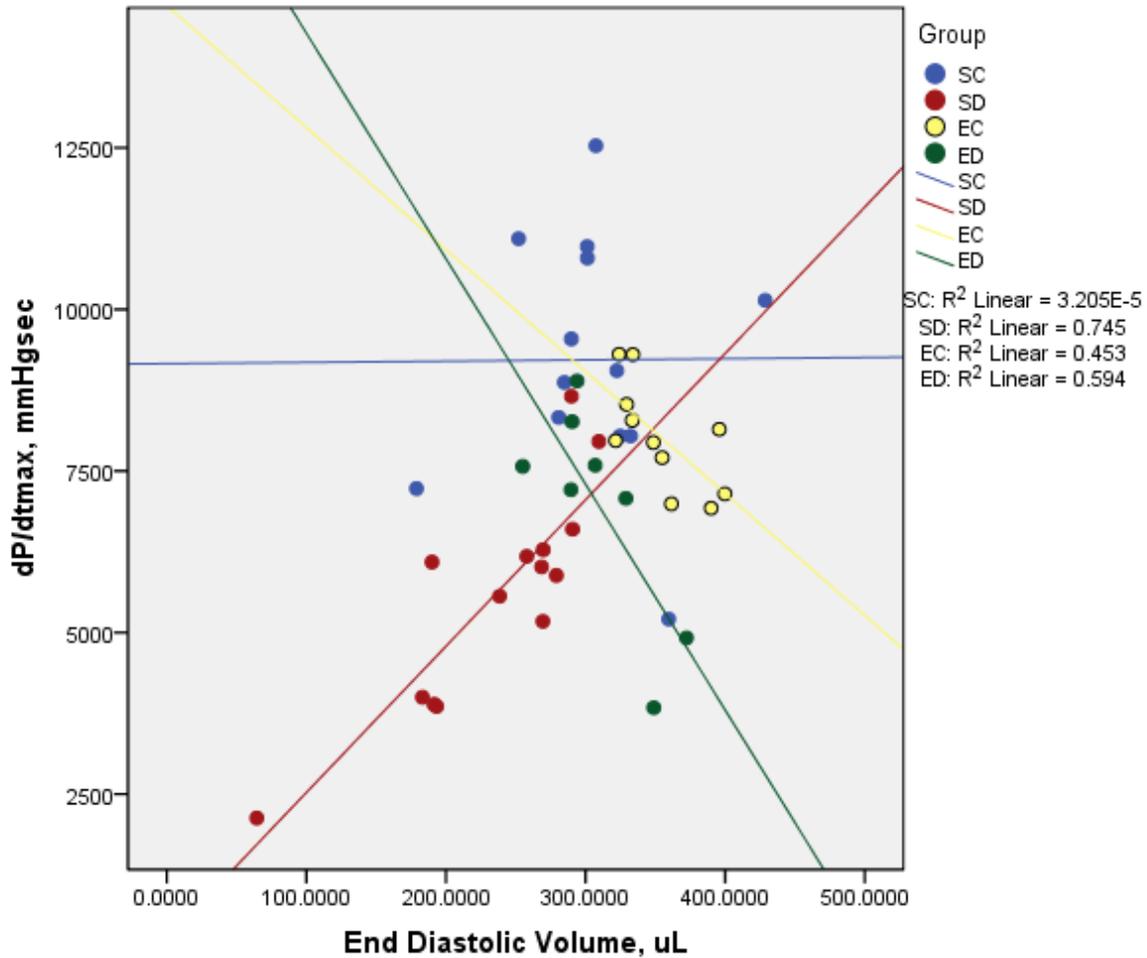


Figure 15 Rat dP/dt_{max} and end diastolic volume relationship. Sedentary diabetic (SD, n=14) rats had the lowest dP/dt_{max} and end diastolic volumes, which improved with exercise in the exercised diabetic (ED, n=8) rats. SC - sedentary control (n=13), EC - exercised control (n=11).

3.5 Discussion

Exercise training has proved to be a non-invasive intervention, which can attenuate the progression of diabetic heart disease. **Diabetes had a negative impact on 15 of the 24 hemodynamic parameters** evaluated in our study. Table 6 shows that **exercise training restored 13 of those 15 hemodynamic parameters** in our model. Our findings supported the hypothesis that exercise training could reduce pressure volume changes indicative of diabetic heart disease in the ZDF rat model.

<i>Hemodynamic Parameters</i>	<i>Affected by diabetes</i>	<i>Improved with exercise</i>
Heart rate	Yes	Yes
SVI	Yes	Yes
COI	Yes	Yes
SWI	Yes	Yes
Maximal power	No	Yes*
Preload adjusted maximal power	No	No
Arterial elastance	No	No
<i>Diastolic filling</i>		
P_{min}	No	No
Minimum volume	Yes	Yes
dV/dt_{max}	No	No
$P@dV/dt_{max}$	No	No
End diastolic pressure	No	No
End diastolic volume	Yes	Yes
<i>Isovolumic Contraction</i>		
dP/dt_{max}	Yes	No
$P@dP/dt_{max}$	Yes	Yes
$V@dP/dt_{max}$	Yes	Yes
dV/dt_{min}	No	No
<i>Ejection</i>		
P_{max}	Yes	Yes
End systolic pressure	Yes	Yes
End systolic volume	Yes	Yes
Ejection fraction	Yes	No
<i>Isovolumic Relaxation</i>		
dP/dt_{min}	Yes	Yes
$V@dP/dt_{min}$	Yes	Yes
Tau	No	No

Table 6 Summary of results. Diabetes affected 15 of the 24 hemodynamic parameters. Exercise improved 13 of the 15 parameters. Exercise increased maximal power, although it was not affected by diabetes (*).

3.5.1 The impact of exercise on diabetic bradycardia

Frank-Starling's law of the heart states that the normal heart will respond to pressure and volume overloads. Research has shown that the heart will adjust

hemodynamics to maintain cardiac output (Solaro, 2007). Autonomic regulation and contractility are the principal components of cardiac output. SD rats had lower heart rates compared to SC rats, which has been reported in other investigations of 19 week old ZDF rats (Shoghi et al., 2008). The **low heart rate observed in the SD rats** has been attributed to the sinoatrial node alterations associated with disease progression (De Angelis et al., 2000; Li, Culver, and Ren, 2003; Schafer et al., 2006). Changes in heart rate have also been identified in Zucker lean and ZDF rats from the ages of 12-24 weeks indicating a possible effect of aging. However, an independent effect of diabetes on heart rate was also determined in that study (Cosson et al., 2009). Exercise training induced a tachycardiac response in ED rats possibly due to improved autonomic cardiac regulation (Souza et al., 2007). β 1-adrenoceptor expression has been shown to increase in rat models of type 1 diabetes with exercise and to result in improved cardiac function (Bidasee et al., 2008; LaHaye, 2011). Our investigation revealed that **exercise training returned heart rate in ZDF rats to control rat levels**. The definite mechanism related to the cardioprotective effects of exercise on heart rate is still speculated in the literature.

3.5.2 The impact of exercise on systolic function in diabetes

Natural adjustments in ventricular pressures and volumes during the cardiac cycle are identified as possible resolutions for preserving cardiac function. In Figure 11, SC rats compensated for losses in stroke volumes with elevations of P_{max} and increases in preload. This rightward shift of the pressure volume loop (Figure 2) of the SC rats demonstrates the ability to deliver a larger stroke volume, which maintained cardiac output. However, biochemical and morphological changes in the diabetic heart limited

cardioprotective adaptations to pressure and volume demands (Castoldi et al., 2010; Westermann et al., 2009). We observed a lack of compensation through the Frank-Starling mechanism in the SD pressure volume loop with decays in systolic and diastolic measures, predominantly pressure values in the 19 week old rats (Figures 10, 11, 12a). Contrary to our findings, other authors have reported no difference in cardiac output between 9-11 week old ZDF and lean Zucker rats (Serpillon et al., 2009). An assessment of 16 and 36 week old ZDF rats showed adaptations in the stroke volume of 16 week old rats (Baynes and Murray, 2009). This compensatory mechanism was lost in the older rats (Baynes and Murray, 2009). Our results indicate that the diabetic heart could not maintain function via the Frank-Starling mechanism because the heart was incapable of producing the necessary pressures or contractility. The effect of diabetes on fractional shortening has been debated in the literature. Fractional shortening has not been observed in 18 week old ZDF rats (Shoghi et al., 2008), but was overt in 9 month old rats (Toblli et al., 2010). An inverse relationship between fractional shortening and the type 2 ryanodine receptor function has been described in the left ventricle of STZ rats (Shao et al., 2009). The ryanodine receptor is responsible for the release of calcium, which is needed for contractility (Fill and Copello, 2002). Altered calcium handling impaired preload and adaptive abilities in diabetic heart disease. Exercise training improved systolic function through type 2 ryanodine receptor stability and calcium utilization in diabetic rats (LaHaye, 2011; Shao et al., 2009). An analysis of the cardiac function of 8 month old ZDF rats also revealed reductions in contractility. These rats had significant declines in dP/dt_{max} compared to lean controls (Burgdorf et al., 2009). Exercise was able to return dP/dt_{max} values of the ED rats to

levels comparable to SC rats in our experiments. Our study indicates that **stroke volume and contractility were impaired in 19 week old sedentary ZDF rats, but were improved with 7 weeks of treadmill training** along with subsequent increases in cardiac output.

3.5.3 The impact of exercise on diastolic function in diabetes

Diastolic dysfunction is the earliest cardiac defect seen in diabetes, which is characterized by slower rates of myocardial relaxation and decreased compliance (Boudina and Abel, 2010; Lalande and Johnson, 2008). **Diastolic changes with diabetes were noted in the current study as reductions in end diastolic volume and dP/dt_{\min}** (Figures 8 and 13). Morphological changes are evident in 11 week old ZDF rats as they exhibited increases in LV diameter, which may be due to volume overload (Serpillon et al., 2009). Echocardiographic studies of ZDF rats of the same age (19 weeks old) reported no cardiac dilation based on measurements of inner diastolic diameters (Fredersdorf et al., 2004). However, other researchers have observed differences in inner diastolic diameter due to aging and diabetes in the ZDF and Zucker lean rats (Shoghi et al., 2008). A leftward shift in pressure volume loops (Figure 2) of the SD rats has been suggested to indicate poor ventricular filling (Villars et al., 2004). Impairments of dP/dt_{\min} and tau have been reported in 37 week old ZDF rats without changes in end diastolic volumes and pressures (Schafer et al., 2006). This finding contradicted the reports of increases in end diastolic volumes and pressures in ZDF rats with aging (Baynes and Murray, 2009). The authors postulated that diastolic dysfunction may be contested by LV dilation, which presented as increases in end diastolic volumes and pressures in the ZDF rat (Baynes and Murray, 2009). The **seven week aerobic**

exercise training regimen in our study did reverse diastolic debilities in our model as shown by 30% improvement in end diastolic volumes.

3.5.4 Mechanisms of diabetic heart disease and possible exercise targets

Many factors are associated with diastolic dysfunction in the ZDF rat, but a single causative factor has yet to be determined and is probably unrealistic due to the complexity of the disease. Hyperglycemia has been reported as the primary trigger for ventricular remodeling and dysfunction (Rodrigues, Cam, and McNeill, 1998). Glucose dysregulation in diabetes may be the result of poor protein kinase B regulation in the myocardium (Lajoie et al., 2004). Phosphorylation of protein kinase B was partially improved with 13 weeks of swimming in the ZDF rat. Exercise improved glycemic control (Lajoie et al., 2004), which could delay cardiac deficits and remodeling, however the study did not address cardiac function of the rats. In our model, we did not observe changes in hyperglycemia for the ED rats although the beneficial effect of exercise on heart performance was evident.

Myocardial wall thickness and fibrosis may have attenuated the lusitropic or relaxation ability of the diseased ventricles in our rats as reported in other studies (Shoghi et al., 2008; Toblli et al., 2010). Increased collagen I deposition and perivascular fibrosis has been reported in the myocardium of 19 week old ZDF rats (Fredersdorf et al., 2004). Similar accumulations of collagen I and III have been described in cardiac muscle from 9 month old ZDF rats compared to lean controls (Toblli et al., 2010). Cardiomyocyte diameters are also widened according to histological assessments (Fredersdorf et al., 2004). However, aging has been associated with increases in left ventricular mass in 19 week old lean and ZDF rats. A difference

between control and diabetic heart weights was not reported for ZDF rats at 19 weeks of age (Shoghi et al., 2008).

Pressure volume loop analysis has been widely used for collecting in vivo left ventricular hemodynamic parameters. Although peripheral resistance was similar across SC and SD animal groups, hyperglycemia could increase oxidative stress leading to endothelial dysfunction of coronary vasculature (Fatehi-Hassanabad, Chan, and Furman, 2010; Wong et al., 2010). Therefore, direct measurements of peripheral vasculature changes in the ZDF rat model due to exercise should also be investigated in future studies. The cross-sectional design of the study limited the outcomes and knowledge of early diabetic heart disease and the cardioprotective effects of an exercise program. However, **our study was the first to investigate and identify improvements in pressure volume relationships with exercise in the ZDF rat model.** Additional explorations are needed to identify the mechanism(s) by which exercise training improved cardiac function.

3.6 Conclusion

This study shows that exercise can alleviate some of the detrimental functional changes that occur in the diabetic heart in a rat model of T2D. Aerobic exercise proved successful in reversing the negative impact of diabetes in 13 of 15 left ventricular hemodynamic parameters altered by diabetes. The restorative impact of aerobic exercise addressed the systolic and diastolic function commonly seen in diabetic heart disease.

Chapter 4

**Does myocardial fluid imbalance leading to edema exist
in the Zucker diabetic fatty (ZDF) rat? A hierarchical analysis**

4.1 Abstract

Introduction: Myocardial edema has been suggested as a trigger for the development and progression of cardiovascular diseases. Myocardial fluid imbalances have been implicated in the fibrosis and hypertrophy associated with common cardiovascular diseases. Diabetic heart disease is one of the primary complications of type 2 diabetes (T2D) and accounts for a significant proportion of diabetes related deaths. However, it is unknown if diabetes causes myocardial edema. Increased vascular permeability has been observed in humans and animals with diabetes through quantification of vascular endothelial growth factor C (VEGF-C). Little is known about the impact of diabetes on the lymphatic system, including effects on the principal receptors, VEGFR-3 and lymphatic endothelial receptor 1, LYVE-1. These receptors are responsible for the binding of their respective ligands, VEGF-C and hyaluronan. The expression of both receptors is regulated by prospero homeobox protein 1 (PROX-1). Through the PROX-1 signaling pathway, the cardiac lymphatic system maintains cardiac fluid homeostasis. We chose to investigate whether myocardial edema was present in the Zucker diabetic fatty (ZDF) rat through a hierarchical analysis of factors directly and indirectly suggestive of myocardial fluid imbalance.

Methods: Male ZDF and Zucker lean (control) rats were assigned to two groups. By 12 weeks of age, this animal model is characterized by marked hyperphagia, obesity, hyperlipidemia, polyuria, and polydipsia similar to the type 2 diabetes seen in humans. At the organismal level, we measured body weight and analyzed systemic inflammatory cytokine levels with a multiplex bead immunoassay. Hemodynamic alterations were determined using left ventricular catheterization and analysis of pressure volume

relationships for alterations at the organ level. We determined left ventricle dry weight to wet weight ratios for further identification of myocardial edema. Subsequently, we explored myocardial edema at the tissue level with measurements of lymphatic vessel area and levels of VEGF-C and hyaluronan in left ventricular (LV) tissue using ELISA. Finally, we explored the lymphatic signaling pathway by analyzing mRNA and protein levels of VEGFR-3, LYVE-1, and PROX-1 with quantitative RT-PCR and immunoblotting, respectively. We also determined localization of the lymphatic transcription factor, PROX-1, and examined its DNA binding activity utilizing a transcription factor filter plate assay.

Results and Conclusion: We did not observe changes in ventricular weights, a direct measure of myocardial edema, or alterations in the levels of VEGF-C, VEGFR-3, LYVE-1, or hyaluronan. However, we were able to observe systemic changes in plasma interleukin (IL)-2 levels, reductions in dP/dt_{max} , increases in lymphatic vessel area, and changes in PROX-1 protein levels and DNA binding activity that were suggestive of the presence of myocardial edema in the ZDF rat. However, these alterations are indirect measures of myocardial edema. Therefore we conclude that in the 19 week old ZDF rat, myocardial edema is not evident and may not play a role in the diabetic heart disease associated with this model.

4.2 Introduction

The heart depends heavily on complex, biological exchanges for fluid homeostasis. Subtle alterations in microvascular permeability can lead to fluid accumulation and possibly cardiac dysfunction and arrhythmias (Laine and Allen, 1991). Hyperpermeability and edema on a cellular level can impact tissue architecture and behavior that can initiate a spiral of devastating events on higher organizational levels (Miller, 1985). **Recent literature has suggested that myocardial edema may play a role in heart disease** (Miller, 2011; Nakamura and Rockson, 2008). Diabetic heart disease is the primary cause of death for persons with type 2 diabetes (T2D) (ADA, 2011). Multiple mechanisms have been identified in the development and progression of diabetic heart disease. Hyperglycemia and its related complications including oxidative stress (Gwechenberger et al., 1999) and impaired calcium handling (Mohamad, Askar, and Hafez, 2011), are implicated in the hypertrophy, contractility deficits, and impaired relaxation of the ventricles. Systolic and diastolic dysfunction can result in poor cardiac performance, physical activity declines, and fatal myocardial events (Abe et al., 2002; Alvarez et al., 2004; Artenie et al., 2003). These poor health outcomes have investigators aggressively exploring mechanisms and possible interventions. Therefore, **research is needed to identify if myocardial edema may be a novel target** for the prevention and attenuation **of diabetic heart disease**.

Myocardial edema can result from structural and biochemical changes in the cardiac vascular and lymphatic systems (Laine and Granger, 1985; Miller, 1985). Diabetes has been associated with leaky blood vessels due to increases in vascular endothelial growth factor C (VEGF-C) (Kivela et al., 2007). VEGF-C is normally

removed from the interstitium by its receptor VEGFR-3, whose expression is regulated by prospero homeobox protein 1 (PROX-1), a lymphatic transcription factor (Partanen and Paavonen, 2001). It is unknown whether diabetes disrupts this defense mechanism and thereby provides an impetus for myocardial edema. External stresses, such as obesity, inflammation, and cardiac dysfunction (Laine and Allen, 1991), can also exacerbate the fluid load of the myocardium. Hyaluronan is a large glycosaminoglycan molecule and component of the extracellular matrix, which plays a role in inflammation and interstitial edema (Laurent and Fraser, 1992). Hyaluronan fragments, appearing after degradation of the glycosaminoglycan by specific proteases, are commonly increased with obesity and diabetes and are associated with inflammation (Lewis et al., 2008; Nanji et al., 1996). The accumulation of hyaluronan fragments can occur from the above mentioned conditions or via decreased uptake by the lymphatic endothelial receptor 1, LYVE-1, whose expression is regulated by PROX-1 (Lewis et al., 2008). However, the impact of diabetes on LYVE-1 binding activity and its relation to inflammation is still unidentified. Chronic inflammation and edema can alter the extracellular matrix composition, induce ventricular remodeling, and cause losses in cardiac function (Rubboli, Sobotka, and Euler, 1994; Kong, Kong, and Wang, 2006). Diabetic weakening or incoordination of ventricles can result in the inadequate removal and propulsion of fluid from the heart (Nakamura and Rockson, 2008). Although **the effects of diabetes on the circulatory system are well described, knowledge about its impact on the lymphatic system and associated signaling pathways is lacking** in the current literature.

The Zucker diabetic fatty (ZDF) rat has shown promise as a model of diabetic heart disease (Russell and Proctor, 2006; Poornima, Parikh, and Shannon, 2006), because at 12 weeks of age, the animal model is characterized by marked hyperphagia, obesity, hyperlipidemia, polyuria, and polydipsia similar to the T2D seen in humans. The animal model has a blending of cardiovascular diseases seen in humans, such as diabetic cardiomyopathy (van den Brom et al., 2010; Forcheron et al., 2009; Boudina and Abel, 2007), hypertension (Tikellis et al., 2004; Oltman et al., 2006; Toblli et al., 2010), and coronary artery dysfunction (Oltman et al., 2006), and can be utilized to investigate diabetic heart disease and therapeutic interventions. We hypothesized that myocardial edema is present in the ZDF rat and investigated this hypothesis through a hierarchical analysis of factors directly and indirectly supportive of myocardial fluid imbalance. We hypothesized that we would observe evidence of myocardial edema from the organismal to the cellular levels. We postulated that diabetes in the ZDF rat would lead to myocardial edema as a result of disruptions to the lymphangiogenic signaling pathway in the ZDF rat heart.

4.3 Methods

4.3.1 Animals

Male Zucker diabetic fatty (fa/fa) rats of 11 weeks of age (n=16) were used in the study with age-matched Zucker lean (fa/+) rats (n=18) serving as non-diabetic controls. Rats were purchased from Charles River Laboratory (Saint Louis, MO) and allowed one week for acclimation. The animals received husbandry services at the Laboratory Animal Resources facility at the University of Kansas Medical Center. The animals were allowed food and water ad libitum and were maintained on a 12:12 light-dark cycle. As

per vendor's recommendations, the animals were fed with Purina 5008 diet during the entire study, for the development of the disease process resembling T2D in humans. All animal procedures were performed according to the IACUC guidelines of the University of Kansas Medical Center.

4.3.2 Animal measurements and sample collection

Body weights and blood glucose levels were measured weekly on all animals from 11 to 19 weeks of age. After seven weeks of diabetes, when animals were 19 weeks of age, pressure volume analysis was completed with left ventricular (LV) catheterization. Three randomly selected animals from each group were designated for LV weight analysis by dry weight to wet weight ratios. For all animals, hearts were excised, rinsed in ice cold phosphate buffered saline (PBS) and blotted dry. Hearts from the three animals per group were processed as described in Section 4.3.4 for dry weight to wet weight ratios. A small section of the apex was placed in 4% paraformaldehyde for histological assessments. The remainder of the heart was frozen in liquid nitrogen and stored at -80° C for protein and mRNA analyses.

Blood samples from all rats were collected with a sterile pipette from the chest cavity after excising the heart. Blood was collected in 4 ml heparin coated tubes (BD, Franklin Lakes, NJ) and kept on wet ice for 30 minutes. Tubes were centrifuged at 3,400 rpm at 4°C for 10 minutes to obtain plasma. Plasma (upper layer of supernatant) was placed in 1.5 ml tubes, frozen in liquid nitrogen, and stored at -80° C for analysis.

4.3.3 Left ventricular hemodynamic measurements

Functional evaluation was performed using LV catheterization through the right carotid artery with a 2 French microtip pressure volume catheter (Millar Instruments, Houston, TX) under ketamine and xylazine anesthesia (80 mg/Kg and 10 mg/Kg, respectively). Rat core temperatures were maintained at 37°C with a heating lamp. Alligator clip electrodes were placed on the upper extremities and left lower extremity to monitor heart rate through electrocardiography during the procedure.

A four centimeter long incision was made from the lower mandible extending to the sternum. The adipose tissue and salivary glands were secured to the sides with retractors. The muscles of the anterior neck region were cleared by blunt dissection to expose the right carotid artery. The artery was ligated superiorly to the site of catheter entry. Subsequently, a loose inferior suture was applied, in addition to a metal clip close to the inferior suture. A fine incision was made close to the superior suture and the catheter was introduced gently through that incision. The metal clip was removed as the catheter slid past the inferior suture to enter the aorta. Then, the inferior suture was tightened around the catheter. The catheter was gently pushed to reach the chamber of the left ventricle and this was monitored by the appearance of the pressure volume loops on the computer monitor.

After allowing the pressure volume loops to stabilize for 3-5 minutes, steady state pressure volume loops were recorded at a sampling rate of 1000 samples per second using the Millar Pressure Volume System (MPVS-400, ADInstruments, Colorado Springs, CO). After recording the measurements for approximately 1 minute, the catheter was gently removed and the inferior suture was tightened.

4.3.4 Left ventricular dry weight to wet weight ratios

Myocardial edema was measured by dry weight to wet weight ratios of the ventricles (Desai et al., 2008). The heart was cut from the aorta through the apex for a butterfly cut. Then the aorta, atria, right ventricle, and other connective tissues were removed. The left ventricle was washed three times in ice cold PBS and blotted dry before measuring wet weight. Ventricles were dried in the laboratory oven (Thelco Model 130, ThermoScientific, Two Rivers,WI) at 60°C for 48 hours. Dried weights were measured at the following time points: 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 24 hours, and 48 hours.

4.3.5 Lymphatic vessel area analysis

For light microscopy, LV apex sections preserved in 4% paraformaldehyde were paraffin embedded. Four micron thick sections were cut from the LV apex with an ultramicrotome. Sections were stained with 0.1% toluidine blue, which stains proteoglycans and glycosaminoglycans, and then analyzed for lymphatic vessel area using light microscopy. Quantification of lymphatic vessel area was performed in nine consecutive fields, allowing for a complete analysis of the tissue section, at a final magnification of X 4. Field one was identified as the upper left portion of the tissue. The viewing field was marked on the photograph and used as a border for the next field. The tissue was read from left to right and upper to lower fields. Six tissue sections were analyzed for each animal; thus a total of 54 fields were analyzed. Lymphatic vessel area was defined as the ratio between the surface area of the lymphatic vessels and the total tissue surface area (Rahier et al., 2011). The surface area (length multiplied by the width, μm^2) of each lymphatic vessel was determined and added together for a total

lymphatic vessel area. Total tissue surface area was measured by the area of cardiac tissue within the field. A ratio was calculated for each field. An average of the ratios was calculated for each animal and expressed as a mean percentage.

4.3.6 Multiplex bead immunoassay

The assay was performed with the Cytokine Rat 10-Plex Panel per vendor's instructions (Invitrogen, Camarillo, CA) using the Luminex 200 (Invitrogen, Camarillo, CA). Briefly, a filter plate was used containing 5.6 μm polystyrene beads each conjugated with an individual antibody against the following antigens: interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- γ , or tumor necrosis factor (TNF)- α , and dyed with a specific fluorophore. The filter plate is a microplate that captures proteins bound to the above mentioned antibodies in the filter bottom of the well. 50 μl of the plasma (as prepared in Section 4.3.2), incubation buffer, and assay diluent was added to each well in triplicate. The plate was allowed to incubate for two hours at room temperature for antibody binding. The plate was washed and aspirated with gentle vacuuming of less than 5 mm Hg. One hundred μl of antigen-specific, biotinylated detector antibody was added to each well followed by an hour of room temperature incubation to bind the detector to the complex. After washing and aspiration, 100 μl of streptavidin R-phycoerythrin was added, followed by 30 minutes of room temperature incubation. After washing and aspiration, the filter plate was analyzed for the fluorescence signal intensity with the Luminex 200 (Invitrogen, Camarillo, CA). A calibration curve was plotted based on seven standards supplied by the vendor for each antigen and was used to determine the concentration of the antigens in the samples.

4.3.7 Immunoblotting

LV tissue samples were analyzed for VEGFR-3, LYVE-1, and PROX-1 total protein levels using immunoblotting. A 50 mg sample of LV tissue was used from seven animals within each of the groups. The tissue was homogenized in a buffer containing 10 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 20 mM Na molybdate, 50 mM NaF, 0.2 mM Na ortho-vanadate, and 1% Triton X-100 with a tissue homogenizer (Arrow Engineering Company, Hillside, NJ). Homogenates were centrifuged at 16,000g for 15 minutes at 4°C. The supernatants were collected and protein concentrations were measured with the BioRad DC Protein Assay (Life Science Research, Hercules, CA). One hundred µg protein samples were loaded per lane on 4-15% gradient polyacrylamide gels (Life Science Research, Hercules, CA). After electrophoretic separation under denaturing conditions in the presence of sodium dodecyl sulfate at 200V, 35 µAmps for 60 minutes, proteins were transferred to polyvinylidene fluoride (PVDF) membranes using sandwich tank transfer at 15V, 45 µAmps overnight at 4°C (Burnette, 1981). Membranes with transferred proteins were stained with Ponceau's solution, which non-specifically stains all proteins, to verify equal protein loading and efficiency of the transfer. Membranes were blocked in 5% non-fat dried milk (Life Science Research, Hercules, CA) in PBS-0.1% Tween 20 for one hour at room temperature, and then incubated overnight at 4°C with appropriate primary antibodies diluted in 5% non-fat dried milk. The following rabbit primary antibodies were used: VEGFR-3, LYVE-1 (both diluted 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) and PROX-1 (1:8,000; Abcam, Cambridge, MA). Membranes were then incubated with secondary anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz

Biotechnology, Santa Cruz, CA) diluted 1:2,000 in 5% non-fat dried milk, for 30 minutes at room temperature. Signal detection was done using chemiluminescent reagent (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL) and X-ray film. Densitometry was used to evaluate the intensity of the specific protein bands on X-ray films. Scanned images of the PVDF membranes stained with Ponceau's solution were collected to use for normalization of intensity of the bands of interest, using the most prominent band on the membrane (O'Neill et al., 2007).

4.3.8 Enzyme-linked immunosorbent assay (ELISA)

Hyaluronan and VEGF-C levels were measured in plasma samples (preparation described in Section 4.3.2) and LV tissue samples (as described in Section 4.3.7) using ELISA. ELISA for hyaluronan (Echelon Bioscience, Salt Lake City, UT) and VEGF-C (eBioscience, San Diego, CA) were performed per manufacturer's instructions in duplicate. Absorbance readings were measured at 450 and 650 nm for the hyaluronan and VEGF-C, respectively using the SpectraMax M5 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). Concentrations were calculated based on manufacturer supplied standards. Plasma concentrations for VEGF-C were undetectable, below the lowest standard concentrations in both control and diabetic samples. The vendor confirmed that VEGF-C levels are not detectable in plasma from healthy rats, but may increase to detectable levels in some disease states. Therefore, plasma VEGF-C results were excluded from analysis and only VEGF-C concentrations in tissue samples are reported.

4.3.9 Quantitative real time polymerase chain reaction

VEGFR-3, LYVE-1, and PROX-1 mRNA levels were analyzed with real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). In order to isolate total RNA, the following procedures were performed at room temperature. A 100 mg LV sample was homogenized in Trizol (Invitrogen, Camarillo, CA) as per manufacturer's instructions. After incubation with Trizol, samples were mixed with chloroform. The mixture rested for phase separation followed by centrifugation at 12,000g for 10 minutes for complete separation. The supernatant was mixed with isopropanol and incubated for 10 minutes. Pelleting was completed with 12,000g centrifugation for 10 minutes. The pellet was air dried and resuspended in diethylpyrocarbonate treated (DEPC) H₂O. RNA concentration and integrity were determined with the Agilent Bioanalyzer 2000 (Agilent Technologies, Santa Clara, CA), and samples with RNA integrity (RIN) values greater than 6 were used for data analysis.

Reverse transcription (RT) was performed in triplicate with a 50 µl mixture of reagents from Invitrogen (Carlsbad, CA) containing 1X First strand buffer, 400 µM of deoxyribonucleotide triphosphate, 0.1µg random primer per µg of total RNA (2 µg prepared as described in the preceding paragraph), 4mM dithiothreitol, 4 U/µl Moloney Murine Leukemia Virus reverse transcriptase, and 0.5 U/µl "RNAase out" solution. RT was performed for 15 minutes at 42°C and then the RT was inactivated at 95°C for five minutes in the Peltier Thermal Cycler 100 (BioRad, Life Science Research, Hercules, CA). Complementary DNA (cDNA) samples, prepared as a result of the above described reaction, were stored at 4°C until needed for qPCR. cDNA samples were diluted 5 times with DEPC-H₂O. The PCR reaction system included 5 µM primers (Table

1), Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and diluted cDNA samples. Power SYBR Green PCR Master Mix is a premix of SYBR Green dye, which binds to double stranded DNA and acts as the detection signal for the 7300 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Cycling conditions consisted of 40 cycles of 30 seconds at 94°C, 30 seconds at 58 °C, and 1 minute at 72°C. Expression levels of the genes of interest were normalized with the levels of 18S ribosomal RNA (rRNA) (Fontaine and Guillot, 2003), a reference gene supplied by Applied Biosystems (Foster City, CA).

	Primer sequences	Product size
LYVE-1	Forward 5'- TGCAGACTTCACCAGAAGGA-3' Reverse 5'-ATGTGCCTGGTTCCAAAGAG-3'	302 bp
VEGFR-3	Forward 5'-AGCCTTTCATCAACAAACCT-3' Reverse 5'-GGGTACAGCTGGATGTCATA-3'	298 bp
PROX-1	Forward 5'-GAGGAGCCTGTGTTCTGTCC-3' Reverse 5'-AGAGGCCTAGCCAGTGTGTA-3'	299 bp

Table 1 Primer sequences for qRT-PCR. Primers were designed with PrimerQuest from Integrated DNA Technologies.

Cycle threshold (C_t) indicates the cycle at which an increase in amplification is observed above the threshold. The threshold is the background fluorescence intensity. The higher the copy number of the mRNA of interest, then the amplification occurs earlier and is reported as a lower C_t value, preferably below 35. Fold differences in our study were calculated by the comparative C_t method = $2^{-\Delta\Delta C_t}$. First, the C_t value of the reference gene, 18S rRNA, is subtracted from the C_t value of the target gene (i.e., PROX-1, LYVE-1 or VEGFR-3) for each replicate of all samples within the two groups (i.e., control, n = 8, and ZDF diabetic, n = 7). This value, ΔC_t , is then averaged for each sample, and is used to calculate the mean ΔC_t for each group (control and diabetic). The mean ΔC_t values for each gene target are then compared between the diabetic and

control conditions to obtain the $\Delta\Delta C_t$ value. Fold differences are calculated by substituting the appropriate values into $2^{-\Delta\Delta C_t}$. The mathematical equations and processes are summarized below:

Step 1: For each replicate, $\Delta C_t = C_t^{\text{target gene}} - C_t^{18S \text{ reference gene}}$ (where target gene = PROX-1, LYVE-1, or VEGFR-3)

Step 2: Average the ΔC_t value from replicates for each sample

Step 3: Average the ΔC_t values for the samples within both groups

Step 4: Compare mean ΔC_t values between diabetic and control groups for each target gene by: $\Delta\Delta C_t = \Delta C_t^{\text{diabetic}} - \Delta C_t^{\text{control}}$

Step 5: Fold difference = $2^{-\Delta\Delta C_t}$

4.3.10 PROX-1 localization

For localization studies, 50 mg of LV tissue were used. Subcellular fractionation to obtain cytosolic and nuclear extracts was completed with the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific Inc, Rockford, IL). Briefly, the tissue was homogenized with a tissue homogenizer (Arrow Engineering Company, Hillside, NJ) in the CER I buffer, based on vendor recommended volumes. The microcentrifuge tube was vortexed for 15 seconds at the maximum setting, followed by incubation on ice for 10 minutes. CER II buffer was added to the microcentrifuge tube based on vendor recommended volumes. The tube was vortexed for five seconds and incubated on ice for one minute. Then the tube was vortexed for another five seconds, followed by centrifugation for five minutes at 16,000g and 4°C. The supernatant was collected in a pre-chilled tube and labeled as the cytosolic extract. The remaining pellet was suspended in ice-cold NER buffer. The tube was vortexed for 15 seconds and then

incubated for 10 minutes on ice. This process was repeated for four cycles for a total incubation time of 40 minutes. The tube was centrifuged for 10 minutes at 16,000g and 4°C. The supernatant was collected in a pre-chilled tube and labeled as the nuclear extract. Protein concentrations were measured with the Pierce Bicinchoninic acid (BCA) Protein Assay (Thermo Scientific, Rockford, IL) for the cytosolic and nuclear fractions. One hundred µg of protein for each sample were loaded per lane on 4-15% gradient polyacrylamide gels (Life Science Research, Hercules, CA). PVDF membrane preparation and PROX-1 detection were completed as described in Section 4.3.7.

4.3.11 PROX-1 transcription filter plate assay

A custom transcription factor filter plate assay was designed (Signosis, Sunnyvale, CA) to evaluate the DNA binding activity of PROX-1. The filter plate was made based on the consensus recognition sequence of the *Drosophila* transcription factor prospero gene, T A/T AG N C/T N (Choksi et al., 2006), which is highly homologous with PROX-1. The assay was performed in duplicate as per manufacturer's instructions.

A DNA complex was formed with transcription factor binding buffer mix, transcription factor probe mix, and cytosolic and nuclear extracts (as prepared in Section 4.3.10) as the PROX-1 source, in PCR tubes. The mixture was incubated at 16°C for 30 minutes in the Peltier Thermal Cycler 100 (BioRad, Life Science Research, Hercules, CA). Filter binding buffer was added to the transcription factor DNA complex and the mixture was transferred to a filter plate for 30 minutes of incubation on ice. The filter plate was washed with wash buffer and centrifuged at 600 g for two minutes for a total of four washes. Then elution buffer was added and allowed to incubate for five

minutes at room temperature. The sample was collected in a 96 well PCR plate after centrifuging for two minutes at 600g. The samples were then transferred to a PCR tube for denaturing of the double stranded DNA at 95°C for three minutes in the Peltier Thermal Cycler 100 (BioRad, Life Science Research, Hercules, CA) to prepare for hybridization. The samples were transferred to the transcription factor hybridization plate, which was covered with the PROX-1 probe, along with hybridization buffer for overnight hybridization at 42°C. The hybridization plate was washed three times with wash buffer. A blocking buffer was added to each well and incubated at room temperature for 15 minutes on the Max Q2000 shaker (Thermo Fisher Scientific Inc, Rockford, IL). The blocking buffer was removed and diluted streptavidin conjugated horse radish peroxidase was added to each well and incubated at room temperature for 45 minutes on a shaker followed by three washings. Substrate solution was added to each well and luminescence intensities were measured over the hour in the microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA).

4.3.12 Statistical analysis

Descriptive statistics were utilized to characterize the animal groups. For differences between two groups, unpaired t-tests were conducted with PASW Version 18 software (IBM, Somers, NY). Significance was determined at $p < 0.05$. Results are described as means \pm standard errors (SEs). Pressure volume loops were not recorded for all animals due to death during the procedures or unstable waveforms. Only those animals with recordable pressure volume loops (control, $n=13$ and diabetes, $n=14$) were included in the data analysis.

4.3.13 Hierarchical analysis

To determine if myocardial edema was present in the ZDF rat, we chose to use a hierarchical analysis to evaluate our hypothesis. The hierarchical analysis was based on the levels of organization for organisms (Nurse and Hayles, 2011). We explored alterations on the organismal, organ, tissue, and cellular levels that would support or suggest the presence of myocardial edema in the ZDF rat. Figure 1 details the workflow completed for the hierarchical analysis of myocardial fluid imbalance under the backdrop of diabetes.

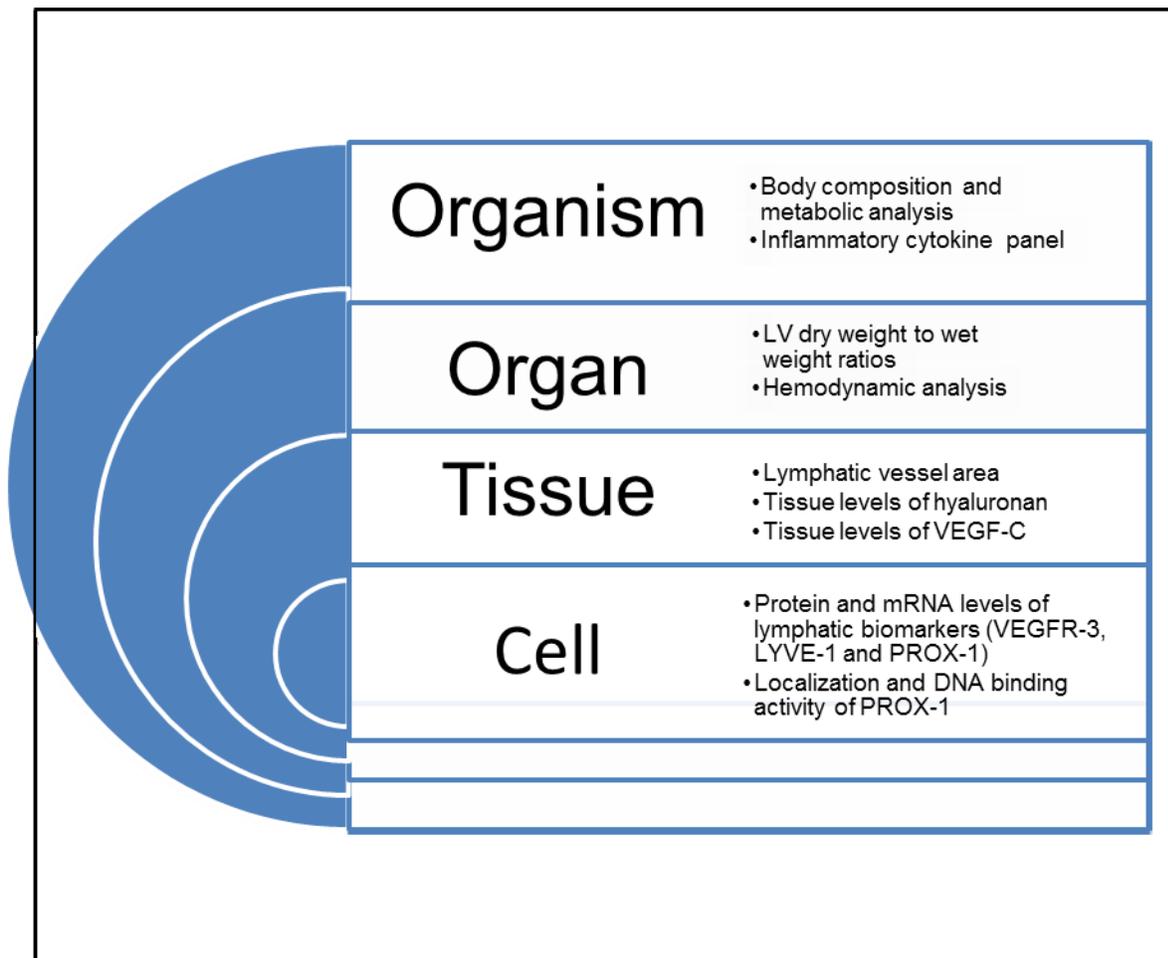


Figure 1 Study workflow based on hierarchical design.

4.4 Results

4.4.1 Investigations of myocardial edema at the organism level

4.4.1.1 Body weight and metabolic analysis

Diabetic rats had higher body weights, blood glucose, and hemoglobin A1C (HbA1C) levels throughout the observation period. At baseline, age of 12 weeks that we considered week 0 of diabetes, diabetic rats weighed 10% more than control rats ($p \leq 0.001$) as shown in Table 2. Blood glucose and HbA1C levels were elevated as expected for diabetic rats compared to controls ($p \leq 0.001$). Diabetic rats were heavier than control rats ($p \leq 0.001$) at the termination of the experiment, at 7 weeks of diabetes when the rats were 19 weeks old. It was unknown if the body composition changes were due to lean mass, fat, or edema. Bioimpedance measurements would address this issue; however they were beyond the scope of this study.

	Baseline (Week 0)		Termination (Week 7)	
	Control (n=16)	Diabetes (18)	Control (n=16)	Diabetes (n=18)
Body weights, g	313±5	401±8 [#]	377±4	460±12 [#]
Blood glucose, mg/dL	138±10	444±11 [#]	180±17	565±15 [#]
Hemoglobin A1C, %	4.6±0.15	11±0.64 [#]	4.3±0.10	12±0.50 [#]

Table 2 Rat characteristics. Diabetic rats weighed more at baseline (week 0) and termination (week 7 of diabetes) than control rats. Blood glucose and HbA1c levels were elevated in diabetic rats compared to control rats.

4.4.1.2 Inflammatory cytokine levels

We investigated levels of inflammatory cytokines in the plasma of ZDF rats to determine whether there may be systemic inflammation, which could initiate an edematous state. Ten cytokines were analyzed, including IL-1 α , IL-1 β , IL-2, IL-4, IL-6,

IL-10, IL-12, GM-CSF, IFN- γ , and TNF- α . Figure 2 illustrates that plasma IL-2 levels were significantly increased under diabetic conditions ($p \leq 0.01$). No statistical difference was observed for the other 9 cytokines (data not shown). The elevation of IL-2 may indicate an initial, systemic response of the immune system to an infection or oxidative stress (Pipkin et al., 2010).

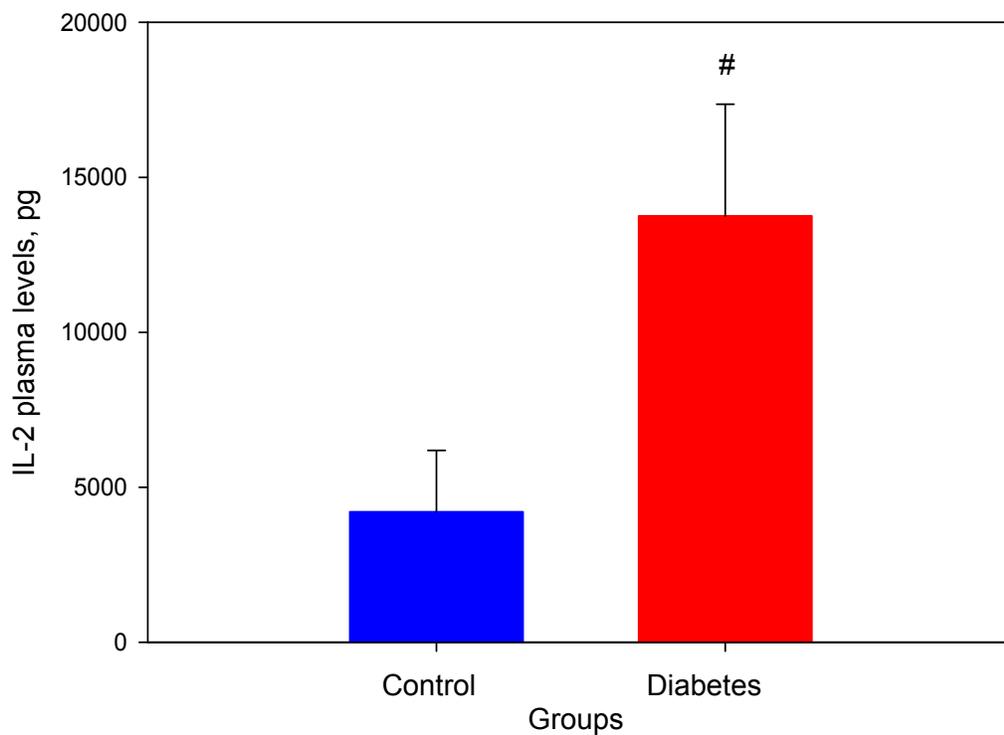
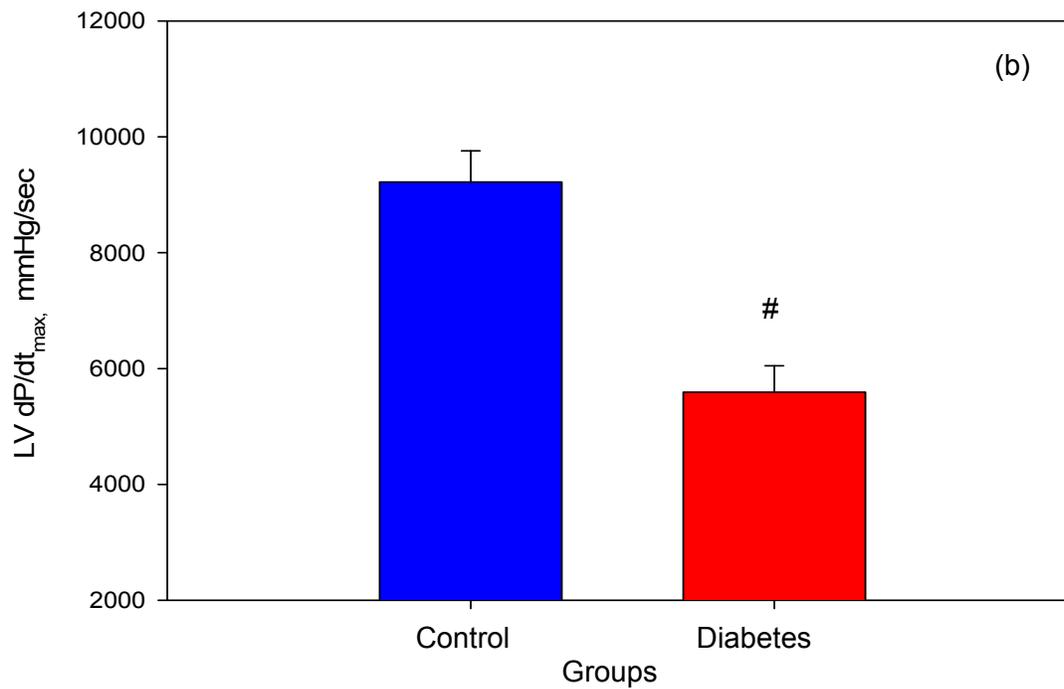
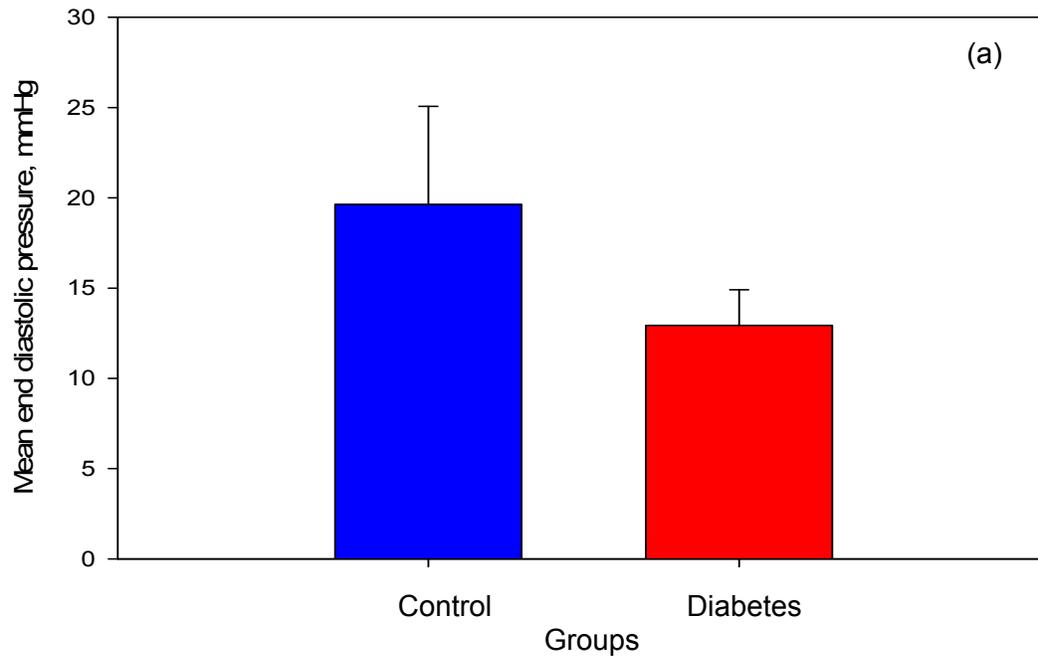


Figure 2 Rat plasma IL-2 levels. ZDF rats had higher circulating IL-2 levels than control rats. Control (n=13), diabetes (n=14).

4.4.2 Investigations of myocardial edema at the organ level

4.4.2.1 Hemodynamic analysis

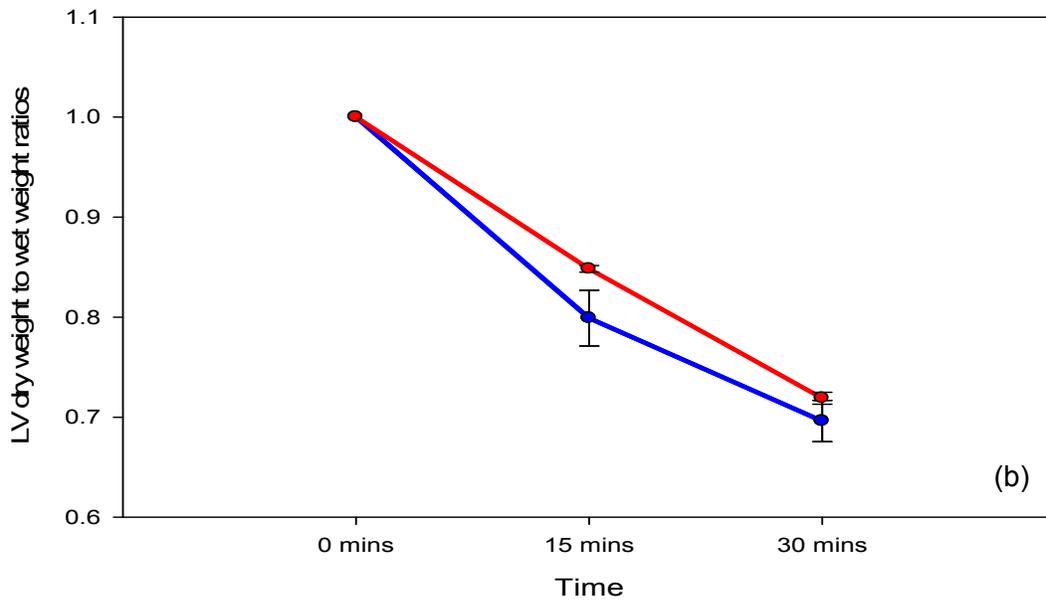
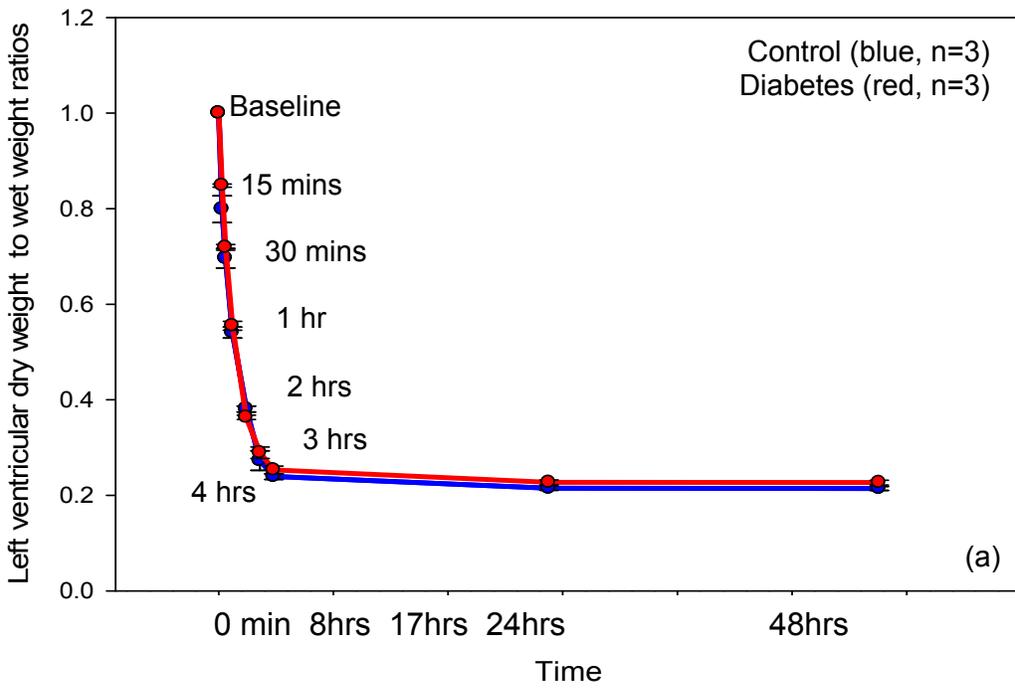
LV end diastolic pressures were unchanged by diabetes ($p=0.14$) as seen in Figure 3a. The index of myocardial microvascular permeability, dP/dt_{\max} (Laine, 1987) is shown in Figure 3b. A 40% reduction in dP/dt_{\max} was observed in diabetic rats ($p\leq 0.001$) compared to control rats. These results suggest that microvascular permeability was increased under diabetic conditions, but perhaps was not sufficient to significantly impact ventricular pressure.



Figures 3 Rat LV end diastolic pressures (a) and dP/dt_{max} values (b). No difference was observed in end diastolic pressures. Diabetic rats had lower dP/dt_{max} values compared to control rats. Control (n=13), diabetes (n=14).

4.4.2.2 Left ventricular dry weight to wet weight ratios

No difference in baseline left ventricular wet weights was observed between control ($1,020\pm 38$ mg) and diabetic rats ($1,050\pm 45$ mg; $p=0.61$). Myocardial edema was evaluated by measuring dry weight to wet weight ratios of the left ventricles (Desai et al., 2008). Dry weight to wet weight ratios for control and diabetic animals were quite similar for both groups throughout the experiment. No statistical significance was observed between control (218 ± 1 mg) and diabetic (238 ± 1 mg) groups after 48 hours of drying ($p=0.22$) as shown in Figure 4a. Although ventricles dried to a comparable constant weight, an interesting observation was noted during the first 30 minutes of drying in Figure 4b. Control samples had a steep decline in ratios during the first 15 minutes (20% loss in weight) compared to the diabetic left ventricles (15% loss in weight). These results suggest that although total LV fluid content was similar between the groups (78-79% fluid loss), there may be morphological differences, such as fibrosis or fat accumulation, which affected the fluid release from the diabetic tissue and thus slowed down the drying of the diabetic ventricles during the initial phases of drying.

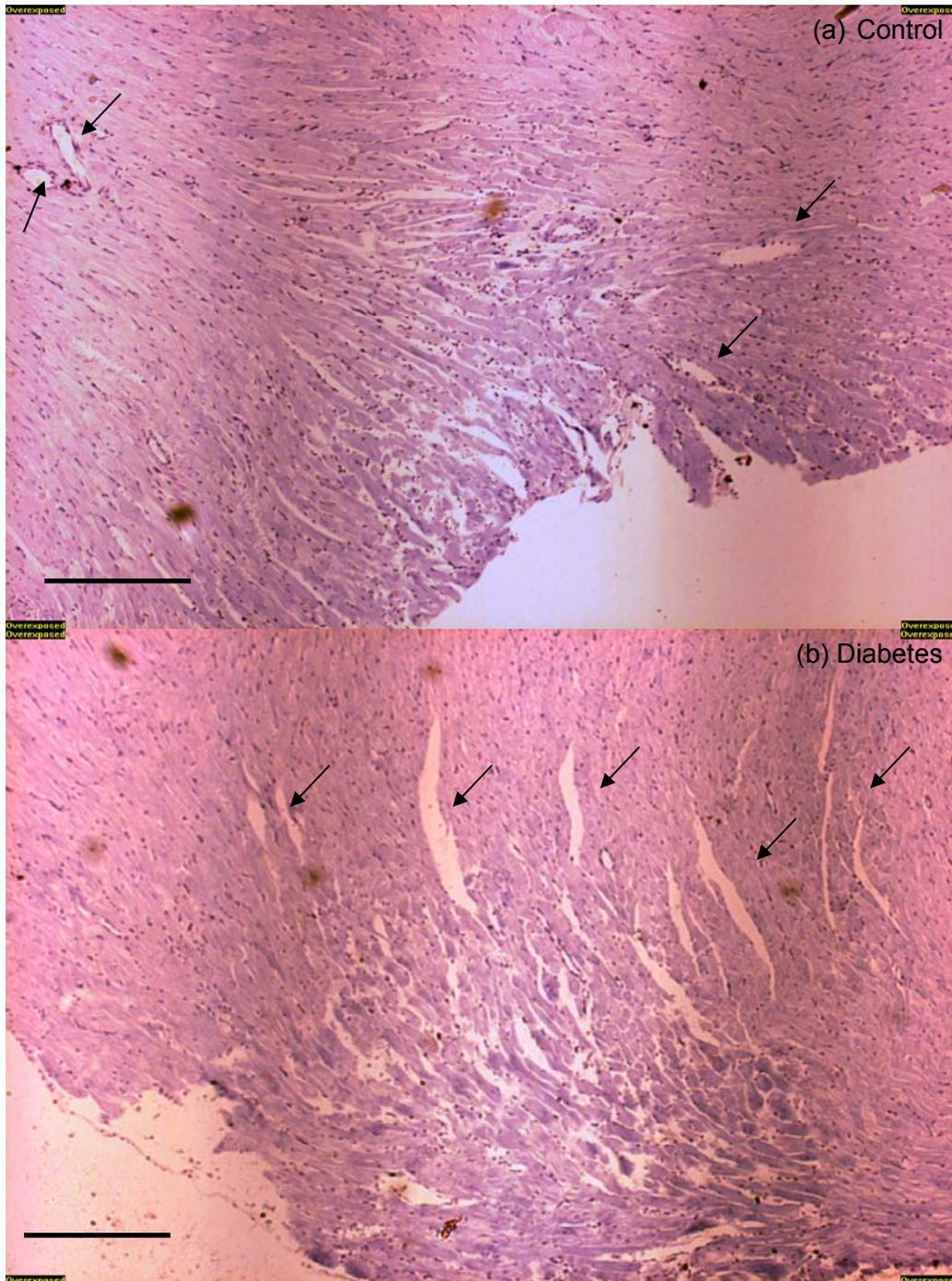


Figures 4 Dynamics of drying of rat LVs over 48 hours (a) and the first 30 minutes (b). Although LVs dried to a similar constant weight, the groups had different drying patterns within the first 30 minutes of drying. Control (n=3) and diabetes (n=3).

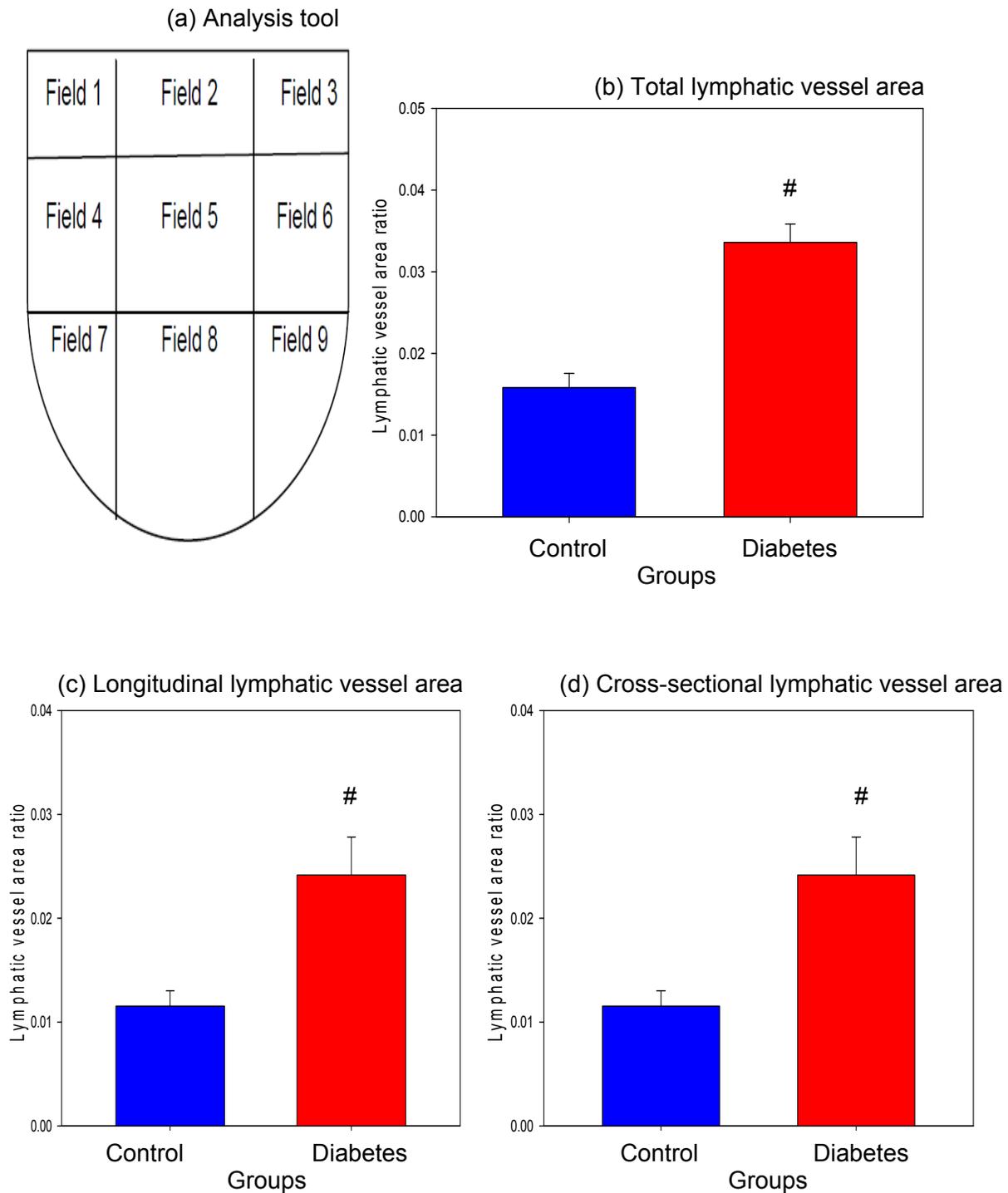
4.4.3 Investigations of myocardial edema at the tissue level

4.4.3.1 Lymphatic vessel area

Cardiac tissue samples were stained with 0.1% toluidine blue, which stains proteoglycans and glycosaminoglycans and allows for lymphatic vessels to be differentiated from blood vessels due to morphological differences in the basement membranes and the lumens of the vessels (Figures 5a-b). The tissue sections were divided into 9 fields for quantification of lymphatic vessel area as shown in Figure 6a. Mean lymphatic vessel area was significantly increased ($p \leq 0.05$) in the diabetic LV samples (Control $1.63 \pm 0.17\%$ and diabetes $3.42 \pm 0.23\%$). The mean vessel area in diabetic samples was 52% higher than control samples (Figure 6b). The increased area indicates a higher number of lymphatic vessels and/or larger size of vessels in the diabetic tissues compared to control tissues. Due to the shape of the heart, we chose to stratify vessels into longitudinal or cross-sectional area for additional analysis. Figures 6c-d shows that longitudinal and cross-sectional vessel areas were also increased in diabetic tissues.



Figures 5 Tissue sections from control (a) and diabetic (b) rat LV samples. These representative samples are from the middle left field of the tissues. Lymphatic vessels lightly stained with toluidine blue have an irregular shaped lumen (arrows indicate a representative sample in the tissues). The vessels present with an incomplete or absent basement membrane. Scale bar equals 1 millimeter.



Figures 6 Diagram of tissue analysis procedure (a). Total (b), longitudinal (c), and cross-sectional (d) lymphatic vessel area ratios. Lymphatic vessel area was increased in diabetic tissues in the three analyses.

4.4.3.2 Hyaluronan levels in LV tissue samples

An analysis of hyaluronan concentrations in LV tissue samples (Figure 7) revealed no difference between control and diabetic rats ($p=0.30$). We did not observe a difference in the plasma samples of control and diabetic groups (data not shown). This finding indicates that hyaluronan accumulation in diabetic LV tissue was not present in diabetic rats after 7 weeks of diabetes.

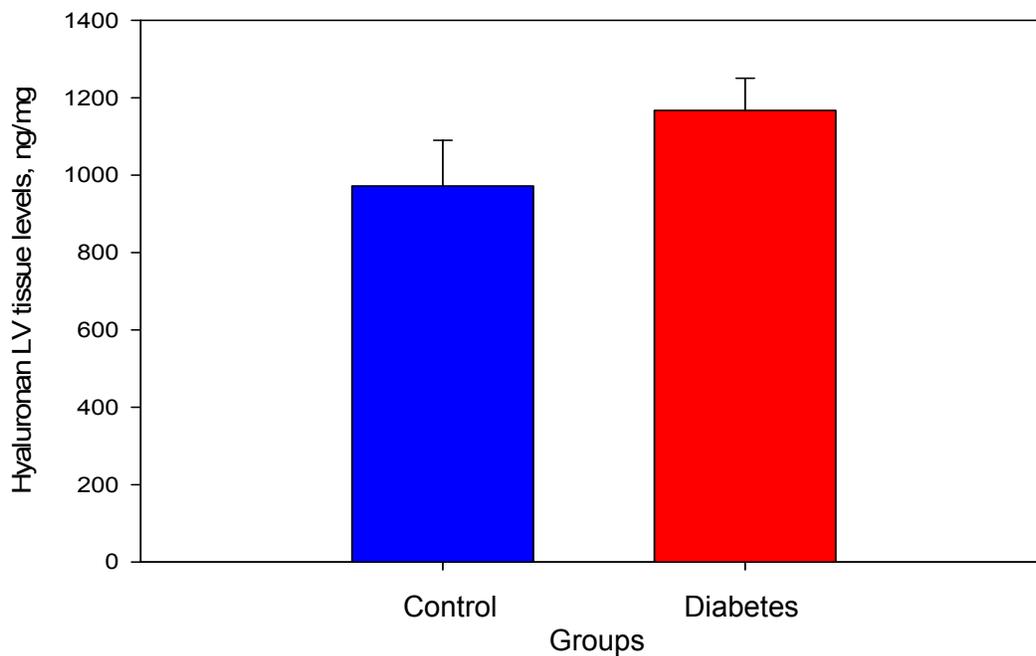


Figure 7 Hyaluronan levels in LV tissue samples. No difference in hyaluronan concentrations was observed between control and diabetic samples. Control (n=6), diabetes (n=6)

4.4.3.3 VEGF-C levels in LV tissue samples

VEGF-C tissue concentrations were similar between the two rat groups ($p=0.58$; Figure 8). We observed no accumulation of VEGF-C, which would trigger increases in vascular permeability in diabetic tissues.

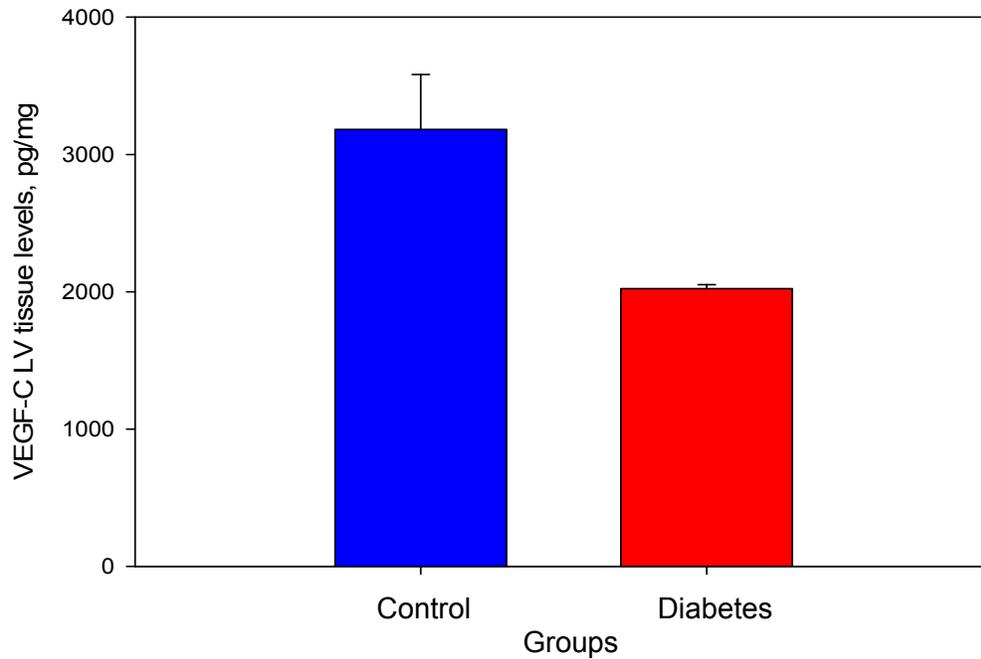
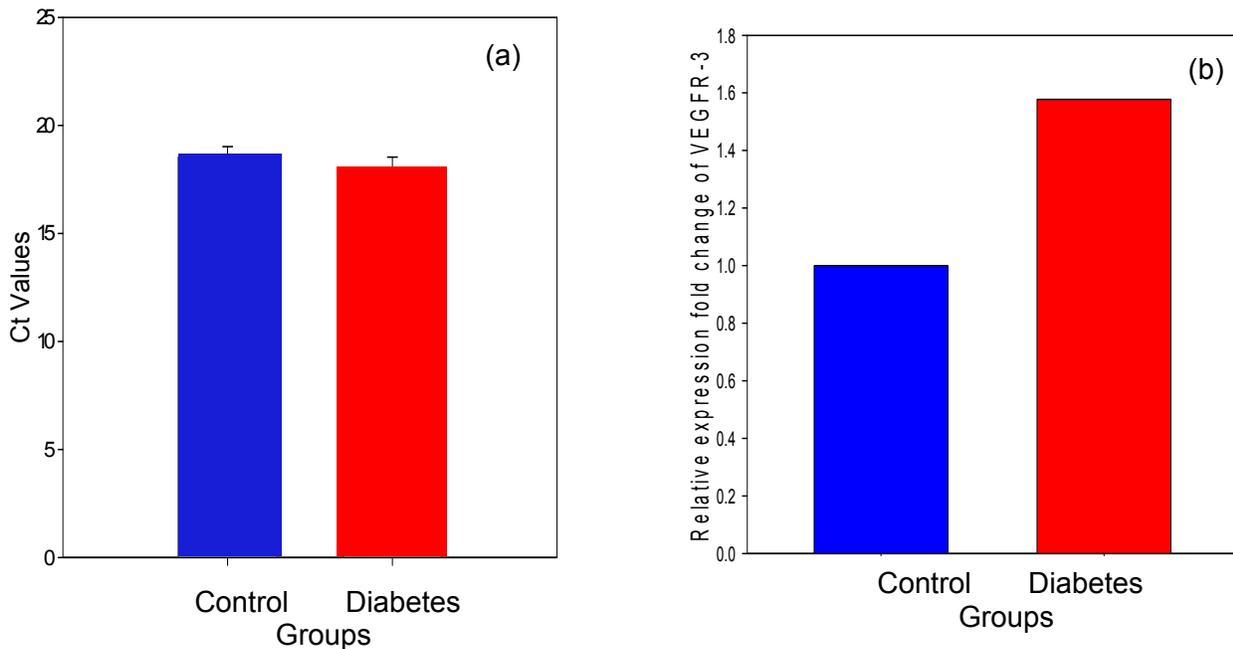


Figure 8 VEGF-C levels in rat LV tissue samples. No differences in VEGF-C amounts were observed between control and diabetic tissue samples. Control (n=6), diabetes (n=6).

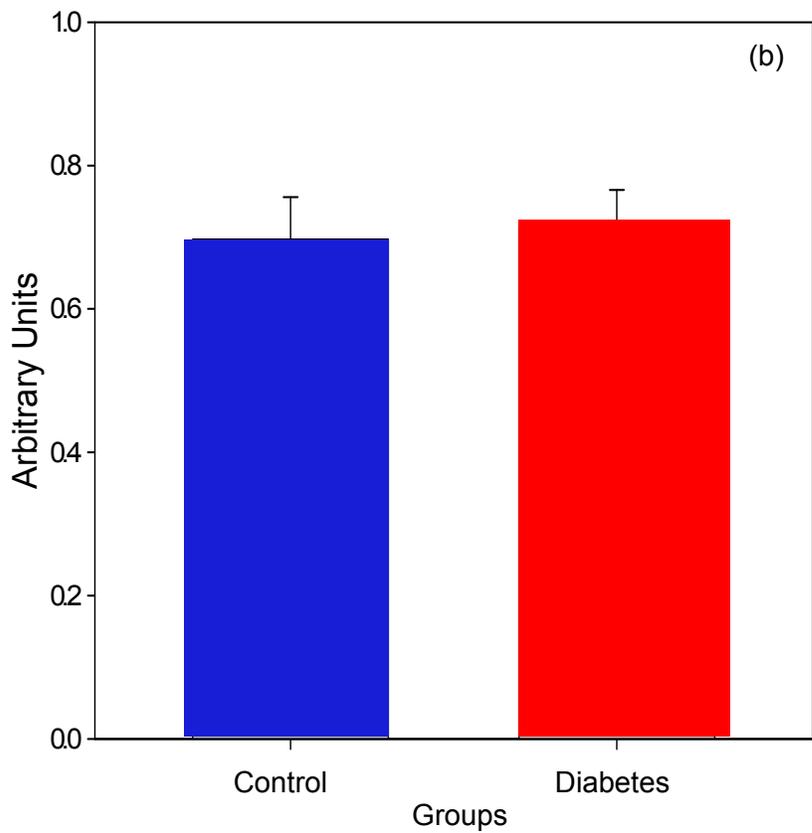
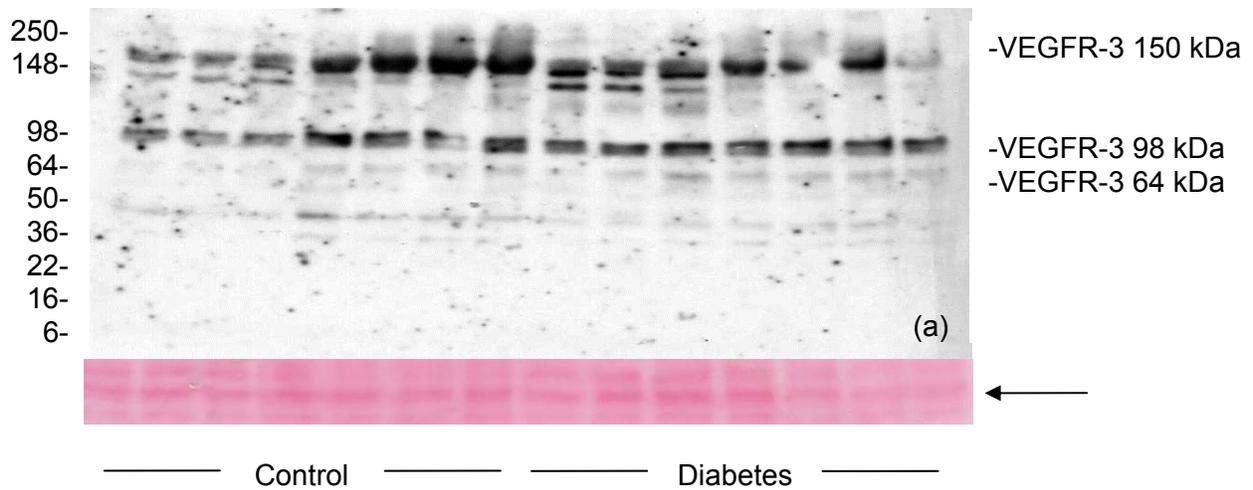
4.4.4 Investigations of myocardial edema at the cell level

4.4.4.1 VEGFR-3 mRNA and protein levels in LV tissue samples

VEGFR-3 is responsible for the uptake of VEGF-C from the interstitium. In Figures 9a-b, VEGFR-3 mRNA levels determined by qRT-PCR were similar between control and diabetic groups ($p=0.45$). Immunoblotting was used to quantify the VEGFR-3 protein levels. Multiple bands were observed in Figure 10a, likely due to varying levels of glycosylation (Zhang et al., 2010). We analyzed the expected 150 (major), 98, and 64 kDa bands for the purposes of this study. No statistical significance was observed in the total VEGFR-3 protein levels between the two rat groups. The protein levels for the 98 kDa band are shown in Figure 10b. The other bands of VEGFR-3 protein had similar patterns (not shown, $p>0.05$).



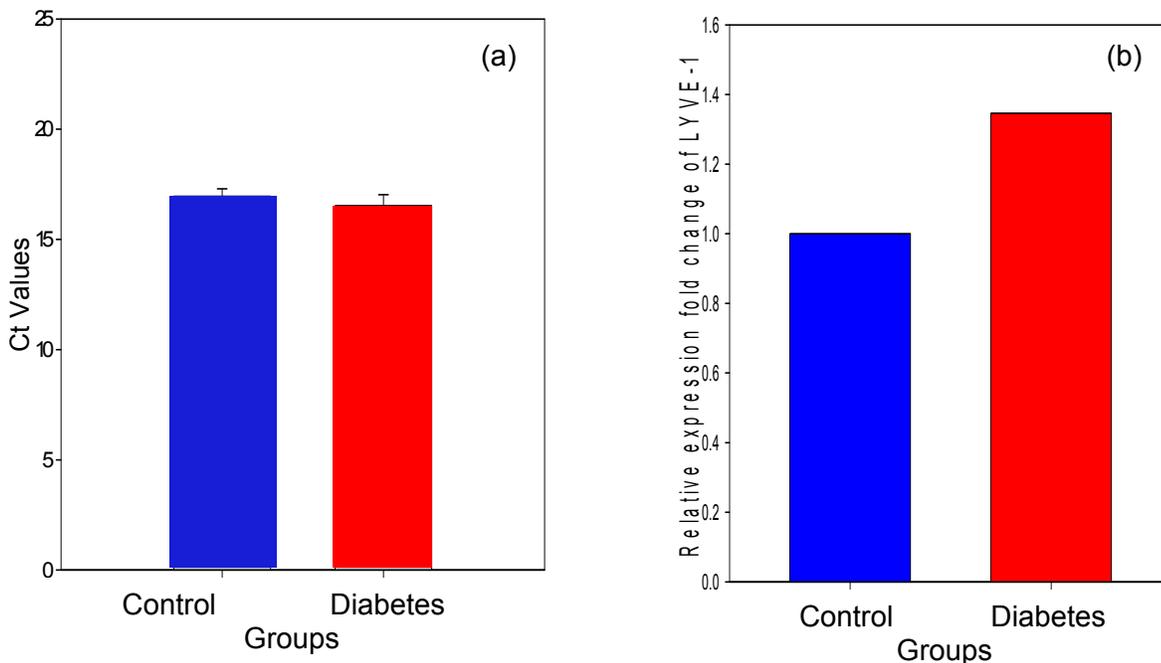
Figures 9a-b VEGFR-3 mRNA levels in LV tissue samples. No difference in C_t values (a) or relative expression fold change of VEGFR-3 (b) was observed between control ($n=8$) and diabetic ($n=7$) samples.



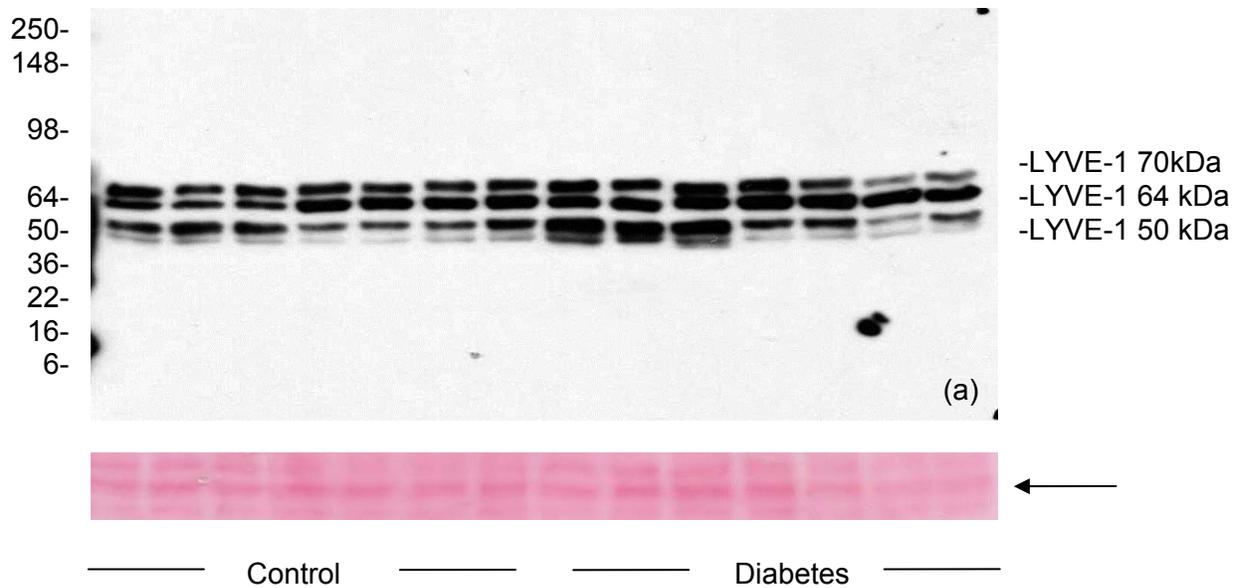
Figures 10 Protein levels of VEGFR-3 with immunoblotting (a) and quantified for the 98kDa band (b) with Ponceau's staining as a loading control. No difference in protein levels (98kDa) was observed between control and diabetic samples. Control (n=7), diabetes (n=7)

4.4.4.2 LYVE-1 mRNA and protein levels in LV tissue samples

LYVE-1 mRNA levels determined by qRT-PCR for diabetic rats were also comparable to control rats as shown in Figures 11a-b ($p=0.48$). Immunoblotting was used to identify the LYVE-1 protein. LYVE-1 has 3 major bands due to post-translational modifications: 70, 64 and 50 kDa (Figure 12a). LYVE-1 is a secondary receptor for hyaluronan, and hyaluronan levels were not affected by diabetes in our study. Likewise, no difference was observed in the protein levels of the three LYVE-1 bands between the two animal groups. Figure 12b shows the protein levels observed for the 70 kDa band of LYVE-1 with similar patterns for the 64 and 50 kDa bands (not shown, $p>0.05$).



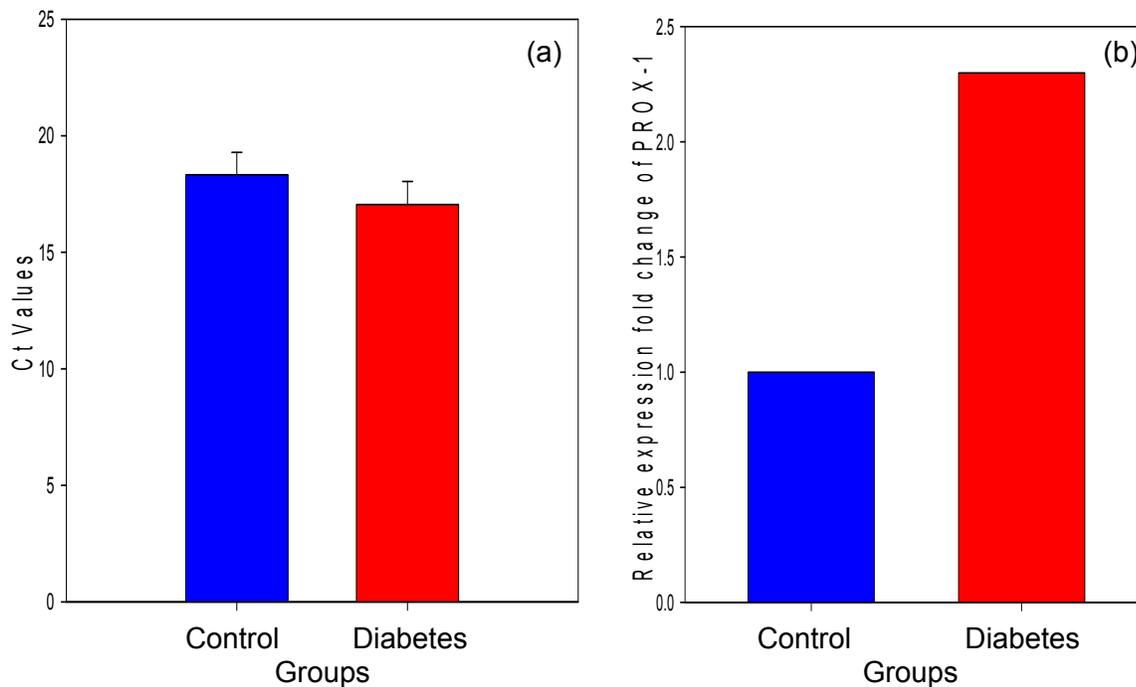
Figures 11a-b LYVE-1 mRNA levels in LV tissue samples. No difference in C_t values (a) or relative expression fold change of LYVE-1 (b) was observed between control ($n=8$) and diabetic ($n=7$) samples.



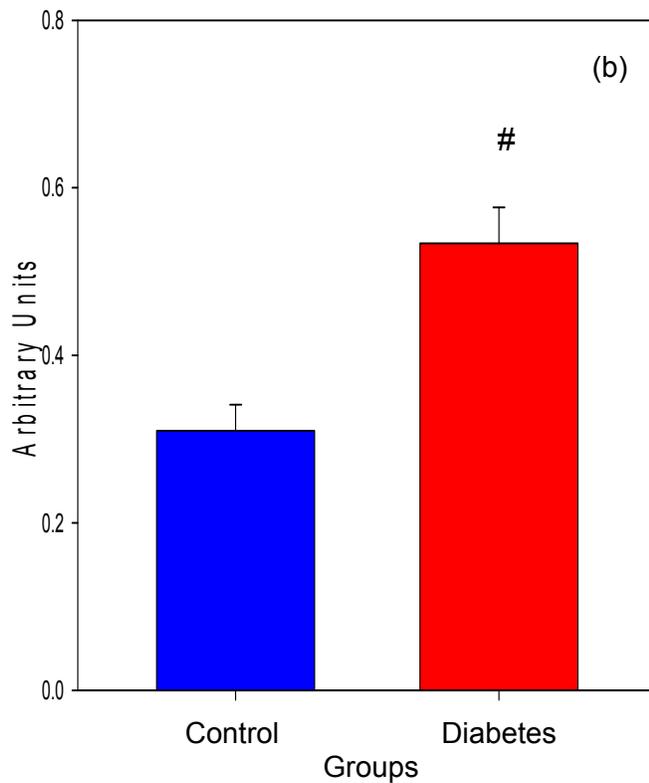
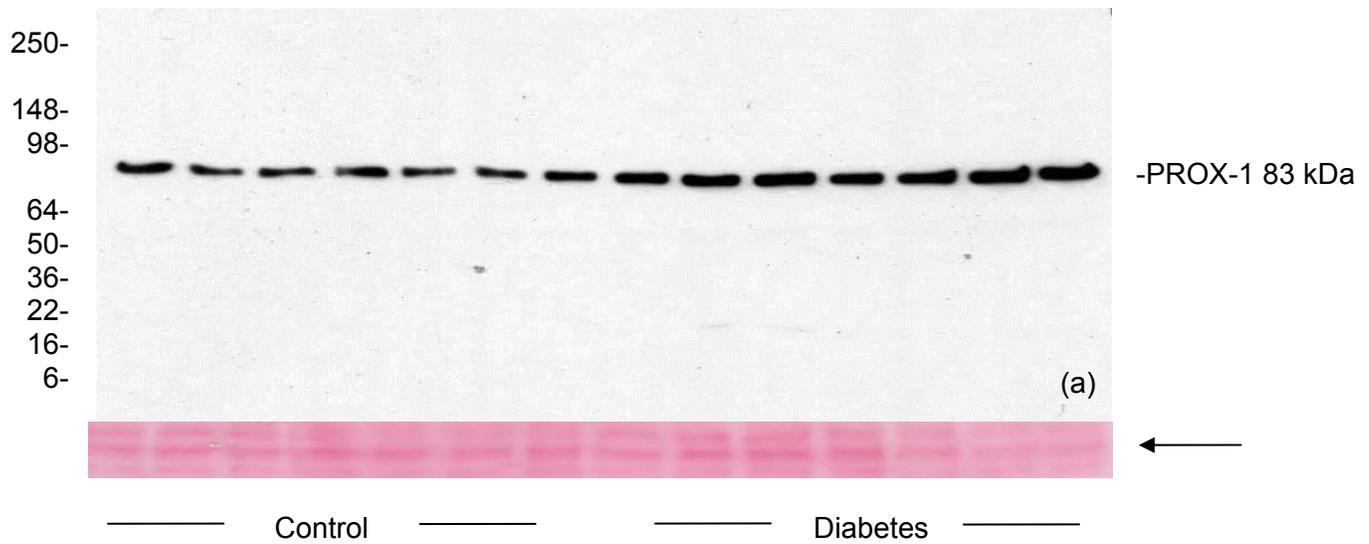
Figures 12 Protein levels of LYVE-1 with immunoblotting (a) and quantified for the 70 kDa band (b) with Ponceau's staining as a loading control. No difference in protein levels (70kDa) was observed between control and diabetic samples. Control (n=7), diabetes (n=7).

4.4.4.3 PROX-1 mRNA and protein levels in LV tissue samples

No significant difference was observed in the mRNA levels of PROX-1 as determined by qRT-PCR ($p=0.37$; Figure 13a-b). Immunoblotting revealed a single band for PROX-1 at 83 kDa corresponding to the molecular weight of this protein (Figure 14a) (Galeeva et al., 2007). Protein levels of the transcription factor, PROX-1, were elevated 12% under diabetic conditions ($p\leq 0.001$; Figure 14b).

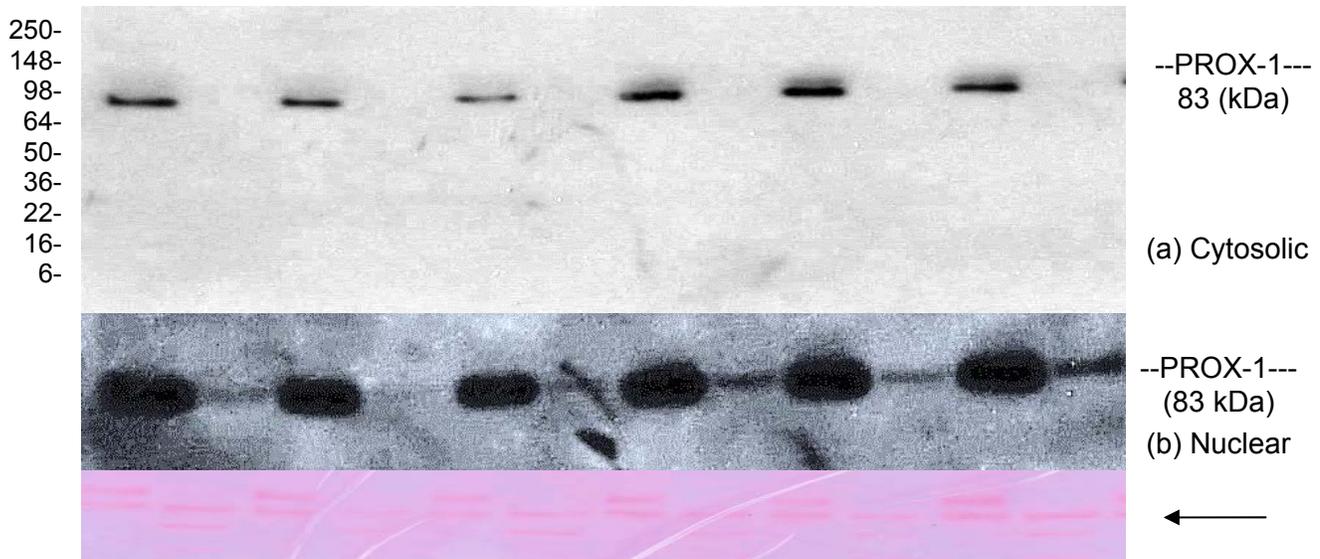


Figures 13a-b PROX-1 mRNA levels in LV tissue samples. No difference in C_t values (a) or relative expression fold change of PROX-1 (b) was observed between control (n=8) and diabetic (n=7) samples.



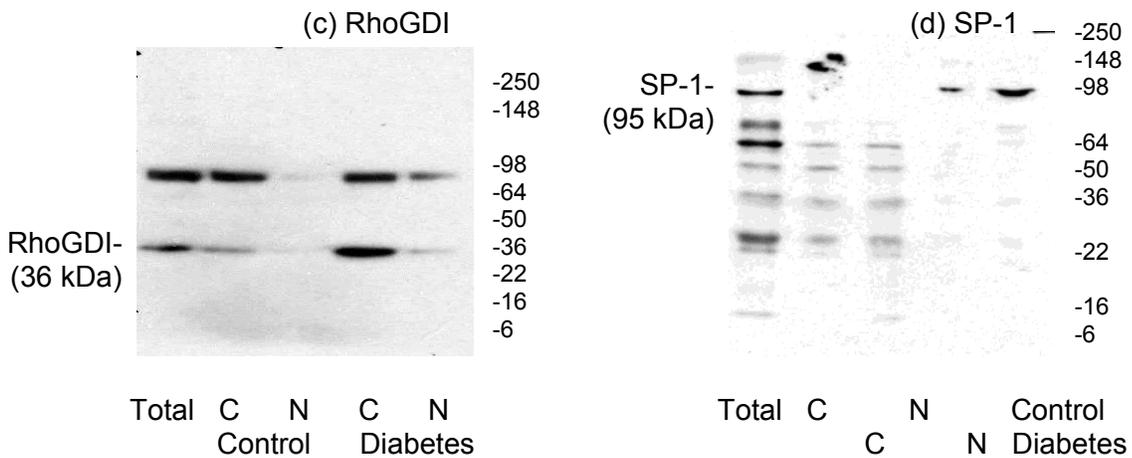
Figures 14 Protein levels of PROX-1 with immunoblotting (a) and quantified (b) with Ponceau's staining as a loading control. Gene expression was unchanged between the two groups. However, protein levels of PROX-1 were increased in diabetic rats compared to controls. Control (n=7), diabetes (n=7).

To investigate the increase in total protein levels of PROX-1 in diabetic samples, we analyzed the cytosolic and nuclear fractions for the presence of the transcription factor in order to determine its subcellular localization. Figure 15a shows that PROX-1 cytosolic levels were higher in diabetic samples compared to controls. Overexposure of the X-ray film was required to produce a visible band in the nuclear fraction as seen in Figure 15b. Quantification showed that PROX-1 cytosolic protein levels were higher in diabetic samples compared to controls ($p \leq 0.05$, Figure 16a). This finding is in agreement with the increased total protein levels of PROX-1 in diabetic rats described in Section 4.4.4.3. Nuclear extracts revealed substantially higher levels of PROX-1 in diabetic rats, approximately 59% difference, compared to control rats ($p \leq 0.05$; Figure 16b). RhoGDI, a cytosolic protein, was used to confirm the quality of the subcellular fractions (Figure 15c). We did observe the presence of RhoGDI in the diabetic nuclear fraction. We postulate that RhoGDI localization may be altered under diabetic conditions. However, SP-1, a nuclear protein, was only detected in the nuclear fractions of control and diabetic samples (Figure 15d). After confirmation of the purity of subcellular fractions, we conclude that PROX-1 nuclear localization is increased under diabetic conditions.

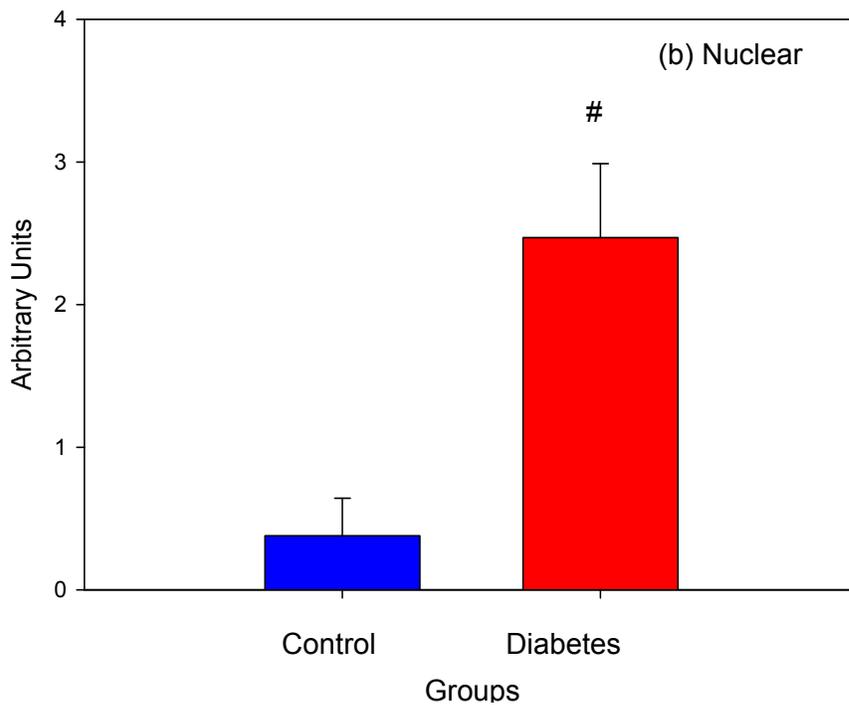
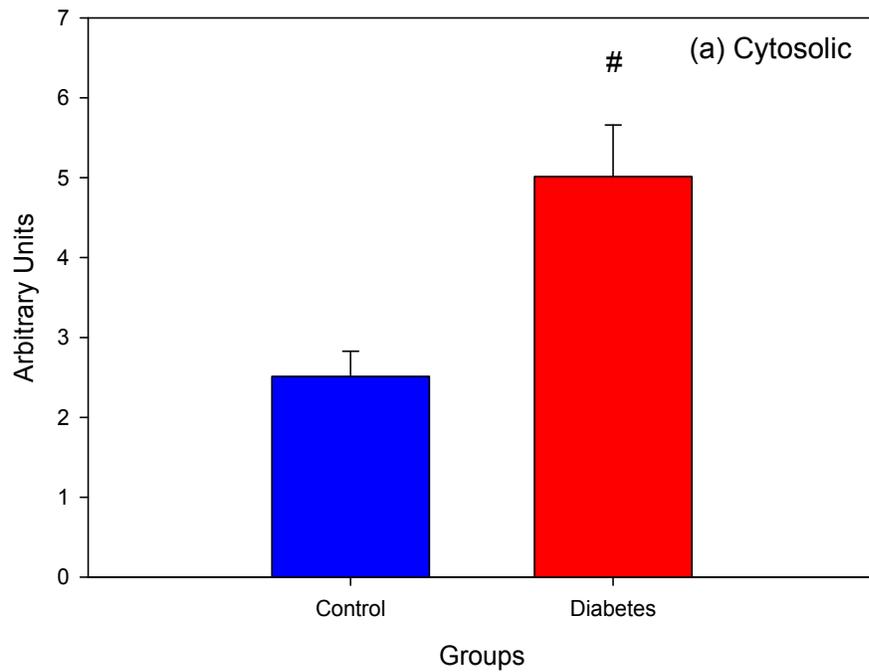


C C C C C C Cytosolic
 N N N N N N Nuclear

Control Diabetes



Figures 15 PROX-1 protein levels in cytosolic (a) and nuclear (b) fractions. Confirmation of subcellular fractionation quality using antibody against RhoGDI, a resident cytosolic protein (c) and SP1, a resident nuclear protein (d). Control (n=3), diabetes (n=3), C (cytosolic fraction), and N (nuclear fraction).



Figures 16 PROX-1 protein levels in cytosolic (a) and nuclear (b) fractions. Cytosolic and nuclear fractions revealed higher protein levels of PROX-1 in diabetic samples. Control (n=3), diabetes (n=3).

4.4.4.4 PROX-1 DNA binding activity

We did not observe differences in PROX-1 DNA binding activity (Figure 17a-b), although subcellular protein levels of PROX-1 were significantly higher in diabetic animals as shown in Figures 16a-b. Although PROX-1 DNA-binding activity is expected to be in the nuclear fraction, we chose to also evaluate the cytosolic fractions due to the high PROX-1 protein levels in diabetic rats (Figure 16a). The binding activity observed in the cytosolic fractions in Figure 17a may be due to non-specific binding to other proteins such as transporters. No difference was observed in PROX-1 specific activity in either nuclear or cytosolic fractions as shown in Figures 18a-b.

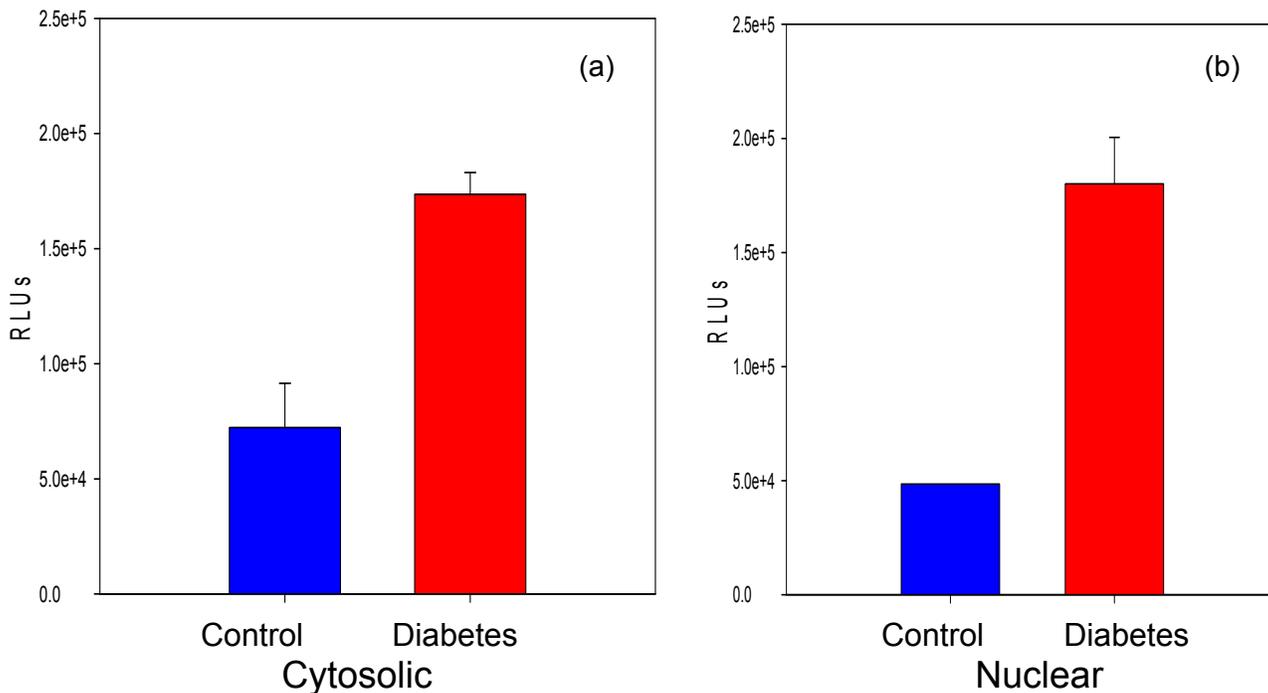
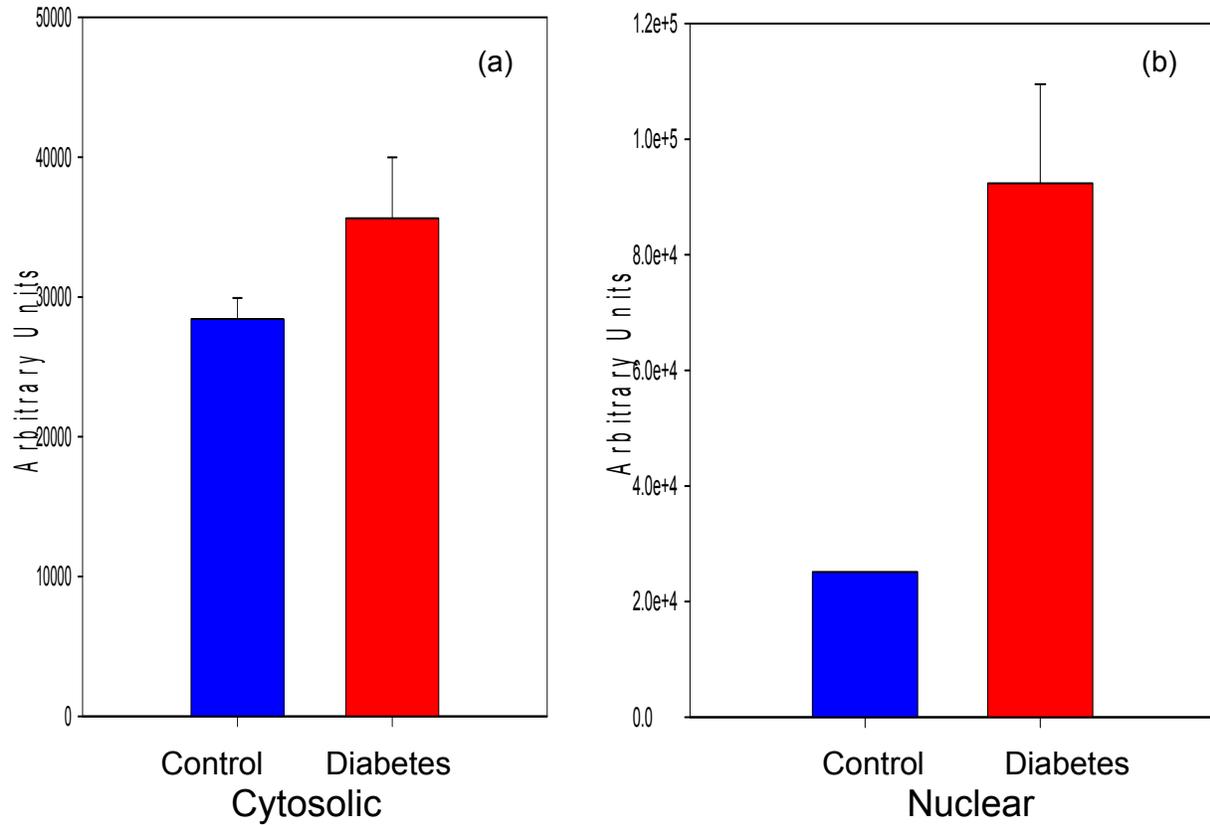


Figure 17 PROX-1 DNA binding activity in cytosolic (a) and nuclear (b) fractions. No difference in DNA binding activity was observed between the control and diabetic samples. RLU (Relative luminescence units). Control (n=3), diabetes (n=3).



Figures 18 PROX-1 specific DNA binding activity in cytosolic (a) and nuclear (b) fractions. No difference in the specific DNA binding activity was observed in cytosolic (a) and nuclear (b) fractions of control and diabetic samples. Control (n=3), diabetes (n=3) for cytosolic activity analysis and control (n=1), diabetes (n=2) for nuclear activity analysis.

4.5 Discussion

Myocardial edema has been suggested as an instigator of the development and progression of cardiomyopathy (Miller, 2011; Nakamura and Rockson, 2008). Animal models have shown that cardiac fluid imbalance results in edema, organelle damage, and declines in cardiac function (Davis et al., 1995; Rohn et al., 1995). However, it is unknown if diabetes in the ZDF rat induces an edematous state in the heart. **Our results did not provide a clear picture as to whether myocardial edema is present in the ZDF model.** A direct measure of myocardial edema, left ventricular dry to wet weight ratios failed to show a significant difference between control and diabetic groups. However, this measurement is fraught with preparation errors. Our power analysis indicated that 112 animals would be required to reach statistical significance. Due to this limitation, we had to rely on an indirect measure, declines in dP/dt_{max} in diabetes, which were suggestive of myocardial edema as an indirect measure.

4.5.1 Diabetes causes metabolic changes in the ZDF rat

We began our investigation for evidence of myocardial edema at the organismal level. Our **ZDF rats had significantly higher body weights** than lean controls as shown in Table 2. This finding is well supported in the literature due to the hyperphagia associated with the phenotype (Belobrajdic et al., 2011; van den Brom et al., 2010). We were unsure if the gains in body weight were the result of accumulations of edema and/or adipose due to unavailability of bioimpedance equipment.

Hyperglycemia, common in the ZDF rat, resulted in increased activity of nuclear factor (NF)- $\kappa\beta$ in rat mesangial cells (Ha et al., 2002). NF- $\kappa\beta$ expression is increased in ZDF rat heart tissue leading to upregulation of several pro-inflammatory cytokines

(Huang et al., 2005). Systemic inflammation has been reported in the ZDF rat with circulating IL-6 and TNF- α levels elevated in 8 week old ZDF rats (Teixeira de Lemos et al., 2009). Increases in TNF- α plasma levels persisted with age in reports of elevated TNF- α levels in 26-32 week old ZDF rats (Gao, Picchi, and Zhang, 2010). However, other studies have shown no change in circulating IL-10 and TNF- α (Mito et al., 2000), or decreases in IL-6 and TNF- α plasma levels (Lamas, Martinez, and Marti, 2004) under inflammatory conditions of obesity and hyperglycemia. We observed no change in TNF- α , IL-6, or IL-10 in our study. In fact, nine of the ten inflammatory cytokines investigated were not impacted by diabetes. **Only circulating IL-2 was elevated with diabetes** in our investigations as shown in Figure 2.

IL-2 has predictive value in cardiovascular disease and may be an initial responder of pro-inflammatory cytokines (Mazzone et al., 1999). Elevations of circulating IL-2 in humans indicated an increased immune response to infections or atherosclerotic lesions (Frostedgard et al., 1999; Simon et al., 2001). In humans, increases in circulating IL-2 had a direct correlation with carotid artery intima media thickness, a predictor of cardiovascular disease (Elkind et al., 2005). Therefore, IL-2 plasma levels are considered a risk factor for cardiovascular disease. However, knowledge regarding the immune response of the ZDF rat is evolving. Although we observed increases in circulating IL2, declines in IL-2 plasma levels have also been reported in this model (Ruth et al., 2008). However, the lack of a robust response of the other inflammatory cytokines may indicate a deficiency in the inflammatory response of our cohort. If our observations are confirmed in future studies, an impaired inflammatory

response may predispose the ZDF rat to endothelial dysfunction and increased vessel permeability, which may play a role in the development of diabetic heart disease.

IL-2 has also been associated with organ edema (Welbourn et al., 1991). An evaluation of 60 patients with burns of 20-40% total body surface area revealed elevated circulating IL-2 levels. The severity of edema was correlated to IL-2 levels (Kowal-Vern et al., 1997). High doses of IL-2 for cancer treatment can result in pulmonary edema and injury. IL-2 toxicity causes increased capillary leakage and fluid accumulation in the interstitium (Schwartz, Stover, and Dutcher, 2002). Although we did not observe changes in VEGF-C, the elevation of circulating IL-2 may indicate increased microvascular permeability (Funke et al., 1994) under diabetic conditions.

4.5.2 Diabetic hemodynamic changes suggest increased myocardial fluid imbalance, but direct evidence of myocardial edema is lacking in the ZDF rat

Research has suggested that the dP/dt_{max} may be a sensitive and clinically relevant measure of microvascular permeability (Laine, 1987). Commonly considered a measure of contractility performance, dP/dt_{max} values decrease when the ventricle stiffens and compliance decreases (Laine, 1987; Tong et al., 2007). However, dP/dt_{max} does not change with acute edematous conditions (Stewart, 2002). We observed a **40% decrease in dP/dt_{max} in diabetic rats** compared to the control group, **which is suggestive of chronic ventricular changes and myocardial edema**. We acknowledge that the number of studies investigating dP/dt_{max} and myocardial edema is limited in the current literature. Therefore, additional research is needed to validate this hemodynamic measure as a true measure of myocardial edema.

The hypertension (Tikellis et al., 2004; Oltman et al., 2006; Toblli et al., 2010), fibrosis (Huang et al., 2005), and LV hypertrophy (Morimoto et al., 2006) observed in

the ZDF rat does increase its risk for myocardial edema. The common factor of these pathologies is the predisposition for increased ventricular pressure due to changes in blood pressure and tissue composition. Ventricular remodeling occurs due to fibrosis and hypertrophy related to pressure overload. In our 19 week old ZDF animals, we did not observe differences in end diastolic pressures compared to control animals. This finding supports the **lack of myocardial edema as evident by dry weight to wet weight ratios**. Myocardial edema has presented with moderate or severe hypertrophy or injury (Dall'Armellina et al., 2011; Kozor, Nelson, and Figtree, 2011). At this young age, the ZDF rat may lack hypertrophy and edema, which would require increases in end diastolic pressures for cardiac compensation. However in another study, 19 week old ZDF rats have been reported to have moderate hypertrophy and substantial perivascular fibrosis (Fredersdorf et al., 2004). In summary, at the organ level, we detected evidence in support of myocardial edema in dP/dt_{max} values, but additional support from end diastolic pressures and ventricular weights was not observed.

4.5.3 Diabetes causes increases in cardiac lymphatic vessel area in ZDF rat

Investigations at the tissue level revealed that lymphatic vessel area and alterations in the extracellular matrix may differ based on tissue type. As shown in Figures 5a-b, **diabetic LV samples had greater lymphatic vessel area** than control samples. We observed the difference to be 52% when comparing average values for diabetic and control rats. Our results may indicate that LV tissue differs from other tissues in terms of changes in lymphatic vessel area or density. No difference in lymphatic vessel density was detected in skeletal muscles between control and diabetic subjects (Kivela et al., 2007), while a decrease in lymphatic vessel density was

observed in the cornea of diabetic mice (Maruyama et al., 2007). The decreased response in both studies may have resulted from an insufficient diabetic lymphatic load, which did not trigger a response, or from a possible loss of lymphangiogenesis (as evident by decreased lymphatic vessel density) in the diabetic skeletal and corneal tissues. These differences in vessel area or density may be a result of tissue-specific responses.

Although we observed increased lymphatic vessel area, **we could not correlate these findings to increases in VEGF-C or pro-inflammatory hyaluronan fragments.** Diabetes has been shown to increase vascular permeability through increased VEGF-C expression in diabetic humans (Kivela et al., 2007). VEGF-C expression is also associated with decreased fluid reabsorption through the vascular capillaries (Kumar et al., 2009). However, we did not observe any differences in VEGF-C tissue levels between diabetic and control LV samples. We speculate that this result may be due to our observations being limited to one time point. This limitation may also account for the lack of change observed in hyaluronan levels. At 19 weeks of diabetes, hyaluronan levels showed no difference in ventricular tissue and plasma samples of diabetic and control rats. We could not find literature reporting hyaluronan levels in ZDF cardiac samples. In STZ diabetic rat kidney investigations, hyaluronan content was elevated in diabetic samples from animals of 26 weeks of age or between 211-325 g (Cohen et al., 2008; Melin et al., 2006). The presence of hyaluronan accumulation in the STZ rat suggests that the phenomenon is insignificant and/or the lymphatic system is intact in this ZDF model. An intact lymphatic system would be capable of handling the extravasation of fluid and macromolecules into the myocardial interstitium.

4.5.4 Diabetes causes alterations in the lymphatic signaling pathway in ZDF cardiac tissue

To further explore the function of the lymphatic system, we chose to extend our experiments to the cellular level. We focused on the PROX-1 signaling pathway for lymphangiogenesis, since we observed increases in lymphatic vessel density. PROX-1 regulates the expression of LYVE-1 and VEGFR-3. LYVE-1 removes hyaluronan from the interstitium, which is normally elevated with diabetes (Lewis et al., 2008). LYVE-1 expression may increase through inflammatory signaling due to excessive hyaluronan fragments. **We observed no changes in hyaluronan levels, and reported a similar response in the expression of the LYVE-1 receptor.** To our knowledge, we are the first to study LYVE-1 and VEGFR-3 total protein and mRNA levels in the cardiac tissues of the ZDF rat. We also found that **VEGFR-3 protein and mRNA levels were unaffected by diabetes.** VEGFR-3 expression is upregulated by VEGF-C (Partanen and Paavonen, 2001). This induction occurs through inflammatory cells expressing VEGFR-3 (Saaristo et al., 2006). Since inflammation was minimal in our ZDF rats based on inflammatory cytokines levels, it is reasonable to assume that VEGFR-3 may not be affected by diabetes at this time point.

We did observe **changes in PROX-1 total, cytosolic and nuclear protein levels, which are novel discoveries related to diabetes.** The **increased total and subcellular compartmental protein levels of PROX-1** could be the result of an initial response of the lymphatic system. However, the alterations in PROX-1 protein levels **did not result in increases in VEGFR-3 or LYVE-1 protein levels.** When normalized to the increased PROX-1 protein levels, PROX-1 **DNA binding activity actually appeared to be decreased under diabetic conditions but the sample size was too**

small to indicate significance. The increased total protein levels could be the result of cardiomyocyte development during diabetes due to injury. PROX-1 is crucial to cardiac development (Karunamuni et al., 2010; Oliver et al., 1993) and research has suggested that diabetes may induce fetal protein expression in certain tissues (Taegtmeyer, Sen, and Vela, 2010). Additional studies are needed to identify the source (cardiomyocytes and/or lymphatic endothelial cells) and the mechanisms responsible for the elevations in PROX-1 levels in total and subcellular protein fractions observed under diabetic conditions. Recent studies have suggested that Notch signaling may regulate PROX-1 and VEGFR-3 expression (Kim and Koh, 2010). Cardiomyocyte differentiation is regulated by Notch (Ahuja et al., 2004). Notch signaling has been reported as cardioprotective for valvular disease (Garg et al., 2005), ventricular remodeling, and other myocardial events (Gude et al., 2008).

4.5.5 Clinical implications of myocardial edema in diabetes

Although we cannot conclusively state that myocardial edema was present in our ZDF rats, the literature illustrates the deleterious impact that fluid imbalance has on cardiovascular diseases and health outcomes. Lymphatic vessels are speculated to play a role in multiple cardiovascular diseases, including atherogenesis and congestive heart failure (Miller, 2011; Nakamura and Rockson, 2008). Myocardial edema has reduced the success of heart transplantation (Chandrasekaran et al., 1987; Geissler et al., 2006). Investigations of LV biopsies of persons with terminal heart failure indicated an increased lymphatic vessel density, which was due to increased chamber volume and edema (Dashkevich et al., 2010). In hypertensive rats, researchers have reported fibrotic lymphatic vessels that are unable to maintain fluid flow (Ishikawa et al., 2007; Li

et al., 2009). In contrast, newly formed lymphatic vessels assisted with wound healing after a myocardial infarction in humans (Ishikawa et al., 2007). Although we have no conclusive data to support the presence of myocardial edema in this model of T2D, the literature suggests that myocardial edema may have a major influence on the development and progression of cardiovascular diseases in humans and other animal models.

4.6 Conclusions

The hierarchical analysis provides an organized evaluation of variables related to myocardial edema. We observed systemic changes in plasma IL-2 levels, reductions in dP/dt_{max} , increases in lymphatic vessel area, and changes in PROX-1 total and subcellular compartmental protein levels. These alterations are suggestive of the initiation of an inflammatory process, which could be a trigger for myocardial edema. In contrast, the increased lymphatic vessel area in diabetic tissue may be the response to myocardial edema prior to 19 weeks of age. However, LV weights revealed no differences between control and diabetic animals. Therefore, we conclude that in our study utilizing the 19 week old ZDF rat, **there is no support for myocardial edema playing a substantial role in diabetic heart disease**. Replication studies are recommended in larger animal species to detect the role of myocardial edema in diabetic heart disease.

Chapter 5

Summary

5.1 Summary and findings

The cardiovascular complications related to diabetes have become a major health concern worldwide (ADA, 2011). Type 2 diabetes (T2D) has a detrimental impact on cardiac function due to the concomitant prevalence of obesity (Galinier et al., 2005; Gidding et al., 2004; Radovits et al., 2009; Voller, Schmailzl, and Bjarnason-Wehrens, 2004). The disease has been shown to cause diastolic and systolic dysfunction resulting in decreased cardiac output and loss of physical activity (Barmeyer et al., 2009; Boudina and Abel, 2010; Watts and Marwick, 2003). Due to the complexity of diabetic heart disease, pharmacological management has been difficult and is often targeted toward a specific symptom. However, lifestyle management has proven successful in addressing the multifaceted development and progression of diabetic heart disease. Physical activity or exercise has demonstrated the ability to normalize glucose, manage weight, reduce oxidative stress, and improve cardiac function in human and animal studies (Bidasee et al., 2008; Borghouts and Keizer, 2000; Shao et al., 2009; Snowling and Hopkins, 2006; Teixeira de Lemos et al., 2009). A greater understanding of the mechanisms responsible for the beneficial effects of exercise is needed for public awareness and improvements in pharmacological treatments.

Since this knowledge has often been obtained through invasive or laborious techniques, researchers have utilized diabetic animal models for the investigation of the cardioprotective benefits and mechanisms related to exercise. The ZDF rat has served as a valid model of exploring diabetic heart disease since in this model the disease development is comparable to the T2D progression seen in humans (Clark, Palmer, and Shaw, 1983; Leonard et al., 2005). Although, we understand the disease progression in

the ZDF model, our knowledge of the physiological response of the ZDF rat to exercise is lacking. Therefore, the characterization of diabetic heart disease in the ZDF rat and the response to exercise would be advantageous in further understanding the disease and possible interventional strategies. We chose to investigate electrocardiographic (ECG) changes, because the technique is commonly used for clinical evaluations along with hemodynamic changes, through left ventricular catheterization, which provide detailed information regarding cardiac function. We hypothesized that myocardial edema is present in the Zucker diabetic fatty (ZDF) rat and may play a role in diabetic heart disease.

5.1.1 Animal characteristics and metabolic findings

Our results presented in Chapters 2, 3, and 4 indicated that SD animals had significant weight gain over the 7 weeks of diabetes duration compared to SC animals. A moderate relationship was observed between body weight and blood glucose levels. As expected, blood glucose and HbA1C levels were elevated in the SD animals in comparison to SC rats. Interestingly, we observed increases in blood glucose levels in all animals group from baseline (week 0) to termination (week 7 of diabetes). Development or aging had a statistically significant effect on blood glucose levels (Chapter 2). Exercise decreased the body weight of ED animals compared to SD animals. The mode of exercise did not influence hyperglycemia in the ED animals as blood glucose and HbA1C levels remained comparable to SD rats. This outcome may be the result of the rather moderate intensity of training used in our protocol, and the multiple rest breaks required for the SD rodents to complete 40 min of aerobic exercise. Although the exercise regimen was sufficient to attenuate ECG (Chapter 3) and

hemodynamic (Chapter 4) changes resulting from diabetes, it was not adequate for normalizing blood glucose levels.

5.1.2 Electrocardiographic findings

Although hyperglycemia was observed in SD and ED animals in our study, which most likely played a role in ventricular remodeling (Brownlee, 2001; Degenhardt, Thorpe, and Baynes, 1998), significant differences in heart weight or heart weight/body weight ratios, indicators suggestive of ventricular hypertrophy, were not noted between the groups. The study design limited our observations to the early stage of diabetes, in which the physical manifestations of hypertrophy may have been harder to decipher based on weight measurements. Noteworthy, R wave amplitudes are considered an indirect measure of ventricular hypertrophy (Myers et al., 1987). After seven weeks of diabetes, SD animals had increases in R wave amplitudes compared to the SC rat indicating increases in ventricular remodeling.

These structural changes may have had an impact on cardiac function and conduction. QT_c intervals were widened in the SD group, which suggest an increased risk of arrhythmias and other cardiac events. The increased risk was further supported by the loss of heart rate variability in the SD animals. This finding is consistent with a possible loss of sympathetic response to external stimuli. Diabetic groups, SD and ED, presented with tachycardia at the initiation of the study. Remarkably, heart rates were similar between all groups at the conclusion of the study. This result was in line with research showing that heart rate elevated with increases in blood glucose levels in humans (Kitabchi et al., 2006). Blood glucose levels were increased in all rat groups by

the termination of the study. Diabetes did not appear to have an impact on T or P wave amplitudes. QRS and PR intervals were also unaffected by the disease.

Seven weeks of exercise training were partially beneficial in attenuating the ECG changes described in Chapter 2. ED rats had R wave amplitudes similar to SC and EC rats at the conclusion of the study. However, exercise did not improve QT_c intervals or heart rate variability in our model. These results support the impact of exercise on ventricle remodeling as shown by significant differences in R wave amplitudes between SD and ED rats.

5.1.3 Hemodynamic findings

In Chapter 3, 24 hemodynamic parameters were assessed for diabetes related alterations in the left ventricle. We identified 15 parameters, listed in Table 1 that were adversely impacted by diabetes. Changes in these parameters confirmed the systolic and diastolic dysfunction associated with diabetic heart disease. We observed declines in each phase of the cardiac cycle: diastolic filling, isovolumic contraction, ejection, and isovolumic relaxation. Assessment of pressure volume loops (Figure 2) revealed that diabetes (SD rats) caused a leftward and downward shift compared to SC animals, indicating that SD animals had lower pressure and volumes values throughout the cardiac cycle compared to the SC group. Bradycardia was also detected in the SD

Heart rate
Stroke volume index
Cardiac output index
Stroke work index
Minimum volume
End diastolic volume
dP/dt_{max}
$P@dP/dt_{max}$
$V@dP/dt_{max}$
P_{max}
End systolic pressure
End systolic volume
Ejection fraction
dP/dt_{min}
$V@dP/dt_{min}$

Table 1 Hemodynamic parameters negatively affected by diabetes.

animals with pressure volume loop analysis. However in Chapter 2, we reported tachycardia in the cohort with ECG analysis. The difference may be a cohort effect, but the literature is inconclusive about heart rate changes in the ZDF rat. Reports of tachycardia (Zhou et al., 2000), bradycardia (Wang and Chatham, 2004), or no change (Daull et al., 2006) have been described in this animal model. General performance indices, such as stroke volume, cardiac output, and stroke work were also significantly depressed under diabetic conditions in this study. SD animals were unable to utilize the Frank-Starling mechanism to maintain cardiac function. In addition, we observed no differences in arterial resistance between the SC and SD animals.

Exercise training restored 13 of the 15 parameters to levels comparable to SC and/or EC animals. Significant differences were observed between the bolded parameters in Table 2 in ED animals as compared to SD animals after seven weeks of exercise training. Exercise also doubled the maximal power of the left ventricle of ED rats as compared to SD animals. Exercise proved highly successful in addressing hemodynamic changes related to diabetes. The improvements observed

strongly favored systolic function. We did not observe robust changes in diastolic function with exercise in our model. An assessment of the pressure volume loops did indicate that the compensatory responses related to heart rate and systolic function were sufficient to improve the cardiac function of ED animals to those of the SC group.

Heart rate
Stroke volume index
Cardiac output index
Stroke work index
Minimum volume
End diastolic volume
dP/dt_{max}
$P@dP/dt_{max}$
$V@dP/dt_{max}$
P_{max}
End systolic pressure
End systolic volume
Ejection fraction
dP/dt_{min}
$V@dP/dt_{min}$

Table 2 Hemodynamic parameters normalized by exercise. Parameters affected by exercise are in bold font.

5.1.4 Diabetes related myocardial edema findings

After the ECG and hemodynamic assessment of the development and progression of diabetic heart disease in the ZDF rat, we sought to determine if fluid imbalance and resulting myocardial edema could be a factor and possible target of exercise. We utilized a hierarchical approach to systematically investigate if the hemodynamic burden of ZDF rats coupled with changes in the lymphatic system causes a myocardial fluid imbalance. Because so little is known about the interaction of diabetes and myocardial edema, basic questions had to be answered before any possible effects of exercise training could be evaluated. For this reason, we analyzed sedentary diabetic and control animals only to address this question. We identified changes in body weight and inflammatory markers in diabetes. The increases in body weight of the diabetic animals suggested either an accumulation of body fat or water. Elevation of IL-2 in diabetic plasma samples indicated that the animals may be responding to an infection or inflammatory condition, which could lead to edema.

As we proceeded to the next level of analysis, we observed no difference in wet ventricle weights between the groups, and the drying weights were also similar. We did note that during the first 30 min of drying, the drying patterns varied between control and diabetic animals. Diabetic hearts appeared to be drying slower during these early time periods, which suggested that there may be differences in the tissue structure resulting in a slower release of fluid from the diabetic ventricle. Hemodynamic analysis showed that diabetic animals may have increased myocardial microvascular permeability with significant declines in dP/dt_{max} . Increased filtration into the interstitium could account for the differences in drying patterns, albeit not sufficient to affect overall

ventricular weights. We were interested to identify other changes in diabetic myocardium including cellular and extracellular matrix protein levels and activities associated with the lymphatic system. VEGF-C tissue levels were not affected by diabetes and could not be implicated in increasing the permeability of coronary vessels. We observed only a trend toward increased hyaluronan concentrations in the diabetic myocardium. Elevated hyaluronan levels would have increased the viscosity of the extracellular matrix leading to fluid accumulation in the matrix and possibly triggering edema.

We completed our analysis with an investigation into the receptors for VEGF-C and hyaluronan, VEGFR-3 and LYVE-1, respectively. We observed no changes in mRNA or protein levels for either receptor. Analysis of the transcription factor, PROX-1, which regulates VEGFR-3 and LYVE-1 expression, revealed significantly higher total, nuclear, and cytosolic protein levels in the left ventricles of diabetic animal compared to control animals. An examination of gene expression and DNA binding activity of PROX-1 indicated no difference between the groups. The lack of an increase in DNA binding activity may actually suggest decreased activity in the diabetic animals considering that the protein levels of PROX-1 increased in diabetes. In conclusion, we identified indirect clinical signs of edema with hemodynamic changes, but further investigation at the tissue and cellular levels revealed that there was no indication of myocardial edema in the ZDF rat model. The changes in PROX-1 are of interest and will require further investigation as they may be related to other pathological changes associated with diabetes.

5.2 Clinical implications

Our investigations have supported the use of the ZDF rat model in the study of diabetic heart disease and possible interventions. The ECG presentation of the ZDF rat reported in Chapter 2 (VanHoose et al., 2010) was similar to ECG changes seen in human studies (Airaksinen, 1985; Christensen et al., 2000; Laptev, Riabykina, and Seid-Guseinov, 2009; Stern and Sclarowsky, 2009). Thus **the ZDF rat could be used to study QT intervals and heart rate variability abnormalities along with pressure volume relationships.** The systolic and diastolic dysfunction observed in the ZDF rat was also comparable to the pathological modifications in humans with diabetes (Johnson et al., 2004; Poirier et al., 2003; Poornima, Parikh, and Shannon, 2006). The positive response of the ZDF rat to exercise provided additional support for the use of exercise as a non-pharmacological treatment of diabetic heart disease for people with diabetes. **Even with this relatively short duration of exercise we were able to observe significant impact on diabetic cardiovascular signs and symptoms.** Although, other reports have suggested that diabetes increases vascular permeability (Kumar et al., 2009; Moriguchi et al., 2005) and **researchers have postulated that edema may play a role in cardiovascular disease** (Miller, 1976; Miller, 2011; Miller, DeBoer, and Palmer, 1992; Nakamura and Rockson, 2008) . However, **we were unable to substantiate this claim** in our model under the conditions of the study. We acknowledge that our study analyzed one time point, which limited our conclusions, and we believe that future research is needed to thoroughly address this hypothesis.

5.3 Future directions

Future research should address the limitations of our one time point analysis and the analysis of the fluid composition of the heart in diabetes. Our study design proved adequate to attenuate some of the diabetes related ECG alterations and hemodynamic changes through exercise training. Since hyperglycemia can appear as early as 5-6 weeks in the ZDF animal, it is possible that fluid imbalance may occur much earlier than at the 19 week termination point in our study. If edema presented earlier than when our observation period started, the lymphatic system may have already responded and morphological changes related to edema were already presenting as fibrosis. Since fibrosis can also occur due to hyperglycemia, it would be hard to differentiate the etiology of fibrosis and related cardiac dysfunction in our study design. Therefore, longitudinal studies are needed to identify if myocardial edema is present at other time points along the ZDF rat lifespan. More advanced methods, such as computed tomography or other whole animal imaging techniques could be utilized to answer this question. Echocardiograms would provide additional information about the ventricle, tissue characteristics, and myocardial performance, which may also suggest changes in water content. We would also recommend larger sample sizes since changes in volume may be minuscule. Additional investigations of PROX-1 are warranted due to the increased protein levels seen in diabetic animals. Our lab has shown that diabetes causes reactivation of fetal gene expression in the hearts of T1D animal models (Smirnova, unpublished), as confirmed by other researchers (Taegtmeyer, Sen, and Vela, 2010). The increased levels of PROX-1 could be due to a reactivation of fetal gene expression in the ZDF rat hearts due to its role in fetal heart development. PROX-

1 could be a signal of cardiomyogenesis in diabetes and a possible target for treatment of diabetic heart disease.

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