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Effect of endurance exercise on the combination of streptozotocin induced diabetes and doxorubicin

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UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

**EFFECT OF ENDURANCE EXERCISE ON THE
COMBINATION OF STREPTOXOTOCIN
INDUCED DIABETES AND
DOXORUBICIN**

**A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy**

Stephanie Erin Greufe

**Natural and Health Sciences
Sport and Exercise Science
Exercise Science**

August 2013

This Dissertation by: Stephanie Erin Greufe

Entitled: *Effect Of Endurance Exercise On The Combination Of Streptozotocin Induced Diabetes and Doxorubicin*

has been approved as meeting the requirement for the Degree of Doctor of Philosophy in the College of Natural and Health Sciences in School of Sport and Exercise Science, Program of Exercise Science

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ABSTRACT

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Diabetes is a metabolic disorder that affects over 25 million Americans. Large epidemiological studies have shown that individuals with diabetes are at increased risk of many types of cancer, including those often treated with the cardiotoxic anticancer drug doxorubicin (DOX). The combination of diabetes and DOX can cause severe cardiac dysfunction, possibly from the adverse effects on cardiac metabolism. Diabetes leads to a shift away from glucose metabolism, while DOX shifts to predominately anaerobic glycolysis. The combination could lead to extremely low levels of ATP. Exercise has the potential to attenuate these adverse metabolic effects by increasing ATP synthesis. The purpose of this study was to examine the effect of endurance exercise on the combined effects of streptozotocin (STZ) -induced diabetes and DOX on cardiac function. **METHODS:** Six week old male Sprague Dawley rats were assigned to either STZ or placebo injection. After confirmed diabetes, animals were assigned to either treadmill training (TM) or sedentary (SED) groups. Following the 8 week activity period, animals were treated with either 12.5 mg/kg DOX or saline (SAL) injection. Cardiac function was measured five days post DOX/SAL injections, using both *in vivo* and *ex vivo* assessments. High performance liquid chromatography (HPLC) was used to quantify the amount of phosphometabolites (ATP, ADP, AMP, creatine, and phosphocreatine) in the left ventricular tissue to assess metabolic dysfunction. **RESULTS:** The STZ groups had significant declines in fractional shortening (FS) and

relative wall thickness (RWT), -9% and -31%, respectively, compared to non-diabetic groups ($p < .05$). Left ventricular developed pressure (LVDP) was significantly increased with STZ treatment (16%) and decreased with DOX treatment (-18%). Exercise improved LVDP compared to sedentary animals, although not significantly. Exercise training increased ATP content in STZ treated animals by 27% and 20% in those treated with DOX, $p < .05$. In addition, exercise training increased Cr levels in STZ and DOX treated groups by 62% and 47%, respectively, $p < .05$. **CONCLUSIONS:** Exercise training provides a protective effect against STZ and DOX-induced cardiac dysfunction. In addition, exercise training significantly increased the availability of phosphometabolites in the left ventricle.

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CHAPTER I

INTRODUCTION

Over 25 million Americans have diabetes according to the 2011 National Diabetes Fact Sheet. Large epidemiological studies have shown that individuals with diabetes are at increased risk of many types of cancer (Vigneri, 2009); in addition, diabetic individuals have increased cancer mortality (Suh & Kim, 2011). The cause of this increased cancer mortality in diabetics is not fully understood.

In 1972 diabetic cardiomyopathy was defined as a cardiac dysfunction in the absence of ischemia or valvular dysfunction (Rubler, et al., 1972). The cause is still largely unknown, however, many theories point to alterations in cardiac metabolism. Due to a decrease in glucose uptake, along with an increase in circulating fatty acids, diabetic metabolism experiences a shift toward predominately fatty acid oxidation (Stanley et al., 1999). In the healthy heart, fatty acid oxidation accounts for approximately 60-70% of its total energy production (Neeley & Morgan, 1974). The shift seen in diabetic hearts increases fatty acid oxidation to 90%-100% of its total energy production (Lopaschuk, 1996) which has been theorized to lead to cellular and structural alterations often described as diabetic cardiomyopathy.

Doxorubicin (DOX) is an FDA approved chemotherapy agent for the treatment of a variety of cancers including non-Hodgkin's lymphoma, acute leukemia, multiple myeloma and cancers of the breast, adrenal cortex, endometrium, lung, and ovary. Doxorubicin enters cancerous cells and inhibits DNA, thereby reducing further cellular

growth. The use of DOX is hindered by its adverse effects on the heart. In fact, 26% of patients who received a cumulative dose of 550 mg/m² experienced congestive heart failure (Swain, Whaley, and Ewer, 2003). Doxorubicin-induced cardiac dysfunction presents itself as decreased ejection fraction (Nousiainen, Jantuen, Vanninen, & Hartikainen, 2002), increased oxidative stress damage (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004), and DNA damage (L'Ecuyer et al., 2006). Theories of DOX-induced dysfunction include impaired abilities of the mitochondria, sarcoplasmic reticulum, and sarcolemma (Pelikan et al., 1989, Zorzato, Salviati, Facchinetti, & Volpe, 1985, and Boucek et al., 1987); however, research is ongoing in this area and further details regarding known mechanisms of cardiotoxicity as well as new mechanisms associated with DOX cardiotoxicity continue to be elucidated.

There are currently no studies that have investigated the effects of DOX treatment on cardiac mitochondrial function in a diabetic model. One study evaluated the pharmacokinetics of DOX in diabetic rats and found higher levels of DOX in diabetic rats' serum compared to non-diabetic rats. Serum creatine phosphokinase activity was also significantly higher in diabetic rats compared to non-diabetic rats, suggesting an increase in anaerobic metabolism. The researchers concluded that diabetes may have an effect on DOX clearance in cardiac tissue, which exacerbates cardiac dysfunction (Al-Shabanah, El-Kashef, Badary, Al-Bekairi, & Elmazar, 2000).

Exercise training has often been used as a protective measure against such adverse cardiac effects. Metabolically, exercise increases both glucose and fatty acid utilization and thus increases available ATP. Several animal models have found that exercise training mitigates cardiac dysfunction and reduces apoptotic markers in diabetic animals

(Lumini-Oliveira, Magalhaes, Pereira, Moreira, Oliveira, & Ascensao, 2001) and in models of DOX cardiotoxicity (Ascensao et al., 2005, Ascensao et al., 2006, Chicco, Schneider, & Hayward 2005, Chicco, Hydock, Schneider, & Hayward, 2006).

Statement of Purpose

The purpose of this study was three-fold: (1) to examine the combined effects of streptozotocin (STZ)-induced diabetes and DOX treatment on cardiac function, (2) to investigate the effects of exercise training on the cardiac dysfunction induced by the combination of diabetes and DOX, and (3) determine the effects of STZ and DOX on cardiac phosphometabolites and the impact exercise may have on this effect.

Research Hypotheses

- H1 Combined treatment of STZ and DOX will lead to an increase in cardiac dysfunction, evidenced by declines in fractional shortening, left ventricular developed pressure and rates of pressure development.
- H2 Combined treatment of STZ and DOX will lead to an increase in cardiac metabolic dysfunction, evidenced by declines in phosphometabolites in left ventricular tissue.
- H3 Endurance exercise training will attenuate the increased cardiac dysfunction and metabolic dysfunction associated with the combination of STZ and DOX, evidenced by improvements in fractional shortening, left ventricular developed pressure, rates of pressure development and phosphometabolite availability in left ventricular tissue.

Need for the Study

Diabetes prevalence is increasing every year, and with the recent finding that diabetes increases cancer risk and cancer mortality, understanding the effects of chemotherapies in the presence of diabetes has never been more important (Vigneri et al., 2009 and Suh, et al., 2011). Diabetes alone will increase cardiac dysfunction (Galderisi, Anderson, Wilson, and Levy, 1991, Bugger et al., 2010, and Shao et al., 2011) causing metabolic shifts and impaired Ca^{2+} homeostasis (Camps et al., 1992, Oliveria, et al., 2003

and Jweied, et al., 2005) which will ultimately lead to decreased ATP production.

Doxorubicin treatment leads to decreases in all oxidative processes, increasing reliance on anaerobic metabolism (Abdel-Aleem et al., 1997 and Gratia et al., 2012). With diabetes, cardiac ATP production comes almost exclusively from fatty acid oxidation, while after DOX treatment there is a shift towards cardiac glycolytic metabolism. At this time, the ramifications of the cardiac metabolic effects of these two conditions are unclear.

Cardiac dysfunction caused by both diabetes and DOX have been attenuated with the inclusion of exercise training, when assessed individually; however, no study has investigated the effects of exercise training on the combined effects of DOX and diabetes on cardiac function. In addition, no studies have investigated the cardiac metabolic effects of the combined treatments, DOX and STZ-induced diabetes. With the prevalence of diabetes increasing and the continued use of DOX, this study was an important first step in understanding the cardiac effects of DOX treatment in diabetic cancer patients.

Table 1

Abbreviations.

ATP - adenosine triphosphate	LVDPd - LVDP during diastole
Ca ²⁺ - calcium	LVDs - LV end systolic diameter
CIT - citrate buffer	NADH - nicotinamide adenine dinucleotide dehydrogenase
CO - cardiac output	PWd - posterior wall thickness during diastole
DNA - deoxyribonucleic acid	PWs - posterior wall thickness during systole
DOX – doxorubicin	ROS - reactive oxygen species
dP/dt _{max} - maximum rate of developed pressure	RONS - reactive oxygen and nitrogen species
dP/dt _{min} - minimum rate of developed pressure	RPLC - reversed phase liquid chromatography
EDP - end diastolic pressure	RWT - relative wall thickness
ESP - end systolic pressure	SED - sedentary
EF - ejection fraction	SOD - superoxide dismutase
ET - ejection time of aortic blood flow	STZ - Streptozotocin
FS - fractional shortening	SV - stroke volume
HPLC - high performance liquid chromatography	SWd - septal wall thickness during diastole
HR - heart rate	SWs - septal wall thickness during systole
i.p. – intraperitoneal	TM - treadmill
LV - left ventricular	A-Vmax - maximal aortic flow velocity
LVDD - LV end diastolic diameter	M-Vmax - maximal mitral flow velocity
LVDP - LV developed pressure	VTI - velocity time integral
LVDs - LV end systolic diameter	

Delimitation of the Study

Samples in this study included 5-7 week old male Sprague Dawley rats (150-200 g). Diabetes was induced using a 45 mg/kg bolus intraperitoneal (i.p.) injection of STZ. The exercise training groups ran on a motorized treadmill five days a week for 8 weeks. Cardiotoxicity was induced using a 12.5 mg/kg bolus i.p. injection of DOX, following treadmill training.

Definition of Terms

Cardiac Efficiency – the ratio of energy output to energy input.

Cardiomyopathy – disease of the heart when the myocardium becomes enlarged, thick, or rigid and can lead to heart failure.

Cardiotoxicity – damage to the heart from cytotoxic drugs.

Diabetic Cardiomyopathy – a ventricular dysfunction in diabetes in the absence of coronary artery disease and hypertension.

Diastole – the filling phase in the cardiac cycle.

Doxorubicin – chemotherapy agent approved by the FDA for the treatment of a variety of cancers including non-Hodgkin's lymphoma, acute leukemia, multiple myeloma and cancers of the breast, adrenal cortex, endometrium, lung, and ovary.

Echocardiogram – a sonogram of the heart used to measure cardiac chamber dimensions and blood flow velocities.

Ejection Fraction – the percentage of blood pumped out of the left ventricular with each cardiac cycle.

Fractional Shortening – the left ventricular diastolic dimension that is lost during systole, reported as a percentage.

High performance liquid chromatography – an instrument used in analytical chemistry to separate compounds in order to identify and quantify the desired compound.

Systole – the contraction phase of the cardiac cycle.

Streptozotocin – a chemical toxic to the beta cells of the pancreas. It is currently approved by the FDA for treatment of pancreatic cancer and is commonly used in research to induce Type 1 diabetes.

CHAPTER II

REVIEW OF LITERATURE

Worldwide, 171 million people have diabetes and that number is expected to rise to 366 million by 2030 (Wild, Sigree, Roglic, King, & Green, 2004). Diabetes is known to lead to atherosclerosis in large and coronary arteries, increasing the risk of heart attack and stroke (Tarquini, Lazzeri, Pala, Rotella, & Gensini, 2011). The incidence of cancer is also increased in diabetic individuals, with relative risk ranging from 0.81 to 2.51 across various cancers (Vigneri et al., 2009). In addition, cancer mortality is increased among the diabetic population compared to the non-diabetic population (Suh & Kim, 2011). DOX is one of the most effective chemotherapy drugs used today; however, it is also one of the most dangerous. Doxorubicin-induced cardiomyopathy is so prevalent that physicians assess a patient's risk of heart disease prior to treatment with DOX, and in incidences where risk of cardiovascular disease is present, DOX is not used for cancer treatment. Currently, diabetes is not a risk factor evaluated by physicians prior to treatment; however, one study found that diabetic patients receiving DOX had an increased likelihood of congestive heart failure (Hershman, McBride, Eisenberger, Tasi, Grann, & Jacobson, 2008).

Diabetic Cardiomyopathy

It is widely believed that cardiac dysfunction in diabetics is the product of hypertension and coronary atherosclerosis. However, large epidemiological studies have

shown that the risk of cardiovascular disease remains significantly higher in diabetic individuals after controlling for age, blood pressure, weight, cholesterol, and a history of coronary artery disease. Diabetic cardiomyopathy is a clinical term used to describe cardiac dysfunction in diabetics in the absence of hypertension and coronary atherosclerosis (Aneja, Tang, Bansilal, Garcia, & Farkouh, 2008).

The cardiac consequences of diabetes include decreased systolic and diastolic function accompanied by increases in cardiac mass (Poornima, Parikh, & Shannon, 2006). Idiopathic dilated cardiomyopathy is also common. In fact 75% of those with unexplained idiopathic dilated cardiomyopathy were found to have diabetes (Tarquini et al., 2011). As part of the Framingham Heart Study, it was found that diabetic patients have increased left ventricular mass, left ventricular end diastolic dimension, left ventricular wall thickness and relative wall thickness, and a decrease in fractional shortening (Galderisi et al., 1991). After removing confounding factors such as obesity, hypertension, and smoking, left ventricular mass and wall thickness remained significantly and independently increased in diabetic patients (Galderisi et al., 1991). Diabetic animals present decreased heart rates, fractional shortening and ejection fractions (Bidasee, Zheng, Shao, Parbhu, Rozanski, & Patel, 2008), in addition to prolonged relaxation times and decreased flow velocity during the filling phase (Shao et al., 2011). Human and animal studies alike have found that diabetes, regardless of cause, results in significant alterations to cardiac function.

Cell death by apoptosis has been shown to increase in diabetic cardiac tissue contributing to diabetic cardiomyopathy (Cai, Li Wang, Guo, Jiang, & Kang, 2002, and Li et al., 2007). The mechanisms are unknown; however, two developing theories

involve mitochondrial and endoplasmic reticulum dysfunction and oxidative stress activating apoptotic pathways leading to cell death. Dysfunction of the mitochondria and endoplasmic reticulum, and an increase in oxidative stress will be discussed below in greater detail.

Diabetes-Induced Metabolic Dysfunction

Considerable changes occur in diabetic energy metabolism which often precede cardiac contractile dysfunction. In the diabetic heart, there is an increase in fatty acid circulation and oxidation, accompanied by low levels of glucose utilization (Stanley, Lopaschuk, and McCormack, 1997). In various studies, STZ-induced type-1 diabetes has resulted in decreased cardiac function, cardiac efficiency, mitochondrial function, and calcium handling, with increases in oxidative stress (Bugger et al., 2010, Shao et al., 2011).

Glucose Metabolism

Myocardial glucose transport, glycolysis, and glucose oxidation are all decreased in diabetic tissues. Myocardial glucose transport has been shown to be defective in both diabetic humans and STZ-induced diabetic animals (Stanley et al., 1999). Glucose transporters, GLUT 1 and 4, are downregulated with diabetes (Camps et al., 1992). Decreased glycolysis is accompanied by an increase of intracellular glucose 6-phosphate and a decrease in the fructose 1,6-biphosphate/fructose 6-phosphate ratio. Phosphofructokinase (PFK) is a key enzyme in glycolysis that catalyzes the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate. With the increase in fatty acid oxidation, there is an increase in citrate, which, along with high ATP/ADP ratios, will inhibit PFK activity and thus inhibit glycolysis further. The rate of glucose oxidation is

also significantly reduced in cardiac tissue due to decreased pyruvate dehydrogenase (PDH) activity (Wall & Lopaschuk, 1989). Pyruvate dehydrogenase is inhibited by high plasma levels of free fatty acid and an increase in fatty acid oxidation. The condition of increased blood glucose, hyperglycemia, can itself lead to cardiac tissue injury.

Hyperglycemia increases the production of reactive oxygen species (ROS) and nitric oxide synthase (NOS), leading to an increase in cytochrome c-mediated caspase 3 activation, which activates pro-apoptotic pathways (Cai, et al., 2002).

Fatty Acid Metabolism

Fatty acid oxidation typically provides 60 – 70% of the heart's energy needs (Neeley & Morgan, 1974); however, in the uncontrolled diabetic heart, that percentage raises to 90 – 100% (Lopaschuk, 1996). Decreased glucose uptake and glucose metabolism, discussed previously, are only a part of the puzzle. Increases in plasma concentration of fatty acids, fatty acid transporters, and fatty acid uptake appear to be large components in the shift to increased fatty acid utilization. Studies using isolated working heart models have shown that significant metabolic alterations occur within the cardiac mitochondria. By perfusing hearts with buffer absent of fatty acids, studies have found that the diabetic heart only utilizes glucose for 20% of its energy requirements (Lopachuk, 1996). Carnitine palmitoyl-transferase 1 (CPT 1) appears to play a significant role in fatty acid metabolism in the diabetic heart. CPT 1 is a key enzyme in the transport of fatty acids from the cytoplasm into the mitochondria facilitating the transfer of fatty acyl CoA into fatty acylcarnitine, and its activity is inhibited by malonyl CoA. The concentration of malonyl CoA is decreased in the STZ diabetic heart due to decreases in acetyl CoA carboxylase (ACC) (Sakamoto, Barr, Kavanagh, & Lopaschuk,

2000). ACC catalyzes the conversion of acetyl CoA into malonyl CoA. In addition, AMP activated protein kinase activity, a known inhibitor of ACC, is increased in STZ diabetic animals.

Cardiac efficiency, the ratio of energy output (pressure: volume area) to energy input (oxygen consumption), is also decreased in STZ treated diabetic animals (How, Aasum, Severson, Chan, Essop, & Larsen, 2006). The shift to fatty acid metabolism leads to increased oxygen demands that outweigh ATP production. One study found that although ATP synthesis is slowed in diabetic animals, the decline in production is not significantly affected (Ferko, M., Gvozdjakova, A., Kucharska, J., Mujkosova, J., Waczulikova, I., Styk, J., et al., 2006). An overview of diabetic metabolism can be seen in Figure 1.

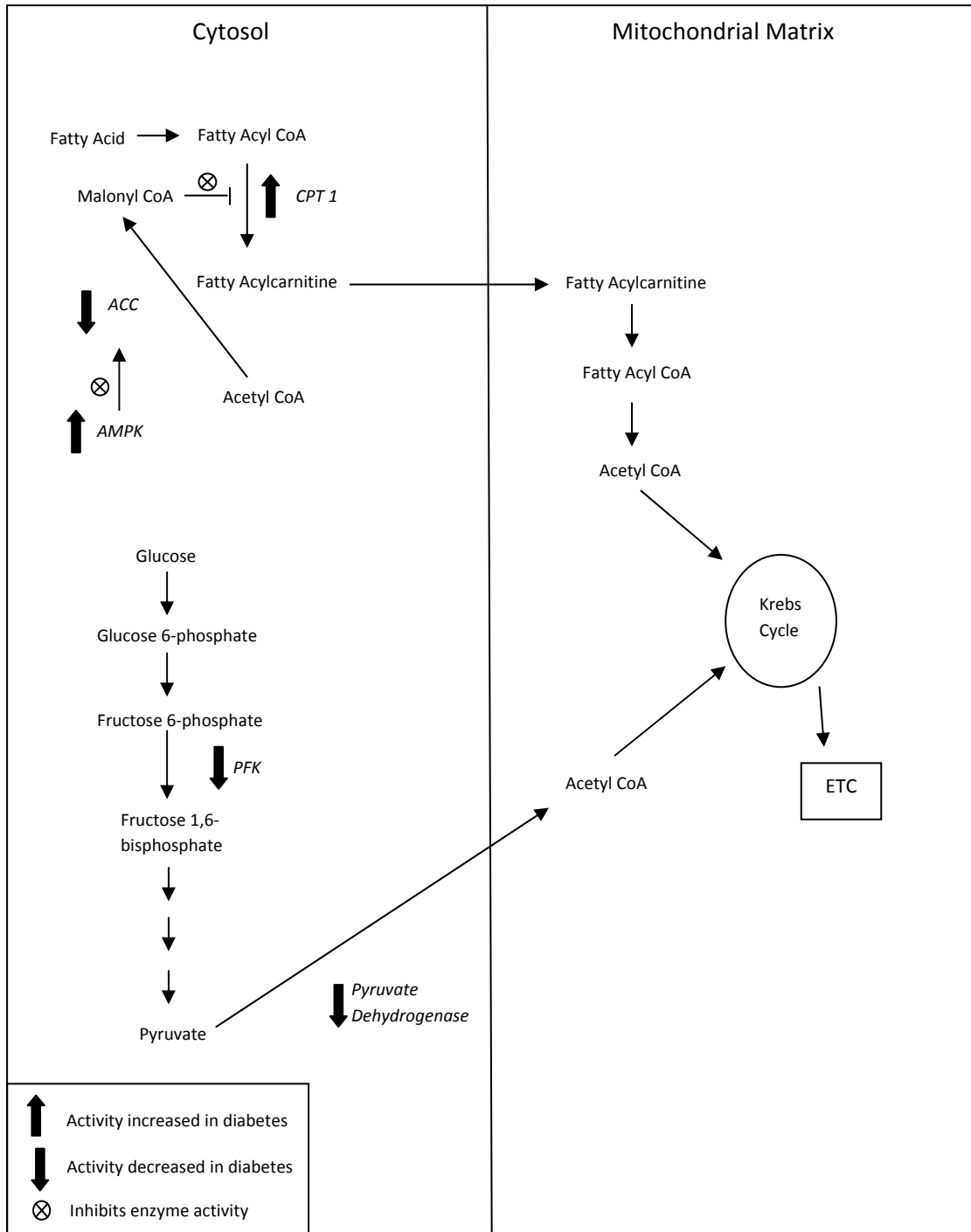


Figure 1: Diabetic metabolism. A shift from glucose metabolism to fatty acid metabolism can be seen by the increase in CPT 1 activity, which increases the transport of fatty acids into the mitochondrial matrix. In addition, AMPK is increased and ACC is decreased leading to a decreased inhibition of CPT 1 by malonyl CoA. PFK and PDH are also decreased, slowing both glycolysis and glucose oxidation.

Oxidative Stress

An increased production of free radicals in heart tissue has been linked to diabetic cardiac complications (Jay, Hitomi, & Griendling, 2006). Evidence suggests increased ROS production is not only found in diabetic cardiac mitochondria, but also cytosolic ROS production is present with hyperglycemia (Jay et al., 2006). Reactive oxygen species, with its short half-life, typically inflicts its damage close to its origin or, in this case, the mitochondria. With further insults on the mitochondria, ROS production is exacerbated. Studies have found that treatment with ROS scavengers (e.g., metallonthionin, catalase and manganese superoxide dismutase) reversed diabetic cardiomyopathy (Liang, Carlson, Donthi, Kralik, Shen, & Epstein, 2002, Matsushima, et al., 2006, Shen, Zheng, Metreveli, & Epstein, 2006).

Calcium Handling

Calcium handling is another theory surrounding altered cardiac function seen in patients with diabetes. Glycolysis typically occurs close to the sarcoplasmic reticulum (SR) and sarcolemma because that is where the necessary enzymes can be found, additionally, ion transporters (such as SERCA2a and Na/K ATPase) located on the SR and the sarcolemma are preferentially fueled by ATP generated from glycolysis (Hattori et al., 2000, Kashihara, Shi, Yu, McNeill, & Tibbits, 2000). If due to decreased ATP, SERCA2a and Na/K ATPase transporters' activity will be decreased and Ca^{2+} homeostasis will be impaired. As a result of impaired Ca^{2+} homeostasis, myofilament contractile function is abated and could lead to the development of cardiomyopathies (Jweied, et al., 2005 and An & Rodrigues, 2006). When a mitochondrial permeability transition pore inhibitor (cyclosporin) was introduced to the STZ diabetic rat, differences

in calcium handling were not observed. Thus, confirming that calcium handling is impaired in diabetic cardiomyocytes, which may possibly result in compromised energy production and reduced myofilament contractility (Oliveria, et al., 2003).

Doxorubicin-Induced Cardiac Dysfunction

Doxorubicin is one of the most prescribed chemotherapies today. It is an anthracycline anticancer treatment used to treat non-Hodgkin's lymphoma, acute leukemias, multiple myeloma and cancers of the breast, adrenal cortex, endometrium, lung, and ovary since the 1960s. However its use is strongly regulated due to its harmful effects on the cardiovascular system. Doxorubicin's mechanism of action is still being studied, but it is believed to preferentially enter cells that are metabolically active. Upon entering, it binds to nuclear DNA and interacts with DNA strands by intercalation. Doxorubicin also inhibits topoisomerase II activity preventing replication and further growth of the tumor.

Despite its potent anticancer effects, DOX's cardiotoxic effects have hindered its clinical usage. In human studies, patients who receive DOX have a decreased ejection fraction and diastolic dysfunction. In animals, DOX treatment decreases left ventricular developed pressure as well as ejection fraction, fractional shortening and $+dP/dt$ and increases diastolic pressure and $-dP/dt$.

Wu and colleagues (2002) investigated the *in vivo* and *in vitro* apoptosis of cardiomyocytes and endothelial cells in cardiomyopathy induced by DOX. Male rats were injected with 4 mg/kg three times per week for two weeks and sacrificed six weeks following the last treatment. DNA laddering, TUNEL assay and caspase 3 expression assays were performed on the cardiomyocytes and endothelial cells of the rats. The *in*

in vitro study cultured cardiomyocytes from 3-5 day old rats and endothelial cells from human umbilical veins and treated them with DOX. Analysis on these cells included: apoptosis measures and expressions of Fas antigen, Bax, Bcl-2 and caspase-3. The *in vivo* study resulted in increased apoptosis and increased expression of caspase-3 in both cardiomyocytes and endothelial cells compared to controls. The *in vitro* study showed an increase in apoptotic cells, Fas, Bax, caspase-3 and a decrease in Bcl-2. The researchers concluded that apoptosis induced by DOX can occur both *in vivo* and *in vitro* in cardiomyocytes and endothelial cells.

Doxorubicin-Induced Metabolic Dysfunction

Glucose Metabolism

Little is understood about the DOX effects on glucose metabolism. One study found that glucose uptake markedly increased with DOX treatment; however, the effect was transient and within three hours, cardiomyocytes showed a marked decrease in glucose uptake (Hrelia et al., 2002). Jeyaseelan and colleagues (1997) saw a decrease in mRNA levels of the key glycolytic enzyme PFK. One research group found that all oxidative processes, including glucose oxidation, were decreased with high levels of DOX (Abdel-Aleem et al., 1997). In addition, decreased activities of both PDH and lactate dehydrogenase (LDH) accompanied DOX treatment, concluding that in addition to glucose, lactate and pyruvate oxidation is reduced in DOX treated cardiomyocytes (Gratia et al., 2012).

Fatty Acid Metabolism

Just as with glucose metabolism, there is conflicting evidence regarding the effect of DOX on fatty acid metabolism. In heart failure, there is a decrease in fatty acid

oxidation and an increase in glucose utilization as a compensatory measure.

Cardiotoxicity induced by DOX has been associated with decreases in both glucose and fatty acid utilization. Wakasugi and colleagues (1993) found that although both glucose and fatty acid metabolism were significantly decreased, fatty acid oxidation was decreased to a lesser extent. CPT 1, as discussed above, is a key enzyme in the transportation of fatty acids and was found to be impaired in DOX treated cardiomyocytes, which would also lead to decreased fatty acid oxidation (Abdel-Aleem et al., 1997). Doxorubicin-induced cardiotoxicity leads to a significant decrease in myocardial ATP, along with decreases in creatine, phosphocreatine and lactate, indicating both aerobic and anaerobic metabolic dysfunction (Wallace, 2003). Fatty acid oxidation was inhibited following DOX administration and compensated for by an increase in aerobic glycolysis with contributions from lactate and pyruvate, which is contrary to Wallace, who, as stated above, found lactate oxidation to be decreased (Carvalho, et al., 2010).

Oxidative Stress

The quinone structure of DOX makes it prone to a one electron reduction forming a semiquinone (Zucchi & Danesi, 2003). This reduction is catalyzed by the exogenous NADH dehydrogenase in the cardiac mitochondria (Nohl, Gile, & Staniek, 1998). The semiquinone then donates an electron to oxygen, forming a superoxide anion (Olson & Muslin, 1990). The superoxide anion can either trigger lipid peroxidation or be converted to hydrogen peroxide (Olson et al., 1990). Mimnaugh, Trush, Bhatnager and Gram (1985) found that lipid peroxidation increased 4-fold after treatment with DOX. Free radicals can also be formed from the interaction of DOX and iron (Minotti, Cairo, &

Monti, 1999). The interaction passes electrons between iron, DOX and oxygen with a final product of one iron (III)-DOX (aldehyde) and two superoxide anions (Olson et al., 1990). Voltage-dependent anion channels release cytochrome *c*, resulting in the activation of caspase-3 and apoptosis (Childs, Phaneuf, Dirks, Phillips, and Leeuwenburgh, 2002).

Clark and colleagues (2002) found that after treatment with DOX, cells showed an increase in Akt activation after hydrogen peroxide-induced oxidative stress. Free radicals impact cardiac function through its effects on the SR. Free radicals lead to release and depletion of calcium from the SR resulting in impaired cardiac contractility and relaxation (Carafoli, 1985). Free radicals may also affect cardiac mitochondria. After administration of DOX to isolated mitochondria, superoxide production rose leading to an increase in cytochrome *c* efflux (Green & Leeuwenberg, 2002). Cytochrome *c* activates caspase-3 which begins the apoptotic pathway, as discussed above.

Calcium Handling

Doxorubicin is hypothesized to increase intracellular levels of calcium. One mechanism is through the altering of the sarcolemmal Na/K-ATPase channels and the Na/Ca exchanger (Boucek et al., 1987). Another mechanism involves the sarcoplasmic reticulum (SR). It is theorized that DOX can alter the release of calcium from the SR, depleting its stores (Zorzato et al., 1985). Solem, Henry and Wallace (1994) found that DOX leads to calcium cycling in the mitochondria and depolarization of the sarcolemma and this effect could be inhibited by cyclosporine A (a known inhibitor of mitochondrial permeability transition pores) or ruthenium red (a mitochondrial Ca²⁺ uniporter inhibitor).

A later study by Solem, Heller and Wallace (1996) found a dose-dependent sensitivity to calcium-induced calcium release and membrane depolarization which induced cellular apoptosis in cardiac mitochondria.

Combined Effects of Diabetes and Doxorubicin

One study conducted by Al-Shabanah and colleagues (2000) investigated the combined effects of STZ and DOX. Animals were injected with 65 mg/kg of STZ, and two weeks after diabetes was induced, animals were injected with 15 mg/kg of DOX. Blood samples were collected and the animals were sacrificed 24 hours following the DOX injection. Streptozotocin-induced diabetic rats, whether treated with DOX or saline, had significantly decreased body weight, heart weight, serum glucose and urine volume, while DOX alone only showed significant decreases in body weight. Serum DOX levels were also measured at seven different time points following the DOX injection. Serum DOX levels in diabetic animals were more than 30% higher than non-diabetic animals. Serum creatine phosphokinase activity was also significantly higher in STZ diabetic rats treated with DOX compared to non-diabetic treated with DOX, signifying an altered metabolic state with combined treatment. The researchers concluded that the pharmacokinetics of DOX is significantly altered in STZ diabetic animals, significantly decreasing the clearance of DOX.

Exercise Training

Exercise training is known to provide cardioprotective effects. Exercise leads to cardiac hypertrophy and increased ejection fraction. Metabolically, exercise increases both glucose and fatty acid utilization and thus increases available ATP. Exercise leads to an increase in AMPK, which in turn inactivates ACC and malonyl CoA, thereby

increasing fatty acid oxidation. AMPK also stimulates glucose uptake (through insulin, which may not be present in STZ diabetic hearts) and glycolysis by activating PFK. Thus both glucose and fatty acid utilization are increased; however, in the DOX heart cardiomyopathies are often present. In the presence of cardiomyopathies, the heart may be in a state of compensated heart failure, in which it is predominately anaerobic due to its ischemic state.

Exercise Training in Diabetic Models

A study conducted by Bidasee and colleagues (2008) has tested the effects of exercise training on cardiac function of STZ-induced diabetic rats. It was found that following seven weeks of diabetes, rats had decreased heart rates, fractional shortening, and ejection fraction. Three weeks of treadmill training was able to improve ejection fraction. A similar study using STZ-induced diabetic rats found that after nine weeks of treadmill training and 7 weeks of STZ-induced diabetes exercise was found to significantly attenuate the magnitude of mitochondrial ultrastructure damage, assessed using transmission electron microscopy (Searls, Smirnova, Fegley, & Stehno-Bittel, 2004). No study has measured the combined effect of exercise and diabetes on cardiac ATP content.

Exercise Training with Doxorubicin Treatment

The use of exercise to attenuate DOX-induced cardiotoxicity has been demonstrated across different modes of exercise, intensities, and durations (Ascensao et al., 2005, Chicco, Schneider, & Hayward, 2005, Chicco et al., 2006 and Wonders, Hydock, Schneider, & Hayward, 2008). All have positively identified exercise as a means of attenuating cardiotoxicity through increased cardiac function, decreased pro-

apoptotic factors, decreased oxidative stress damage, increased antioxidant capacity, and improved calcium homeostasis (Ascensao et al. 2005, Ascensao et al., 2006, Chicco, et al., 2005, Chicco et al., 2006, Hydock, Lien, Jensen, Schneider, & Hayward, 2011, and Wonders, Hydock, Greufe, Schneider, & Hayward, 2009). Although numerous studies have researched the metabolic effects of exercise in DOX treated hearts, no studies have quantified cardiac ATP content as a means of understanding the metabolic effects.

Conclusion

Diabetes has been connected to cancer risk and cancer mortality (Vigneri et al., 2009 and Suh, et al., 2011). Human studies have found increased left ventricular mass, left ventricular end diastolic dimension, left ventricular wall thickness and relative wall thickness and a decrease in fractional shortening in diabetic patients (Galderisi et al., 1991). Animal studies with STZ-induced diabetes have also resulted in adverse effects such as, decreased cardiac function, cardiac efficiency, mitochondrial function, calcium handling and increased oxidative stress damage (Bugger et al., 2010, Shao et al., 2011). Oxidative stress is increased with diabetes both in the mitochondria and cytosol leading to increased cardiac damage (Jay et al., 2006). Further decrements in cardiac function, as well as decreased energy production and contractility can occur from impaired Ca^{2+} homeostasis (Oliveria, et al., 2003 and Jweied, et al., 2005). The shift to fatty acid oxidation is due to decreased glucose transporter and increase fatty acid circulation (Camps et al., 1992). Relying on fatty acids almost entirely for ATP production will lead to a significant decrease in cardiac efficiency due to increased oxygen demand (How et al., 2006).

Doxorubicin treatment leads to decreases in all oxidative processes: fatty acid, glucose, lactate and pyruvate (Abdel-Aleem et al., 1997 and Gratia et al., 2012). With both aerobic and anaerobic metabolic dysfunction and increased percentage of energy production coming from anaerobic pathways, DOX treatments result in significant decreases in ATP production (Wallace, 2003 and Kawasaki, Lee, Shimizu, Ishii, and Ueda, 1996). With the DOX-treated heart preferential to anaerobic metabolism and the diabetic heart being fueled almost entirely by fatty acid oxidation, the combination of a diabetic patient receiving DOX treatments could be detrimental to the heart's ability to generate ATP for contraction and relaxation.

Our lab has successfully attenuated the cardiotoxic effects of DOX treatment with exercise training. Cardiac dysfunction by diabetes has also been attenuated with the inclusion of exercise training. No study has looked at the effects of exercise training on the combined effects of DOX and diabetes on cardiac function. In addition, no study has looked at the cardiac metabolic effects of combined treatments with DOX and STZ-induced diabetes. Exercise has been shown to attenuate the effects of the treatments alone, however, the combination of DOX and STZ may lead to the increase of other substrates (other than glucose and fatty acids) or it may increase the ATP debt. With the prevalence of diabetes increasing and the continued use of DOX, this study provides much needed knowledge about the combined effects of these conditions on the heart and the potential role of exercise in alleviating those effects.

CHAPTER III

METHODOLOGY

Experimental Design

The purpose of this study was to assess the cardiovascular effect of endurance training in type-1 induced diabetic animals treated with DOX. This was accomplished by first inducing diabetes with STZ. Animals were randomly assigned to either STZ or citrate buffer only (CIT) groups. Three days following STZ/CIT injection, diabetes was confirmed via blood glucose testing. Animals then were randomly assigned to either sedentary (SED) or treadmill trained (EX) groups. Twenty-four hours following the last treadmill session animals were again randomly assigned to either SAL or DOX groups. Five days following SAL/DOX injections animals were sacrificed. Cardiac function was assessed using an isolated working heart model and mitochondrial function was assessed by quantifying phosphometabolites (ATP, ADP, AMP, creatine, and creatine phosphate) using HPLC. Figure 2 displays the experimental design, and the following sections include details of each experiment.

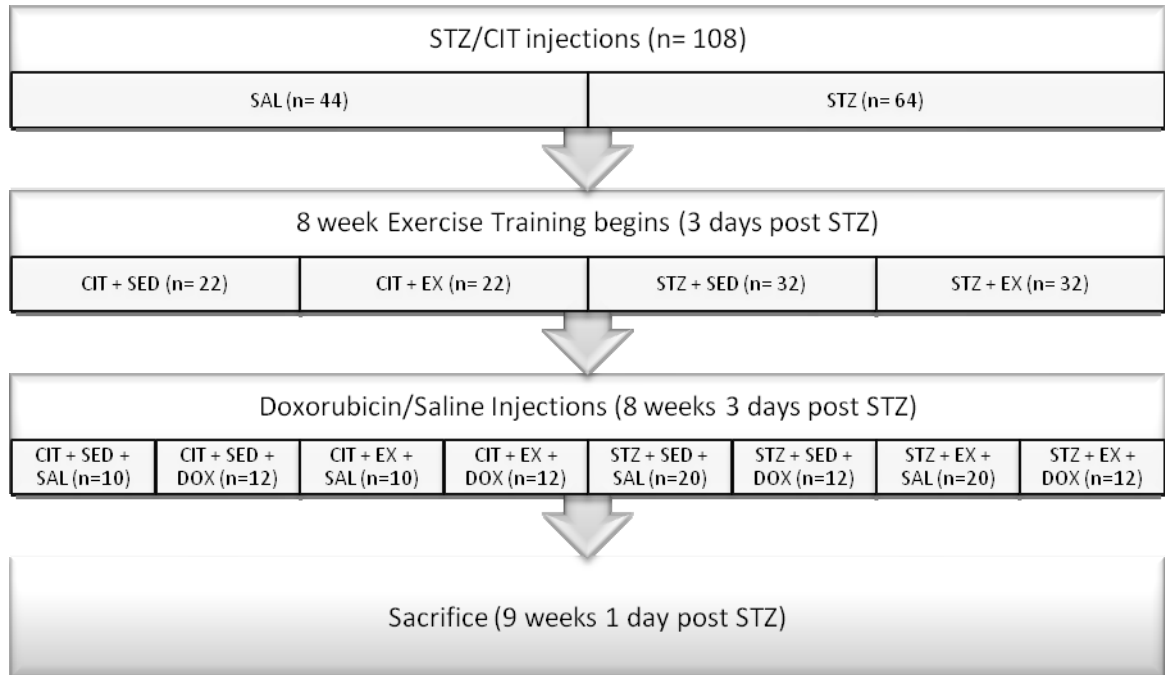


Figure 2: Proposed Study Design

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin.

Subjects

This study used 108 male Sprague-Dawley rats (150-200g) obtained from Harlan Industries (Indianapolis, IN). Rats were housed two per cage and given free access to standard rat chow and distilled water *ad libitum*. Animals were kept in a temperature controlled facility and kept on a 12:12 hour light-dark cycle. All of the proposed procedures have been approved by the University of Northern Colorado Institutional Animal Care and Use Committee (IACUC) and were in compliance with the Animal Welfare Act guidelines.

Streptozotocin-Induced Diabetes

Male Sprague-Dawley rats weighing 150-200g were chosen because they have been shown to rarely develop ketosis following STZ-induced type I diabetes. Streptozotocin was used to induce type I diabetes with an i.p. injection of 45 mg/kg in a 0.01 M citrate buffer at a pH of 4.5, the injection was prepared immediately prior to injection. Control animals received citrate buffer only. Animals were fasted 6-8 hours prior to STZ injection and given free access to food for the remainder of the study. There is a high risk of death immediately following STZ injection due to the rapid release of insulin from necrotic islet β -cells causing life threatening hypoglycemia. To combat this, forty-eight hours following STZ injection animals were given free access to 10% sucrose water. Non-fasting blood glucose was tested 3 days post STZ injection to confirm the presence of diabetes. A blood glucose value >250 ml/dL was used to confirm the diabetic condition. Animals with lower blood glucose (<250 ml/dL) were excluded from the experiment (Bidasee et al., 2008).

Blood glucose, body weight and food intake were measured weekly. Blood glucose was measured with an Accu Chek blood glucose meter. The tail was first cleaned with ethanol, and a lancet was used to stick the tip of the tail. The tail was then milked until at least 0.6 microliters of blood was absorbed into the testing strip (Bidasee et al., 2008). In the cases where blood glucose exceeds 600 ml/dL the test was performed a second time. If blood glucose tests higher than 600 mg/dL on the second test, the animal was euthanized.

Exercise Training

Animals in STZ and SAL groups were further divided into the following groups; CIT+SED, CIT+EX, STZ+SED, and STZ+EX. Animals in SED groups were restricted to cage activity for 8 weeks. Animals in EX groups were trained five days a week (Monday – Friday) for 8 weeks during their dark cycle. Training speed was held constant at 18 m/min for the 8 week training schedule. Exercise training progressed to a maximum of 15% incline for 60 minutes at the end of the training period. A summary of the proposed exercise training regimen appears in Table 2.

Table 2: Exercise Training Protocol

Week	1	2	3	4	5	6	7	8
Speed (m/min)	18	18	18	18	18	18	18	18
Incline (%)	0	0	0	3	6	9	12	15
Duration (min)	20	40	60	60	60	60	60	60

Doxorubicin Injection

Twenty-four hours following the last exercise training session, animals in the 4 groups were further divided into DOX and SAL for a total of 8 groups (CIT+SED+SAL, CIT+SED+DOX, CIT+EX+SAL, CIT+EX+DOX, STZ+SED+SAL, STZ+SED+DOX, STZ+EX+SAL, and STZ+TM+DOX). DOX was administered as a bolus i.p. injection at a dose of 12.5 mg/kg. Five days post DOX/SAL injection, animals were sacrificed and cardiac function was assessed using an isolated perfused working heart model.

Echocardiography

In vivo cardiac function was assessed using echocardiography on sedated rats with a commercially available echocardiographic system (Toshiba Nemio 30; 10 MHz transducer). Animals were sedated with ketamine (40 mg/kg, i.p.), and echocardiography was completed within 10-15 minutes after the administration of the sedative. From the

short-axis view, an M-mode tracing of the LV was obtained for measures of septal wall thickness during systole (SWs) and diastole (SWd), posterior wall thickness during systole (PWs) and diastole (PWd), LV end systolic diameter (LVDs), and LV end diastolic diameter (LVDd). Aortic flow was assessed from the five-chamber apical view and mitral flow with a four-chamber apical view with the smallest possible sample volume placed at the tips of the valve. LV mass was calculated as $1.04[(LVDd + PWd + SWd)^3 - LVDd^3]$, relative wall thickness (RWT) was calculated as $(PWd + SWd)/LVDd$, and fractional shortening (FS) was calculated as $(LVDd - LVDs)/LVDd$.

Isolated Perfused Working Heart

Following echocardiography, animals were administered heparinized sodium pentobarbital (i.p., 50 mg/kg). Anesthesia was confirmed by the absence of a tail pinch reflex, at which time the heart was rapidly excised and immersed in ice-cold Krebs Henseleit buffer (120 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl, 25 mM NaHCO₃, 17 mM glucose, and 0.5 mM EDTA). The aorta was cannulated within one minute, and the heart was subjected to retrograde perfusion with Krebs-Henseleit buffer saturated with 95% O₂/5% CO₂. Temperature was maintained in a water-jacketed column at 37°C for the duration of the experiment. The pulmonary vein was then cannulated and flow was re-directed to enter through the left atrium and exit through the aorta to measure cardiac function using the working heart method. The heart was then given 15 minutes to equilibrate before data were collected. Preload and afterload was set at 10 cmH₂O and 100 cmH₂O above the cannula, respectively.

Quantification of Cardiac Phosphometabolites

Following isolated heart experiments, the left ventricle was isolated, frozen in liquid nitrogen, and stored at -80°C for the subsequent measurement of ATP, ADP, AMP, phosphocreatine, and creatine using high performance liquid chromatography (HPLC). Frozen ventricular samples were weighed and homogenized immediately with 500 μL cold (4°C) perchloric acid (7%). The homogenate was centrifuged at 8,800g in an eppendorf centrifuge for 5 minutes. The supernatant was extracted and the pH adjusted to 7.0 with potassium hydroxide and centrifuged again at 8,800g for 5 minutes. The supernatant was removed and filtered, and the pellet was discarded. Once filtered, the sample (filtered supernatant) was injected into a reversed phase C-18 column, 15 cm in length with a $2\mu\text{m}$ particle size. An isocratic mobile phase was used to measure quantities of ATP, ADP, AMP, Cr, and PCr. The running buffer during the isocratic mobile phase consisted of 215 mM K_2HPO_4 , 2.3 mM TBAHS, 3.5% acetonitrile, pH to 6.25 with KOH and filtered through a $2\mu\text{m}$ cellulose acetate filter. The spectrophotometer was set at 204 nm with a flow rate of 0.25 ml/min. All phosphometabolites (ATP, ADP, AMP, phosphocreatine, and creatine) eluted from the column within 10 minutes. Retention times are located in Table 3.

Table 3: HPLC Phosphometabolite Retention Times

Compound	Retention Time (min)
Creatine	1.5
Phosphocreatine	1.8
AMP	2.4
ADP	2.8
ATP	3.7

Statistical Analysis

Three-Factor Analysis of Variance was used to determine factor effects and interaction effects of the three treatments, citrate buffer/STZ, sedentary/treadmill training, and saline/DOX. If a significant F-value was observed, a Tukey post-hoc pairwise comparisons were used to determine which groups were significant. A family alpha level of 0.05 was considered significant.

CHAPTER IV
MANUSCRIPT

Introduction

Over 25 million Americans have diabetes according to the Centers for Disease Control and Prevention. Large epidemiological studies have shown that individuals with diabetes are at increased risk of many types of cancer (Vigneri, 2009) along with increased cancer mortality (Suh & Kim, 2011). The cause of this increased cancer mortality in diabetics is not fully understood.

Diabetic cardiomyopathy is defined as a cardiac dysfunction in the absence of ischemia or valvular dysfunction (Rubler, et al., 1972). The cause is still largely unknown, however, many theories point to alterations in cardiac metabolism. Decreased glucose uptake, along with increased circulating fatty acids causes the diabetic individual to experience a shift toward predominately fatty acid oxidation (Stanley et al., 1999). In the healthy heart, fatty acid oxidation accounts for approximately 60-70% of total cardiac energy production (Neeley & Morgan, 1974). The shift seen in diabetic hearts increases fatty acid oxidation to 90%-100% of its total energy production (Lopaschuk, 1996) which may lead to cellular and structural alterations often described as diabetic cardiomyopathy.

Doxorubicin (DOX) is an FDA approved chemotherapy agent for the treatment of a variety of cancers including non-Hodgkin's lymphoma, acute leukemia, multiple myeloma and cancers of the breast, adrenal cortex, endometrium, lung, and ovary. Doxorubicin enters cancerous cells and inhibits DNA function, thereby reducing further

cellular growth. The use of DOX is hindered by its adverse effects on the heart. In fact, 26% of patients who received a cumulative dose of 550 mg/m² experienced congestive heart failure as a result (Swain, Whaley, and Ewer, 2003). Doxorubicin-induced cardiac dysfunction presents as decreased ejection fraction (Nousiainen, Jantuen, Vanninen, & Hartikainen, 2002), increased oxidative stress (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004), and DNA damage (L'Ecuyer et al., 2006). Theories of DOX-induced dysfunction include impaired abilities of the mitochondria, sarcoplasmic reticulum, and sarcolemma (Pelikan et al., 1989, Zorzato, Salviati, Facchinetti, & Volpe, 1985, and Boucek et al., 1987); however, research is ongoing in this area and further details regarding known mechanisms of cardiotoxicity as well as new mechanisms associated with DOX cardiotoxicity continue to be elucidated.

Exercise training has often been used as a protective measure against such adverse cardiac effects. Metabolically, exercise increases both glucose and fatty acid utilization and thus increases available ATP. Several animal models have studied this effect with great success and have found that exercise training mitigates cardiac dysfunction and mitigates cardiac injury in diabetic animals (Lumini-Olieveia, Magalhaes, Pereira, Moreira, Oliveira, & Ascensao, 2001) and in models of DOX cardiotoxicity (Ascensao et al., 2005, Ascensao et al., 2006, Chicco, Schneider, & Hayward 2005, Chicco, Hydock, Schneider, & Hayward, 2006).

The purpose of this study was to examine the combined effects of streptozotocin (STZ)-induced diabetes and DOX treatment on cardiac function, phosphometabolite levels and the effects of exercise training on the cardiac and metabolite dysfunction induced by the combination of diabetes and DOX.

Methods

Subjects

A total of 108 male Sprague-Dawley rats (150-200g) were obtained from Harlan Industries (Indianapolis, IN). Rats were housed two per cage and given free access to standard rat chow and distilled water *ad libitum*. Animals were kept in a temperature controlled facility and kept on a 12:12 hour light-dark cycle. All of the proposed procedures were approved by the University of Northern Colorado Institutional Animal Care and Use Committee (IACUC) and are in compliance with the Animal Welfare Act guidelines.

Streptozotocin-Induced Diabetes

Streptozotocin (STZ) was used to induce type I diabetes in the STZ group with an i.p. injection of 45 mg/kg in a 0.01 M citrate buffer at a pH of 4.5. Control animals (CIT) received an equivalent volume citrate buffer as a control. Animals were fasted 6-8 hours prior to STZ injection and given free access to food for the remainder of the study. There is a high risk of death immediately following STZ injection due to the rapid release of insulin from necrotic islet β -cells causing life threatening hypoglycemia. To combat this, forty-eight hours following STZ injection animals were given free access to 10% sucrose water. Non-fasting blood glucose was tested 3 days post STZ injection to confirm the presence of diabetes and a blood glucose value >250 ml/dL was used to confirm the diabetic condition (Bidasee et al., 2008). Animals with blood glucose values <250 ml/dL were excluded from the study.

Blood glucose, body weight and food intake were measured weekly. Blood glucose was measured with an Accu Chek blood glucose meter. The tail was first

cleaned with ethanol, and a lancet was used to stick the tip of the tail. The tail then was milked until at least 0.6 microliters of blood was absorbed into the testing strip (Bidasee et al., 2008). In cases where blood glucose exceeded 600 mg/dL the test was repeated. If blood glucose tested higher than 600 mg/dL on the second test, the animal was euthanized. Animals were given free access to food and water for the remainder of the study.

Exercise Training

Animals in CIT and STZ groups were further divided into the following groups; CIT+SED, CIT+TM, STZ+SED, and STZ+TM. Animals in SED groups were restricted to cage activity for 8 weeks. Animals in TM groups trained five days a week (Monday – Friday) for 8 weeks during their dark cycle. Training speed remained at 18 m/min for the entire 8 weeks, while the treadmill incline increased to a maximum of 15% and exercise duration reached a maximum of 60 minutes by the end of the training period. A summary of the exercise training regimen appears in Table 1.

Table 1: Exercise Training Protocol

Week	1	2	3	4	5	6	7	8
Speed (m/min)	18	18	18	18	18	18	18	18
Incline (%)	0	0	0	3	6	9	12	15
Duration (min)	20	40	60	60	60	60	60	60

Doxorubicin Injection

Twenty-four hours following the last exercise training session, animals in the 4 groups were further divided into DOX and SAL for a total of 8 groups (CIT+SED+SAL, CIT+SED+DOX, CIT+TM+SAL, CIT+TM+DOX, STZ+SED+SAL, STZ+SED+DOX, STZ+TM+SAL, and STZ+TM+DOX). Doxorubicin was administered as a bolus i.p.

injection at a dose of 12.5 mg/kg. Five days post DOX/SAL injection animals cardiac function was assessed using echocardiography and the isolated perfused working heart.

Echocardiography

In vivo cardiac function was assessed by echocardiography using a commercially available echocardiographic system (Toshiba Nemio 30; 10 MHz transducer). Animals were sedated with ketamine (40 mg/kg, i.p.) and echocardiography was completed within 10-15 minutes after the administration of the sedative. From the short-axis view, an M-mode tracing of the LV was obtained for measures of septal wall thickness during systole (SWs) and diastole (SWd), posterior wall thickness during systole (PWs) and diastole (PWd), LV end systolic diameter (LVDs), and LV end diastolic diameter (LVDd). Aortic flow was assessed from the five-chamber apical view and mitral flow with a four-chamber apical view with the smallest possible sample volume placed at the tips of the valve. LV mass was calculated as $1.04[(LVDd + PWd + SWd)^3 - LVDd^3]$, relative wall thickness (RWT) was calculated as $(PWd + SWd)/LVDd$, and fractional shortening (FS) was calculated as $(LVDd - LVDs)/LVDd$.

Isolated Perfused Working Heart

Following echocardiography, animals were administered heparinized sodium pentobarbital (i.p., 50 mg/kg). Anesthesia was confirmed by the absence of a tail pinch reflex, at which time the heart was rapidly excised and immersed in ice-cold Krebs Henseleit buffer (120 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl, 25 mM NaHCO₃, 17 mM glucose, and 0.5 mM EDTA). The aorta was cannulated within one minute and the heart was subjected to retrograde perfusion with Krebs-Henseleit buffer saturated with 95% O₂/5% CO₂ and temperature was maintained in a water-jacketed

column at 37°C for the duration of the experiment. The pulmonary vein was then cannulated and flow was re-directed to enter through the left atrium and exit through the aorta to measure cardiac function using the working heart method. The heart was then given 15 minutes to equilibrate before data were collected. Preload and afterload were set at 10 cmH₂O and 100 cmH₂O above the cannula, respectively. A microtip catheter pressure transducer (Scisense, Ontario, Canada) was inserted into the LV cavity via the apex for measurement of end systolic pressure (ESP), end diastolic pressure (EDP), left ventricular developed pressure (LVDP), maximum rate of pressure development (dP/dt_{max}) and the minimum rate of pressure development (dP/dt_{min}).

Quantification of Cardiac Phosphometabolites

Following isolated heart experiments, the left ventricle was isolated, frozen in liquid nitrogen, and stored at -80°C for subsequent measurement of ATP, ADP, AMP, phosphocreatine, and creatine using high performance liquid chromatography (HPLC). Frozen samples were weighed and homogenized immediately with 500 µL cold (4°C) perchloric acid (7%). The homogenate was centrifuged at 8,800g for 5 minutes. The supernatant was extracted and the pH was taken to 7.0 with potassium hydroxide and centrifuged again at 8,800g for 5 minutes. The supernatant was removed and filtered, and the pellet was discarded. The filtered supernatant was injected into a reversed phase C-18 column, 15 cm in length with a 5µm particle size and a 3 cm guard. An isocratic mobile phase was used to measure quantities of ATP, ADP, AMP, Cr, and PCr. The running buffer used during the isocratic mobile phase consisted of 215 mM K₂HPO₄, 2.3 mM TBAHS, 3.5% acetonitrile, pH to 6.25 with KOH and filtered through a 2 µm cellulose acetate filter. The spectrophotometer was set at 204 nm with a flow rate of 0.5

mL/min. All phosphometabolites (ATP, ADP, AMP, phosphocreatine, and creatine) were eluted from the column within 20 minutes.

Prior to sample measurement, standard curves were developed for each of the phosphometabolites at 5 different concentrations within a physiological range. Those standard curves were used to determine the μg of phosphometabolite per mg left ventricle tissue. Due to the degree of daily error seen with HPLC, each metabolite quantity was then divided by the metabolite quantity of the daily control and reported as a percentage of that control. Sedentary animal samples were controlled with the average of the sedentary control samples run that day; exercise trained animal samples were controlled with the average of the exercise control samples.

Statistical Analysis

Means and standard deviations were calculated for each variable. A Three-Factor Analysis of Variance was used to determine main effects and interaction effects of the three treatments, citrate buffer/STZ, sedentary/treadmill, and saline/DOX. If a significant F-value was observed, Tukey post-hoc pairwise comparisons were used to determine significance between all treatment combinations (8 groups). A family alpha level of less than 0.05 was considered significant.

Results

General Observations

Of those animals injected with STZ, 31% did not survive the 8 week study. In addition, the success rate of STZ inducing diabetes was 73% of injected animals. Survival rates were not different between sedentary and exercise trained animals. Only

two animals (1 sedentary and 1 treadmill trained) were lost following the DOX injection, resulting in less than 5% mortality following the DOX injection.

Main effects testing found that diabetic animals weighed significantly less than non-diabetic animals at time of sacrifice, 221 ± 49 grams and 364 ± 55 grams, respectively ($p < .05$). Diabetic animals' heart masses were lower than non-diabetic animals, 1.0 ± 0.2 grams and 1.4 ± 0.2 grams, respectively ($p < .05$). Diabetic animals had significantly higher blood glucose levels, 546 ± 45 ml/dL compared to 110 ± 14 ml/dL. No significant differences in body mass, heart mass, or blood glucose were seen as a result of the exercise training. Animals in the DOX treatment groups had significantly lower body masses compared to saline injected animals, 286 ± 64 grams and 332 ± 101 grams, respectively ($p < .05$). In addition, they had significantly lower heart weights, 1.2 ± 0.2 grams in the DOX treated groups and 1.3 ± 0.3 grams in the SAL groups ($p < .05$). DOX treated diabetic animals did not have further significant declines in either body or heart mass compared to SAL injected diabetic animals.

Demographic data are displayed in Table 2 and significant differences between the 8 groups are indicated. Groups CIT.SED.SAL (407 ± 43) and CIT.TM.SAL (403 ± 26) had significantly higher body mass compared to STZ.SED.SAL (223 ± 72), STZ.TM.SAL (230 ± 32), CIT.SED.DOX (338 ± 44), CIT.TM.DOX (306 ± 26), STZ.SED.DOX (204 ± 21), and STZ.TM.DOX (223 ± 47 , $p < .05$, Table 2). Similarly, CIT.SED.SAL (1.6 ± 0.3) and CIT.TM.SAL (1.5 ± 0.1) had significantly higher heart mass compared to STZ.SED.SAL (1.1 ± 0.2), STZ.TM.SAL (1.0 ± 0.1), CIT.TM.DOX (1.3 ± 0.1), STZ.SED.DOX (1.0 ± 0.1), and STZ.TM.DOX (1.0 ± 0.2 , $p < .05$, Table 2). However, the body and heart mass loss was not proportional across all groups,

STZ.SED.DOX (0.46 ± 0.02), STZ.TM.DOX (0.45 ± 0.05), and STZ.SED.SAL (0.5 ± 0.07) had significantly higher relative heart mass compared to both the SED and TM controls ($p < .05$). As mentioned with the main effects results, all STZ groups had significantly higher blood glucose compared to the CIT groups. Although not significant, both CIT.TM.SAL (119 ± 15) and CIT.TM.DOX (113 ± 15) groups had higher blood glucose compared to their sedentary counterparts (105 ± 12 and 103 ± 10 , respectively).

Table 2: Subject Demographics

		N	Body Mass (g)	Heart Mass (g)	Relative Heart Mass (%)	Blood Glucose (mL/dL)
CIT.SAL	SED	11	407 (43)	1.6 (0.3)	0.38 (0.04)	105 (12)
	TM	14	403 (26)	1.5 (0.1)	0.38 (0.03)	119 (15)
STZ.SAL	SED	9	223 (72)*#	1.1 (0.2)*#	0.50 (0.07)*#	543 (70)*#
	TM	7	230 (32)*#	1.0 (0.1)*#	0.44 (0.02)	552 (32)*#
CIT.DOX	SED	14	338 (44)*#	1.4 (0.1)	0.41 (0.05)	103 (10)
	TM	11	306 (26)*#	1.3 (0.1)*#	0.42 (0.04)	113 (15)
STZ.DOX	SED	8	204 (21)*#	1.0 (0.1)*#	0.49 (0.02)*#	561 (21)*#
	TM	7	223 (47)*#	1.0 (0.2)*#	0.45 (0.05)*#	531 (24)*#

Data are represented as mean (standard deviation). CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. Significance level set at $p < .05$, * compared to CIT.SED.SAL, # compared to CIT.TM.SAL

Echocardiography

Main effects testing resulted in significant changes to the left ventricle were seen as the result of STZ-induced diabetes, including decreased LV mass (-35%), as well as the left ventricle dimensions SWs (-20%), SWd (-20%), PWs (-23%), PWd (-34%), LVDs (-15%), and LVDd (-9%), ($p < .05$, Table 3). In addition, calculated variables RWT and FS were decreased -31% and -9%, respectively ($p < .05$), in the STZ groups compared to CIT groups (Figure 1). Exercise training did not have an effect on M-mode variables. Doxorubicin treatment however, resulted in significant declines in LV mass (-15%), as well as decreased PWs (-8%) and LVDd (-9%), ($p < .05$, Table 3).

Table 3: M-mode Echocardiography Treatment Effects

	SWs (mm)	SWd (mm)	PWs (mm)	PWd (mm)	LVDs (mm)	LVDd (mm)	LV mass (mg)	RWT (mm)
STZ	-0.7*	-0.4*	-0.8*	-0.7*	-0.4*	-0.2*	-328*	-0.2*
TM	0.0	-0.1	0.1	0.1	-0.1	-0.1	-42	0.0
DOX	-0.1	0.0	-0.3*	0.0	-0.1	-0.6*	-129*	0.1

STZ = Streptozotocin-induced diabetes; TM = treadmill exercise trained; DOX = doxorubicin; SWs = septal wall thickness at systole; SWd = septal wall thickness at diastole; PWs = posterior wall thickness at systole; PWd = posterior wall thickness at diastole; LVDs = left ventricular dimension at systole; LVDd = left ventricular dimension at diastole; LV mass = left ventricle mass; RWT = relative wall thickness. Data are the difference in mean values when subtracted from its control; STZ effect = STZ – CIT; TM effect = TM – SED; DOX effect = DOX – SAL. * denotes significance, $p < .05$.

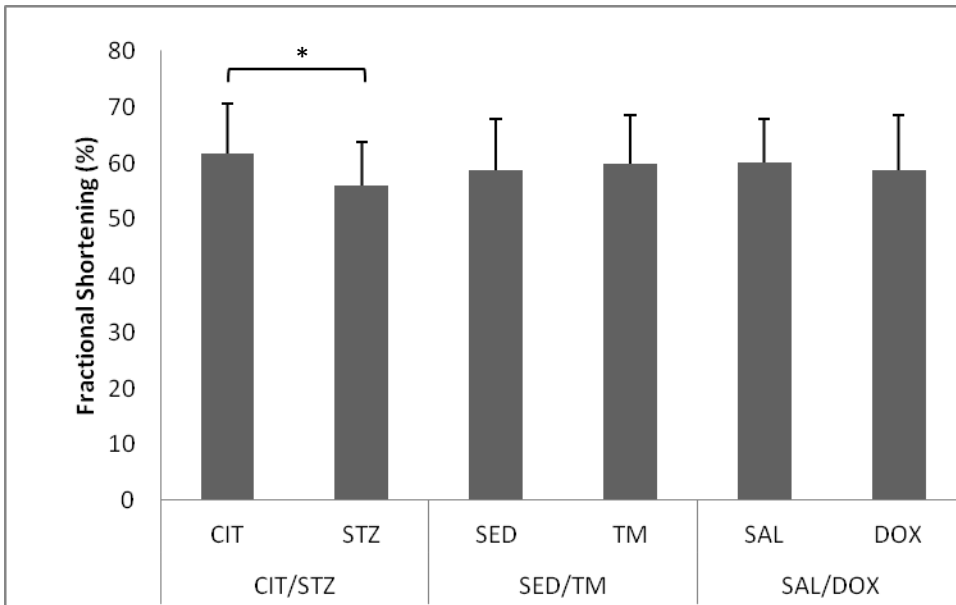


Figure 1: Fractional Shortening Treatment Effects

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. Fractional shortening = $(LVDd - LVDs)/LVDd$ * denotes significance, $p < .05$.

Table 4 presents the mean and standard deviation of each of the groups on analyzed M-mode images. Septal wall thickness during systole was significantly decreased in STZ.SED.SAL (2.8 ± 0.6), STZ.SED.DOX (2.9 ± 0.6), and STZ.TM.DOX (2.9 ± 0.6) compared to CIT.SED.SAL (3.8 ± 0.6) and CIT.TM.SAL (3.7 ± 0.5 , $p < .05$). Posterior wall thickness during systole was significantly decreased in STZ.SED.SAL (2.8

± 0.5), STZ.TM.SAL (3.0 ± 0.5), STZ.SED.DOX (2.5 ± 0.5), and STZ.TM.DOX (2.8 ± 0.4) compared to CIT.SED.SAL (3.8 ± 0.5 , $p < .05$). Compared to CIT.TM.SAL (3.7 ± 0.5), STZ.SED.SAL (2.8 ± 0.5), STZ.SED.DOX (2.5 ± 0.5), and STZ.TM.DOX (2.8 ± 0.4) were significantly decreased ($p < .05$). In addition, STZ.SED.DOX (2.5 ± 0.5) was significantly decreased compared to CIT.SED.DOX (3.5 ± 0.6) in PWs ($p < .05$). Groups STZ.SED.SAL (1.4 ± 0.4), STZ.TM.SAL (1.5 ± 0.2), STZ.SED.DOX (1.5 ± 0.3), and STZ.TM.DOX (1.5 ± 0.3) had significantly decreased PWd compared to groups CIT.SED.SAL (2.2 ± 0.7), CIT.TM.SAL (2.2 ± 0.4), CIT.SED.DOX (2.2 ± 0.6), and CIT.TM.DOX (2.2 ± 0.3 , $p < .05$). No significant differences were seen in LVDs among the 8 groups. LVDd was significantly decreased in the CIT.TM.DOX (5.1 ± 0.8) group compared to the CIT.TM.SAL (6.5 ± 1.1) group ($p < .05$, Table 4).

Table 4: M-mode Echocardiography

		SWs		SWd		PWs		PWd		LVDs		LVDd		
		N	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
CIT.SAL	SED	11	3.8	0.6	2.3	0.5	3.8	0.5	2.2	0.7	2.6	0.7	6.5	1.1
	TM	14	3.7	0.5	2	0.5	3.7	0.5	2.2	0.4	2.5	0.6	6.5	0.6
STZ.SAL	SED	9	2.8*#	0.6	1.7	0.3	2.8*#	0.5	1.4*##†	0.4	2.6	0.5	6	0.7
	TM	7	3.1	0.5	1.6*	0.4	3*	0.5	1.5*##†	0.2	2.7	0.9	6.4	0.8
CIT.DOX	SED	14	3.7	0.5	2	0.6	3.5	0.6	2.2	0.6	2.2	0.9	5.9	1.2
	TM	11	3.4	0.5	2.1	0.4	3.5	0.4	2.2	0.3	2	0.7	5.1#	0.8
STZ.DOX	SED	8	2.9*#	0.6	1.9	0.5	2.5*##	0.5	1.5*##†	0.3	2.9	0.7	6.2	1
	TM	7	2.9*#	0.6	1.6	0.3	2.8*#	0.4	1.5*##†	0.3	2.7	0.2	6.1	0.6

STZ = Streptozotocin-induced diabetes; TM = treadmill exercise trained; DOX = doxorubicin; SWs = septal wall thickness at systole; SWd = septal wall thickness at diastole; PWs = posterior wall thickness at systole; PWd = posterior wall thickness at diastole; LVDs = left ventricular dimension at systole; LVDd = left ventricular dimension at diastole; A significance level of $p < .05$ was set, * compared to CIT.SED.SAL, # compared to CIT.TM.SAL, † compared to CIT.SED.DOX and ‡ compared to CIT.TM.DOX

Table 5 represents the means and standard deviations of the 8 groups. Left ventricle mass was significantly decreased among the following groups, CIT.TM.DOX

(743 ± 90), STZ.SED.SAL (553 ± 139), STZ.TM.SAL (632 ± 134), STZ.SED.DOX (658 ± 147), and STZ.TM.DOX (606 ± 154) compared to CIT.SED.SAL (1130 ± 361) and CIT.TM.SAL (1011 ± 233, p <.05). Relative wall thickness was significantly decreased in all of the STZ groups compared to CIT.TM.DOX (p <.05). Although significant as a main effect, FS was not significantly different among the 8 treatment groups (Figure 2). Cardiac function variables, V_{cf}, V_{cfc}, and MPI were not significant among the 8 treatment groups.

Table 5: M-mode Echocardiography

		N	LV mass			RWT		FS		V _{cf}		V _{cfc}		MPI	
			M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	
CIT.SAL	SED	11	1130	361	0.73	0.39	0.61	0.07	0.0088	0.0013	0.0007	0.00012	0.4	0.1	
	TM	14	1011	233	0.66	0.14	0.62	0.08	0.0089	0.0015	0.00074	0.00013	0.33	0.1	
STZ.SAL	SED	9	553*#	139	0.52‡	0.11	0.57	0.07	0.0067	0.0028	0.00045	0.00022	0.65	0.2	
	TM	7	632*#	134	0.5‡	0.11	0.59	0.1	0.0088	0.0082	0.00057	0.00039	1.09	2	
CIT.DOX	SED	14	866	185	0.77	0.34	0.62	0.12	0.047	0.12	0.0033	0.0087	5.02	14.3	
	TM	11	743*#	90	0.88	0.24	0.61	0.1	0.0094	0.0023	0.00068	0.00016	0.68	0.3	
STZ.DOX	SED	8	658*#	147	0.56‡	0.15	0.53	0.08	0.0049	0.001	0.00035	0.00008	0.36	0.1	
	TM	7	606*#	154	0.52‡	0.08	0.56	0.06	0.0058	0.0014	0.00039	0.00011	0.75	0.3	

STZ = Streptozotocin-induced diabetes; TM = treadmill exercise trained; DOX = doxorubicin; LV mass = left ventricle mass; RWT = relative wall thickness. A significance level of p <.05 was set, * compared to CIT.SED.SAL, # compared to CIT.TM.SAL, † compared to CIT.SED.DOX and ‡ compared to CIT.TM.DOX

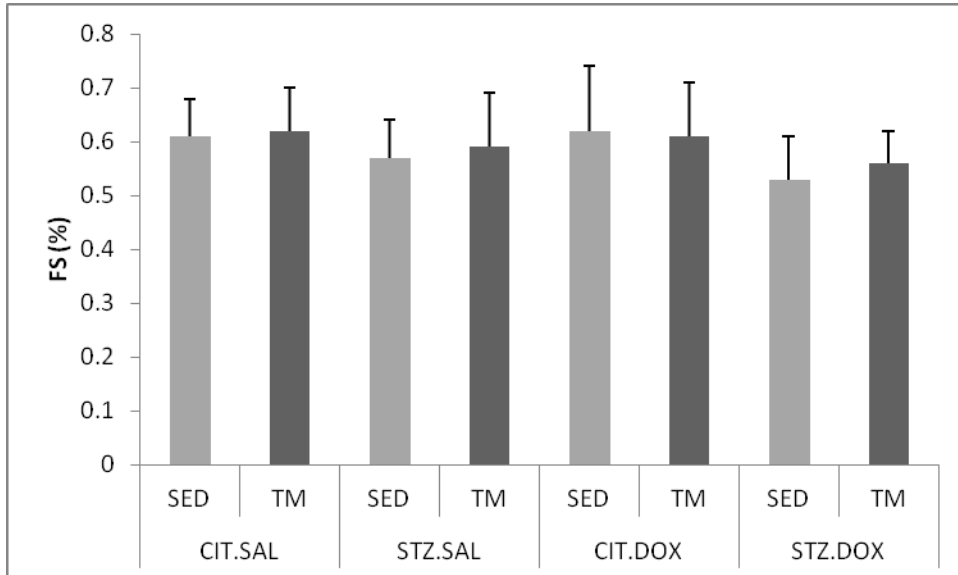


Figure 2: Fractional Shortening Group Comparisons
 CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin; FS = fractional shortening.

Echocardiography Doppler images were used to analyze HR, maximum blood flow through the aortic (Aortic V_{max}) and mitral valves (Mitral V_{max}), filling time (FT), ejection time (ET), isovolumetric relaxation time (IVRT), and isovolumetric contraction time (IVCT). Heart rate was significantly decreased in both STZ and DOX treated animals, -23% and -11%, respectively, ($p < .05$, Table 6). Exercise training did not have an effect on heart rate. Streptozotocin treatment also resulted in a significant 17% decrease in Aortic V_{max} compared to controls, ($p < .05$, Figure 3). Exercise training significantly increased Aortic V_{max} by 17% compared to sedentary animals, ($p < .05$, Figure 3). Ejection time increased by 47% and IVRT increased by 83% with STZ treatment, ($p < .05$, Table 6). Mitral V_{max} was significantly decreased by DOX treatments, declining 18%, ($p < .05$, Figure 4).

Table 6: Doppler Echocardiography Treatment Main Effects

	Mitral V_{max} (cm/s)	FT (ms)	Aortic V_{max} (cm/s)	ET (ms)	HRI (ms)	IVRT (ms)	IVCT (ms)
STZ	-0.5	-2.4	-15.2*	31.5*	50.3*	18.6*	1.2
TM	4.8	0.6	12.9*	-2.5	-7.2	-2.2	0.4
DOX	-16.7*	7.6	-24.4*	0.7	23.3*	5.5	5.1*

STZ = Streptozotocin-induced diabetes; TM = treadmill exercise trained; DOX = doxorubicin; Mitral V_{max} = maximum blood flow through mitral valve; FT = filling time; Aortic V_{max} = maximum blood flow through aortic valve; ET = ejection time; HRI = heart rate interval between beats; IVRT = isovolumetric relaxation time; IVCT = isovolumetric contraction time. Data are the difference in mean values when subtracted from its control; STZ effect = STZ – CIT; TM effect = TM – SED; DOX effect = DOX – SAL. * denotes significance, $p < .05$.

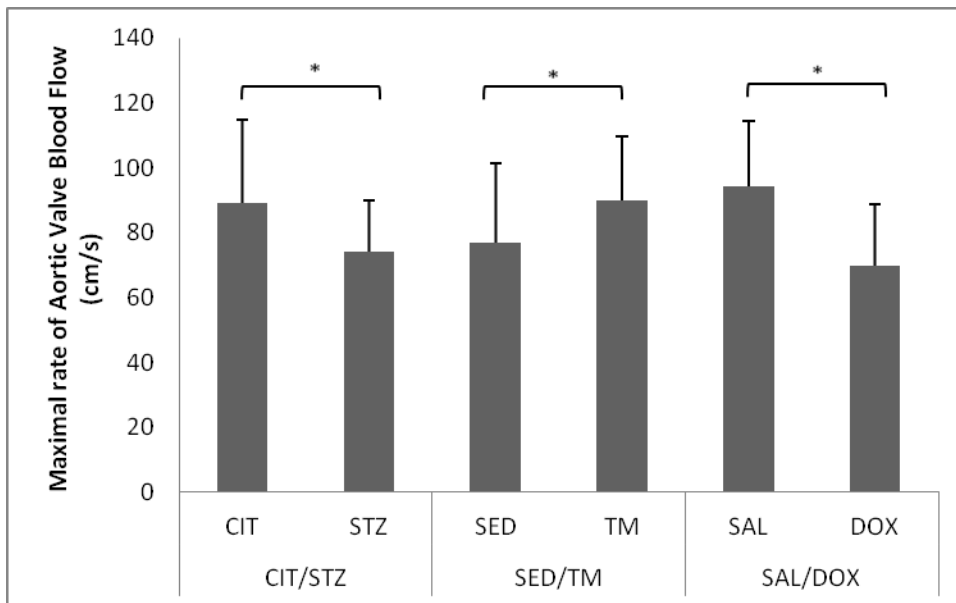


Figure 3: Aortic V_{max} Treatment Main Effects

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. * denotes significance, $p < .05$.

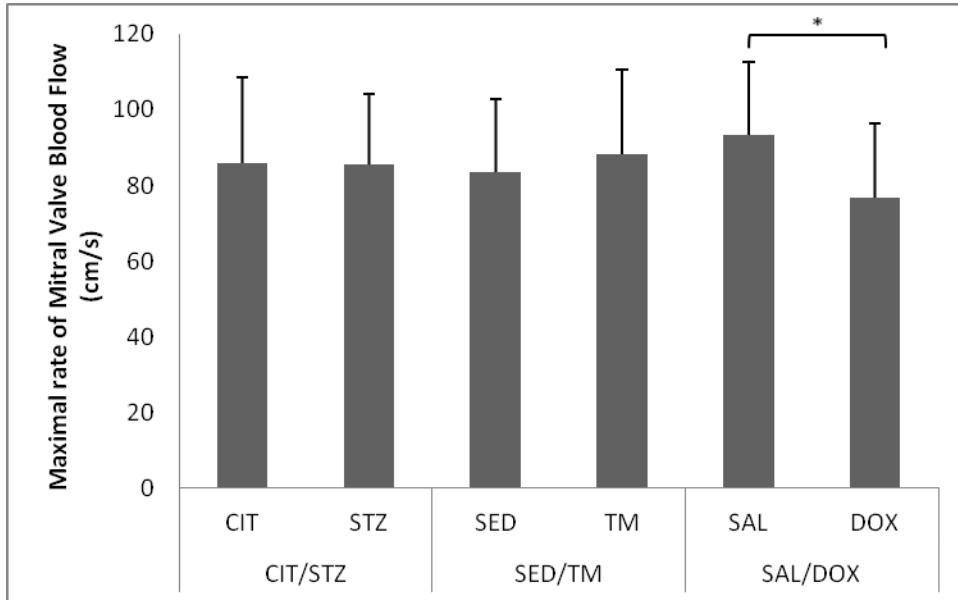


Figure 4: Mitral V_{max} Treatment Main Effects

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. * denotes significance, $p < .05$.

Aortic maximal blood flow was significantly decreased in CIT.SED.DOX (64 ± 27) and STZ.SED.DOX (67 ± 15) compared to CIT.SED.SAL (100 ± 15 , Figure 5). CIT.TM.DOX (80 ± 11), STZ.TM.SAL (81 ± 12), and STZ.TM.DOX (71 ± 11) were all significantly decreased compared to CIT.TM.SAL (109 ± 15 , $p < .05$, Table 7). Ejection time (Table 7) was also significant different among the 8 treatment groups. Group CIT.SED.SAL (70 ± 8) ejection time was significantly less than STZ.SED.DOX (108 ± 13 , $p < .05$). Group CIT.SED.DOX (59 ± 21) was significantly less than STZ.SED.DOX (108 ± 13 , $p < .05$). Group CIT.TM.DOX (66 ± 10) was significantly less than STZ.TM.DOX (101 ± 21 , $p < .05$). Maximal blood flow through the mitral valve was significantly decreased in CIT.SED.DOX (73 ± 19) compared to CIT.SED.SAL (97 ± 17) and CIT.TM.DOX (61 ± 11) was significantly decreased compared to CIT.TM.SAL (105

± 12 , $p < .05$, Table 7). Group CIT.TM.DOX (61 ± 11) Mitral V_{\max} was significantly decreased compared to STZ.TM.SAL (92 ± 19 , $p < .05$, Figure 6). Filling time was not significantly different among the 8 treatment groups. Heart rate was significantly decreased in STZ.SED.SAL (257 ± 45) compared to CIT.SED.SAL (378 ± 34). Groups STZ.TM.SAL (282 ± 57) and STZ.TM.DOX (258 ± 62) had significantly decreased heart rate compared to CIT.TM.SAL (405 ± 29). Group STZ.SED.SAL (51 ± 21) had a significantly longer IVRT compared to CIT.SED.SAL (18 ± 6 , $p < .05$). Groups STZ.TM.SAL (37 ± 16) and STZ.TM.DOX (47 ± 18) were significantly longer IVRT compared to CIT.TM.SAL (15 ± 4 , $p < .05$). STZ.TM.DOX (25 ± 14) had a significantly longer IVCT compared to CIT.TM.SAL (10 ± 4 , $p < .05$).

Table 7: Doppler Echocardiography

		Mitral V_{\max}			FT		Aortic V_{\max}		ET		HR		IVRT		IVCT	
		<i>N</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>
CIT.SAL	SED	11	97	17	69	10	100	15	70	8	378	34	18	6	10	4
	TM	14	105*	12	66	8	109	15	70	8	405	29	15	4	8	4
STZ.SAL	SED	9	74	18	79	21	76	21	96	30	257*	45	51*	21	13	6
	TM	7	92#	19	74	31	81#	12	88	30	282#	57	37#	16	10	7
CIT.DOX	SED	14	73*	19	83	34	64*	27	59	21	308	50	30	7	15	6
	TM	11	61#	11	91	11	80#	11	66	10	312	17	29	13	15	7
STZ.DOX	SED	8	92*	12	65	14	67*	15	108*†	13	285	42	31	10	7	6
	TM	7	84	22	75	35	71#	11	101#	21	258#	62	47#	18	25#	14

STZ = Streptozotocin-induced diabetes; TM = treadmill exercise trained; DOX = doxorubicin; Mitral V_{\max} = maximum blood flow through mitral valve; FT = filling time; Aortic V_{\max} = maximum blood flow through aortic valve; ET = ejection time; HRI = heart rate interval between beats; IVRT = isovolumetric relaxation time; IVCT = isovolumetric contraction time. Data are the difference in mean values when subtracted from its control; STZ effect = STZ – CIT; TM effect = TM – SED; DOX effect = DOX – SAL. * denotes significance, $p < .05$.

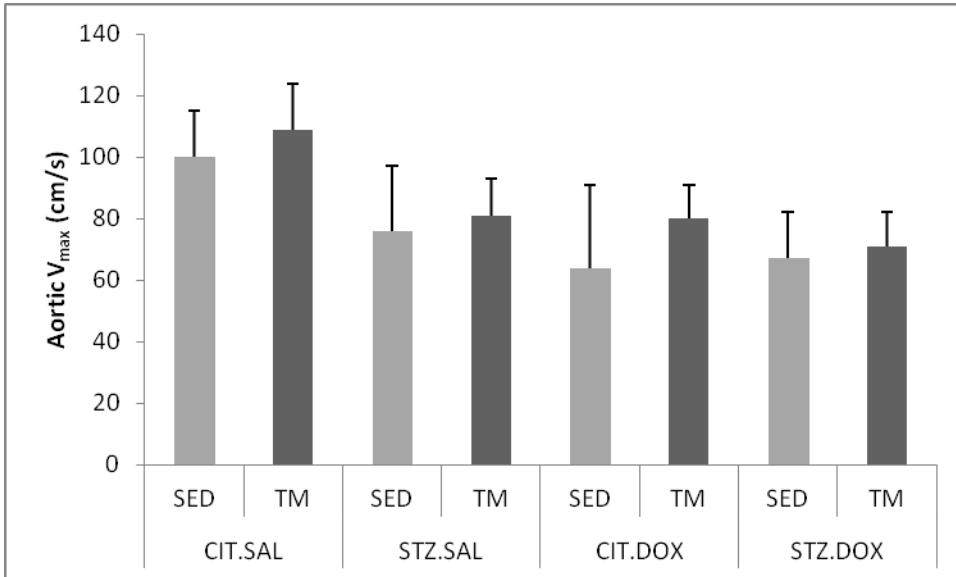


Figure 5: Aortic V_{max} Group Comparisons

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin

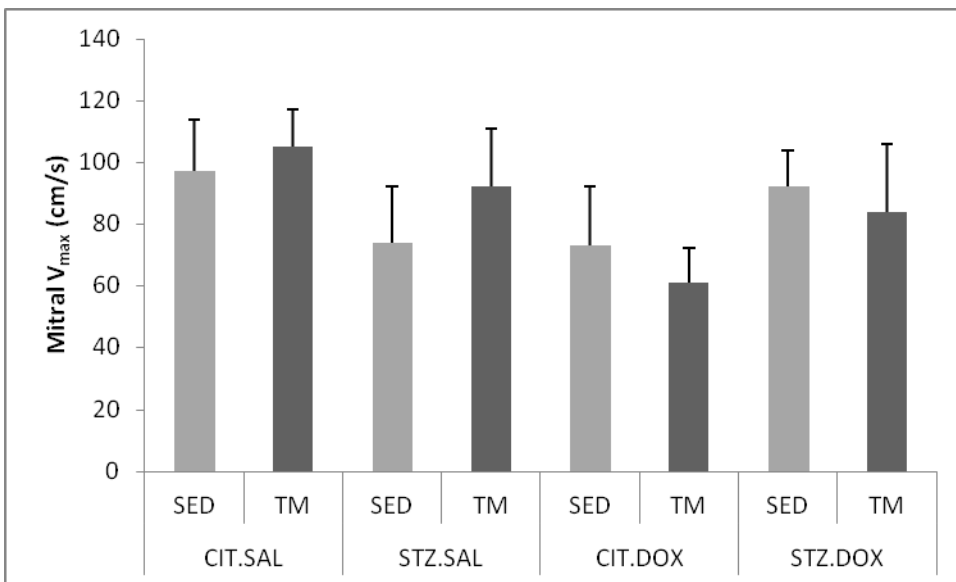


Figure 6: Mitral V_{max} Group Comparisons

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin

Isolated Perfused Working Heart

Ex vivo cardiac function was measured with the isolated perfused working heart model. The main effects treatment with STZ resulted in an increase in ESP (15%), and LVDP (16%, $p < .05$). Doxorubicin treatment significantly decreased ESP (-15%) and LVDP (-18%, $p < .05$). Treatment with DOX significantly affected dP/dt_{max} (-16%) and dP/dt_{min} (-14%, $p < .05$); in addition to increasing EDP ($p < .05$). Treadmill training positively affected cardiac function measured by an increase in ESP (13%), ($p < .05$). Table 8 contains the treatment effects, while Figures 7 and 8 graphically depict the treatments' effects on ESP and LVDP.

Table 8: Isolated Working Heart Treatment Main Effects

	ESP (mmHg)	EDP (mmHg)	LVDP (mmHg)	dP/dT_{max} (mmHg)	dP/dT_{min} (mmHg)	HR (bpm)
STZ	11*	0.0	11*	57	103	-5
TM	8*	0.4	8	204	138	-6
DOX	-12*	2.5*	-14*	-496*	363*	-2

STZ = Streptozotocin-induced diabetes; TM = treadmill exercise trained; DOX = doxorubicin; ESP = end systolic pressure; EDP = end diastolic pressure; LVDP = left ventricular developed pressure; dP/dT_{max} = maximum rate of pressure development; dP/dT_{min} = minimum rate of pressure development; HR = heart rate. Data are the difference in mean values when subtracted from its control; STZ effect = STZ – CIT; TM effect = TM – SED; DOX effect = DOX – SAL. * denotes significance, $p < .05$.

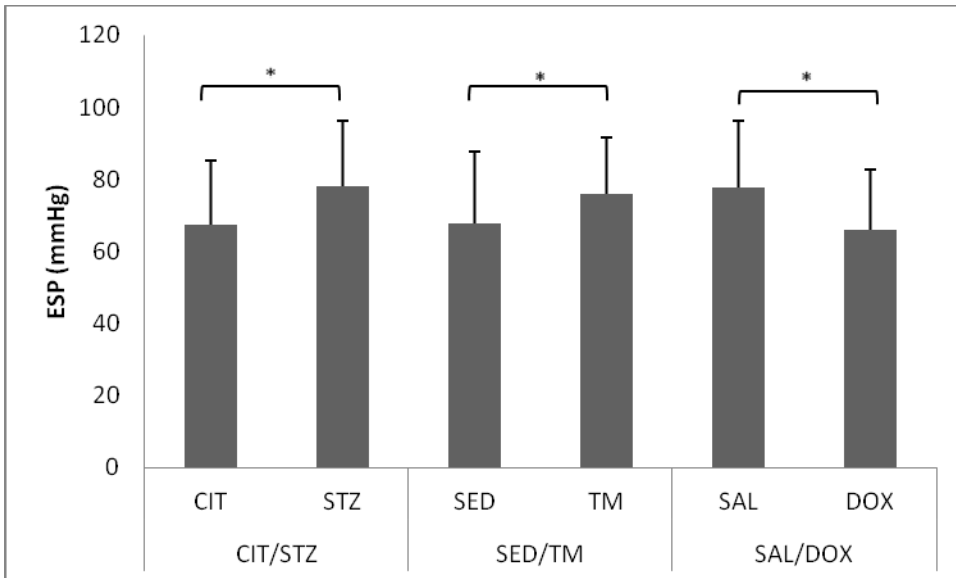


Figure 7: End Systolic Pressure Treatment Main Effects CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. * denotes significance, $p < .05$.

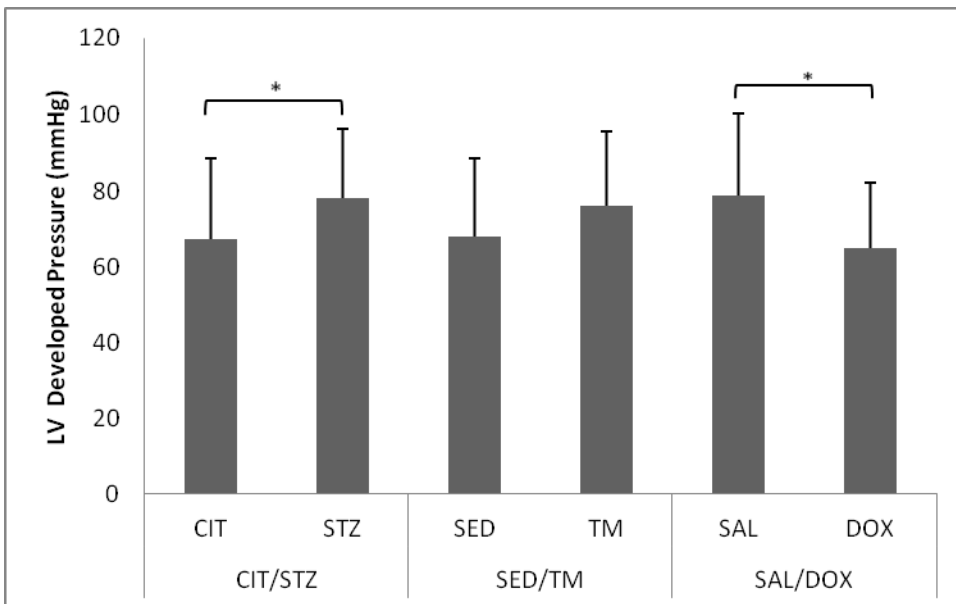


Figure 8: Left Ventricular Developed Pressure Treatment Main Effects CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. * denotes significance, $p < .05$.

Table 9 displays the means and standard deviations from *ex vivo* cardiac assessment of the 8 treatment groups. Statistically, there were no meaningful differences between the 8 treatment groups. However, trends are observed and will be discussed in the following section.

Table 9: Isolated Working Heart

		ESP (mmHg)			EDP (mmHg)		LVDP (mmHg)		dP/dt _{max} (mmHg/s)		dP/dt _{min} (mmHg/s)		HR (bpm)	
		<i>N</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>
CIT.SAL	SED	11	76	22	-0.6	1.7	76	23	3210	771	-2585	436	122	34
	TM	14	76	16	-3	10	79	24	3294	785	-3019	1344	114	29
STZ.SAL	SED	9	73	22	-0.7	0.9	74	22	2927	630	-2651	1060	103	59
	TM	7	89	16	0.9	1.8	88	17	3017	549	-2334	261	106	27
CIT.DOX	SED	14	55	13	1.2	2.8	54	12	2426	418	-2301	487	115	48
	TM	11	66	13	3	3.9	63	16	2589	357	-2304	261	100	35
STZ.DOX	SED	8	73	21	-0.7	1.1	73	21	2698	660	-2270	179	114	36
	TM	7	80	9	1.4	3.6	79	9	3032	503	-2502	615	108	62

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin ESP = end systolic pressure; EDP = end diastolic pressure; LVDP = left ventricular developed pressure; dP/dt_{max} = maximum rate of pressure development; dP/dt_{min} = minimum rate of pressure development; HR = heart rate.

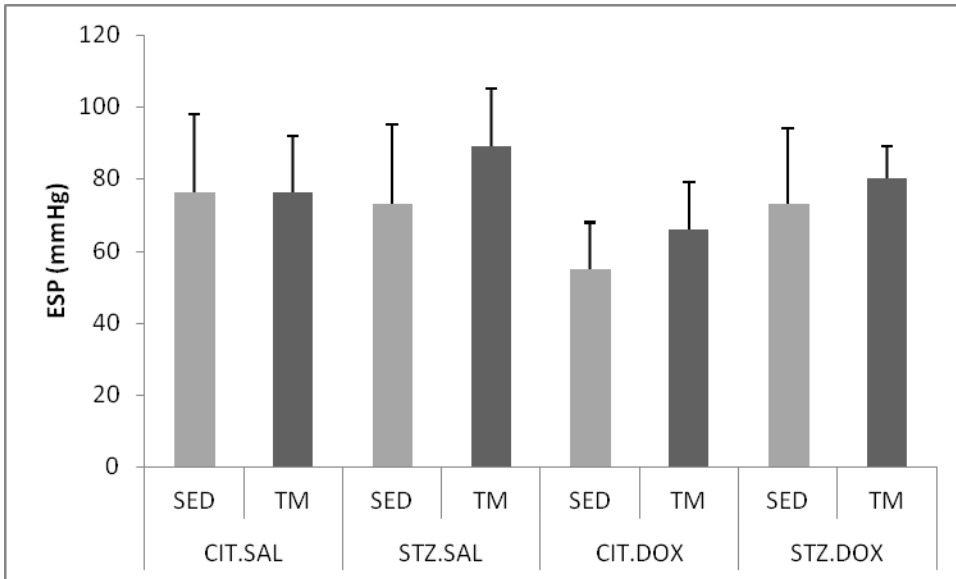


Figure 9: End Systolic Pressure Group Comparisons
 CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary;
 TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin

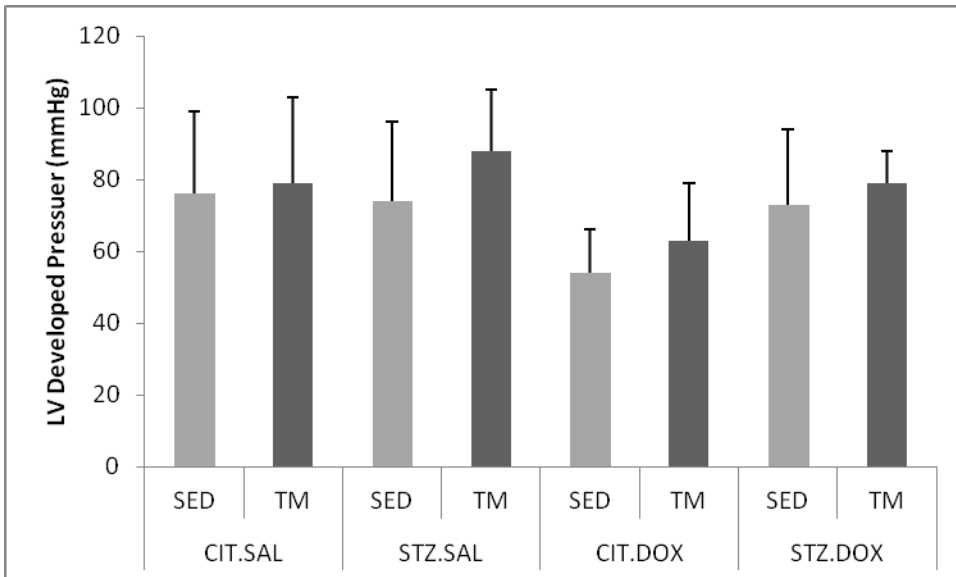


Figure 10: Left Ventricular Developed Pressure Group Comparisons
 CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary;
 TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin

Quantification of Cardiac Phosphometabolites

With main effects testing, all phosphometabolites were significantly increased after treadmill exercise training (Figures 11-15). Exercise training increased ATP content in STZ treated animals by 27% and 20% in those treated with DOX, ($p < .05$). Exercise training increased Cr levels in STZ and DOX treated groups by 62% and 47%, respectively, ($p < .05$). Although no effect is seen in DOX treated groups, exercise training increased PCr levels in STZ treated animals by 32%, ($p < .05$). Exercise training increased ADP levels in DOX treated animals significantly by 15%, ($p < .05$); while significant increases in AMP with exercise training were only seen in STZ treated animals (64%, $p < .05$). Apart from the main effect of exercise, there were no new findings when comparing all 8 groups; Figures 16-20 graph the group means and standard deviations.

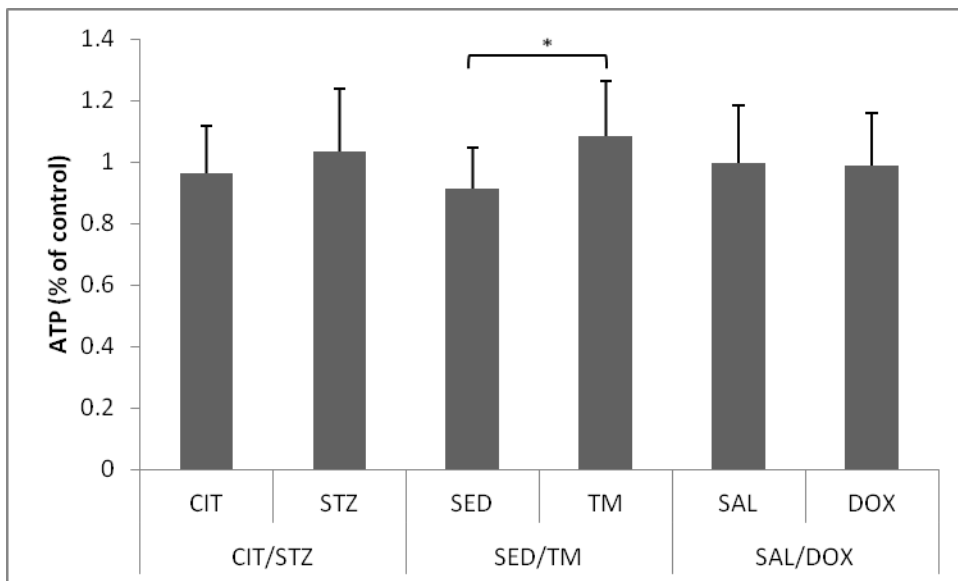


Figure 11: ATP Treatment Main Effects

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. ATP was quantified as % of daily control sample quantity to control for instrument error. * denotes significance, $p < .05$.

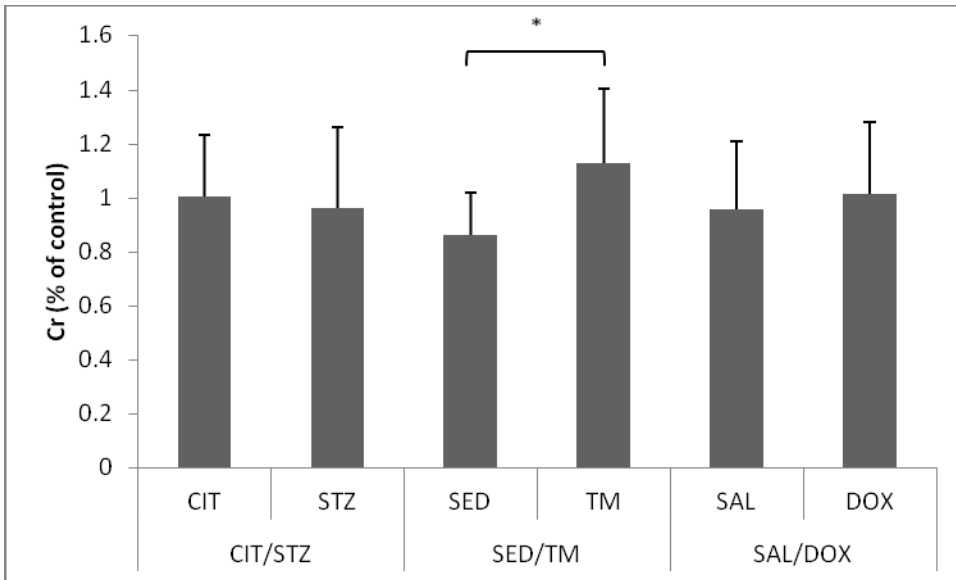


Figure 12: Creatine Treatment Main Effects

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. Cr was quantified as % of daily control sample quantity to control for instrument error. * denotes significance, $p < .05$.

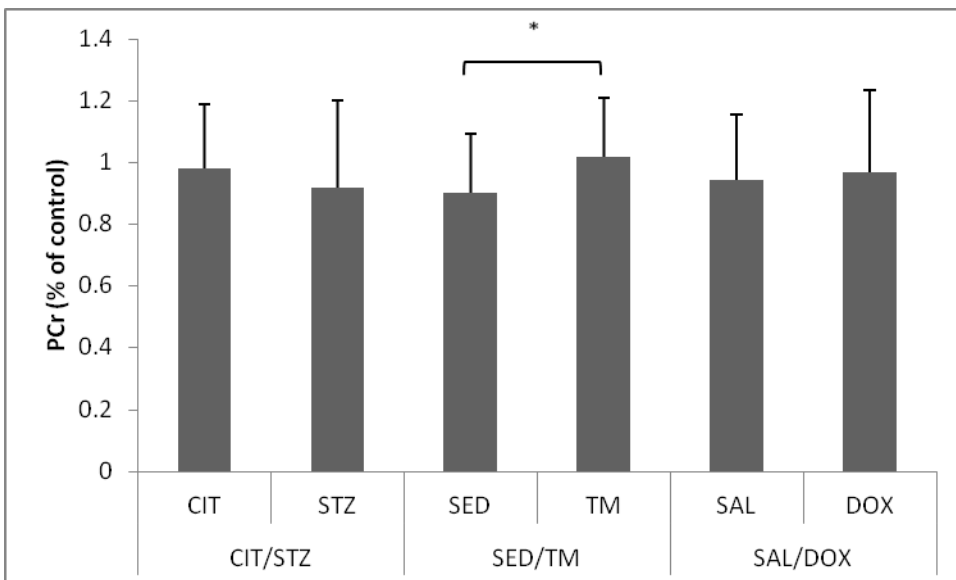


Figure 13: Phosphocreatine Treatment Main Effects

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. PCr was quantified as % of daily control sample quantity to control for instrument error. * denotes significance, $p < .05$.

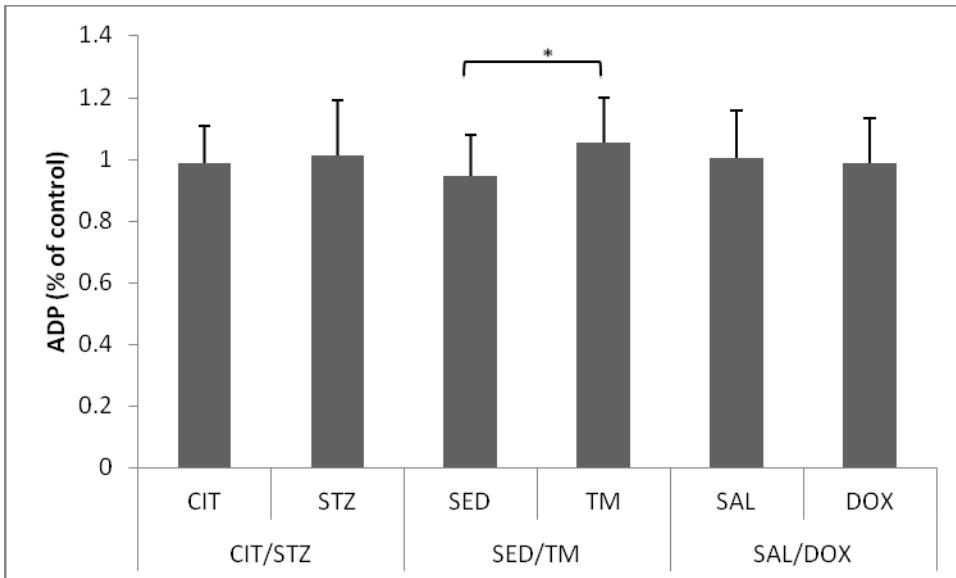


Figure 14: ADP Treatment Main Effects

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. ADP was quantified as % of daily control sample quantity to control for instrument error. * denotes significance, $p < .05$.

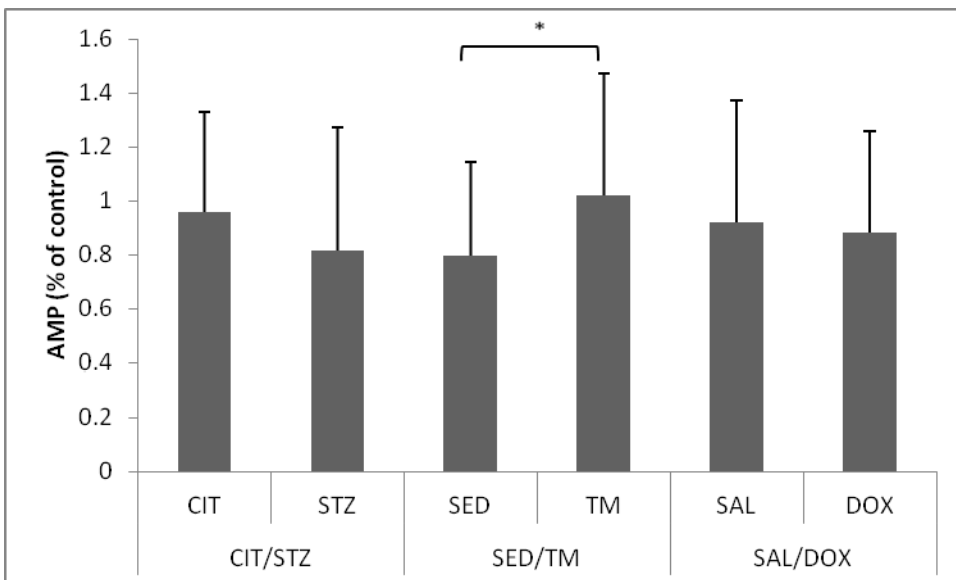


Figure 15: AMP Treatment Main Effects

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. AMP was quantified as % of daily control sample quantity to control for instrument error. * denotes significance, $p < .05$.

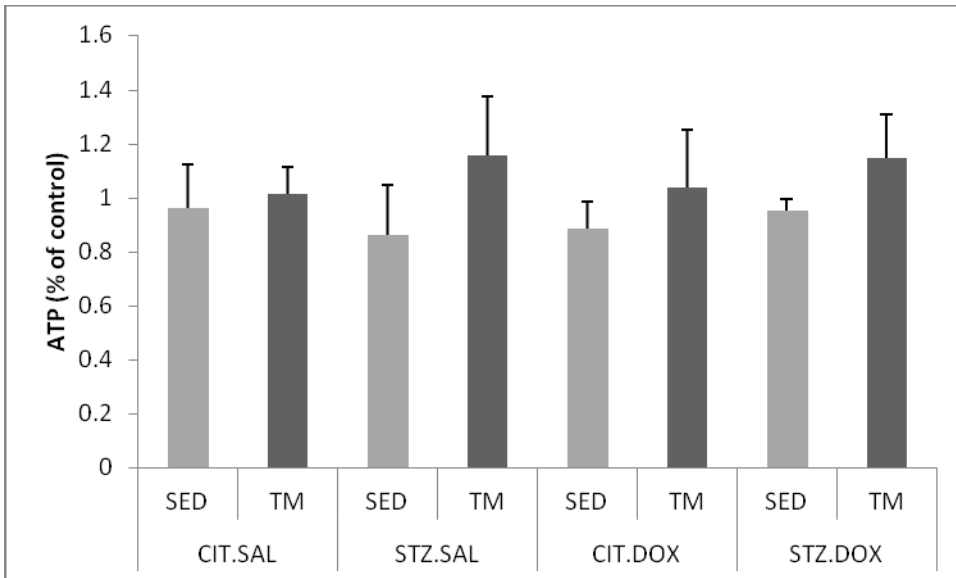


Figure 16: ATP Group Comparisons

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. ATP was quantified as % of daily control sample quantity to control for instrument error.

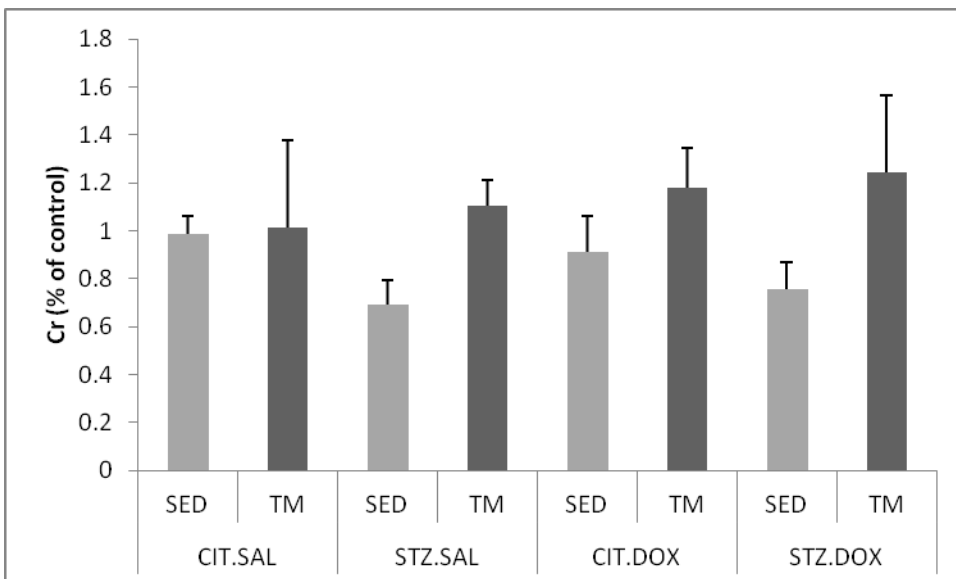


Figure 17: Creatine Group Comparisons

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. Cr was quantified as % of daily control sample quantity to control for instrument error.

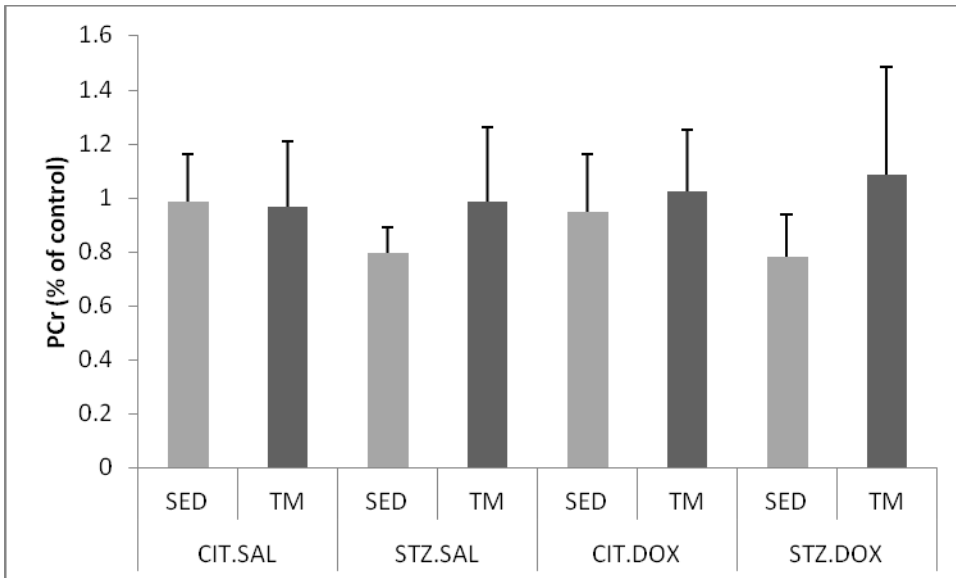


Figure 18: Phosphocreatine Group Comparisons
 CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. PCr was quantified as % of daily control sample quantity to control for instrument error.

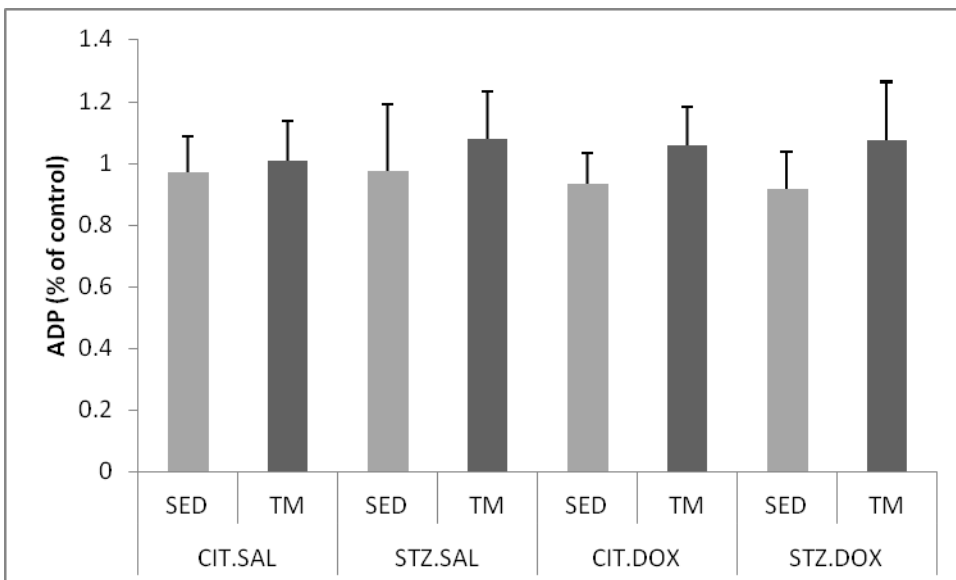


Figure 19: ADP Group Comparisons
 CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. ADP was quantified as % of daily control sample quantity to control for instrument error.

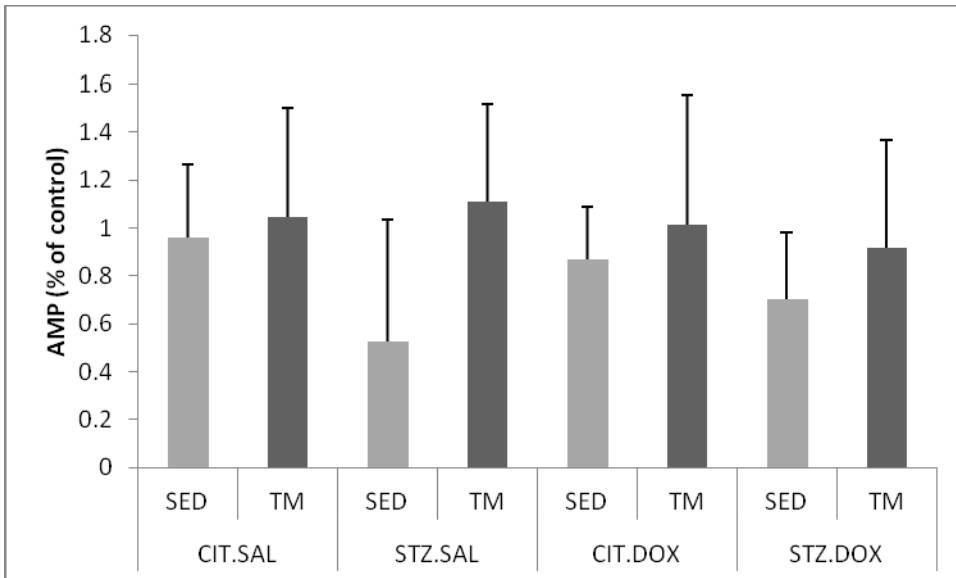


Figure 20: AMP Group Comparisons

CIT = citrate buffer placebo; STZ = streptozotocin-induced diabetes; SED = sedentary; TM = treadmill exercise trained; SAL = saline placebo; DOX = doxorubicin. AMP was quantified as % of daily control sample quantity to control for instrument error.

Discussion

The present study demonstrated the adverse cardiovascular effects of DOX and STZ treatments, individually and in combination. Additionally, exercise training was able to attenuate the cardiac dysfunction induced by DOX and STZ, individually and in combination. Furthermore, exercise training significantly increased cardiac phosphometabolite levels in the left ventricle.

Body and heart masses were significantly decreased with the treatment of STZ and DOX; however, the relative heart mass was increased in animals treated with STZ or the combination of STZ and DOX. Diabetes has been shown to increase blood viscosity due to hyperglycemia (MacRury, et al, 1988) and thus lead to hypertensive hypertrophy.

Cardiac Dysfunction

In the present study, diabetes resulted in decreased left ventricle dimensions during both systole and diastole, left ventricle mass, relative wall thickness, and fractional shortening. Dysfunction was also observed in STZ animals with maximum aortic blood flow and ejection time. Previous studies have found after as little as 5 weeks of STZ-induced diabetes that left ventricle dimensions are decreased during both diastole and systole, along with decreased fractional shortening and peak blood flow velocities (Joffe et al., 1999). In addition, DOX treatment lead to significant declines in left ventricle dimensions, and left ventricle mass. The *ex vivo* analysis of cardiac function, revealed significant dysfunction measured by decreases in ESP and LVDP with DOX treatment. Streptozotocin treatment resulted in an increase in ESP and LVDP, which could be the result of the absence of fatty acids in the perfused buffer. Increases in plasma concentration of fatty acids, fatty acid transporters, and fatty acid uptake appear to be large components in the shift to increased fatty acid utilization seen in diabetics. Fatty acid oxidation typically provides 60 – 70% of the heart's energy needs (Neeley & Morgan, 1974); however, in the uncontrolled diabetic heart, that percentage increases to 90 – 100% (Lopachuk, 1996). By perfusing hearts with lipid-free buffer, studies have found that the diabetic heart increases its utilization of glucose from 0-10% to 20% of its energy requirements (Lopachuk, 1996). Therefore, the unexpected increase in ESP and LVDP, could be the result of a buffer inconsistent with the diabetic condition. In addition, function could have improved due to the improvement in blood viscosity, especially if the heart had experienced hypertensive hypertrophy. The interaction of STZ and DOX on LVSP and ESP has shown that the effect of STZ treatment is exacerbated by

treatment with DOX. One study found that diabetic patients receiving DOX had an increased likelihood of congestive heart failure (Hershman, McBride, Eisenberger, Tasi, Grann, & Jacobson, 2008).

Cardiac Metabolic Dysfunction

The current study did not find altered phosphometabolite content in the left ventricle following treatment with STZ or DOX treatment. Previous research found that ATP synthesis is slowed in diabetic animals, concluded by increased levels of ADP and AMP, however, no significant decline in ATP availability was seen in diabetic cardiac tissue (Ferko, M., Gvozdjakova, A., Kucharska, J., Mujkosova, J., Waczulikova, I., Styk, J., et al., 2006). Contrary to the present findings, DOX-induced cardiotoxicity has been found to significantly decrease myocardial ATP, along with decreases in creatine, phosphocreatine and lactate, indicating both aerobic and anaerobic metabolic dysfunction (Wallace, 2003). Although STZ and DOX did not significantly affect phosphometabolite content, the effect of exercise to increase phosphometabolites was present across all treatments. Exercise significantly increased ATP and Cr in animals treated with STZ and DOX and may partially explain the benefits of exercise training on cardiac function in STZ and DOX treated animals.

Exercise Cardioprotecion

Exercise attenuated cardiac dysfunction induced by STZ and DOX treatments, and in the cases of aortic blood flow and end systolic pressure, had a significant effect over all sedentary groups. Bidasee and colleagues (2008) investigated the effects of exercise training on cardiac function in STZ-induced diabetic rats. It was found that following seven weeks of diabetes, rats had decreased heart rates, fractional shortening,

and ejection fraction. Three weeks of treadmill training was able to improve ejection fraction. A similar study using STZ-induced diabetic rats found that after nine weeks of treadmill training and seven weeks of STZ-induced diabetes exercise was found to significantly attenuate the magnitude of mitochondrial ultrastructure damage, assessed using transmission electron microscopy (Searls et al., 2004).

The use of exercise to attenuate DOX-induced cardiotoxicity has been demonstrated across different modes of exercise, intensities, and durations (Ascensao et al., 2005, Chicco et al., 2005, Chicco et al., 2006 and Wonders et al., 2008). All have identified exercise as a means of attenuating cardiotoxicity through preserved cardiac function, decreased apoptosis, decreased oxidative stress, increased antioxidant capacity, and improved calcium homeostasis (Ascensao et al. 2005, Ascensao et al., 2006, Chicco, et al., 2005, Chicco et al., 2006, Hydock et al., 2011, and Wonders et al., 2009).

Exercise training resulted in an increase in all phosphometabolites, and those increases were seen in animals treated with STZ and DOX. Exercise increases both glucose and fatty acid utilization and thus increases available ATP. Exercise leads to an increase in AMPK, which in turn inactivates ACC and malonyl CoA, thereby increasing fatty acid oxidation, in addition, AMPK also stimulates glucose uptake (through insulin, which may not be present in STZ diabetic hearts) and glycolysis by activating PFK. Thus both glucose and fatty acid utilization are increased.

Summary

Exercise training protects against STZ and DOX –induced cardiac dysfunction. In addition, exercise training significantly increased the availability of phosphometabolites in the left ventricle, which may be decreased with STZ and DOX treatments.

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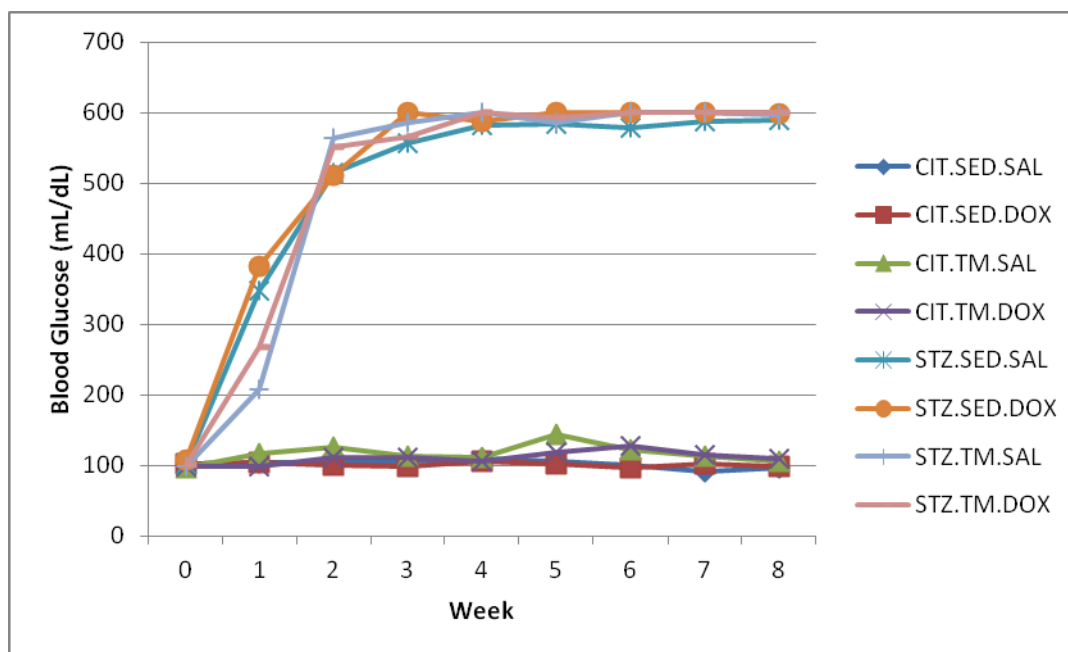
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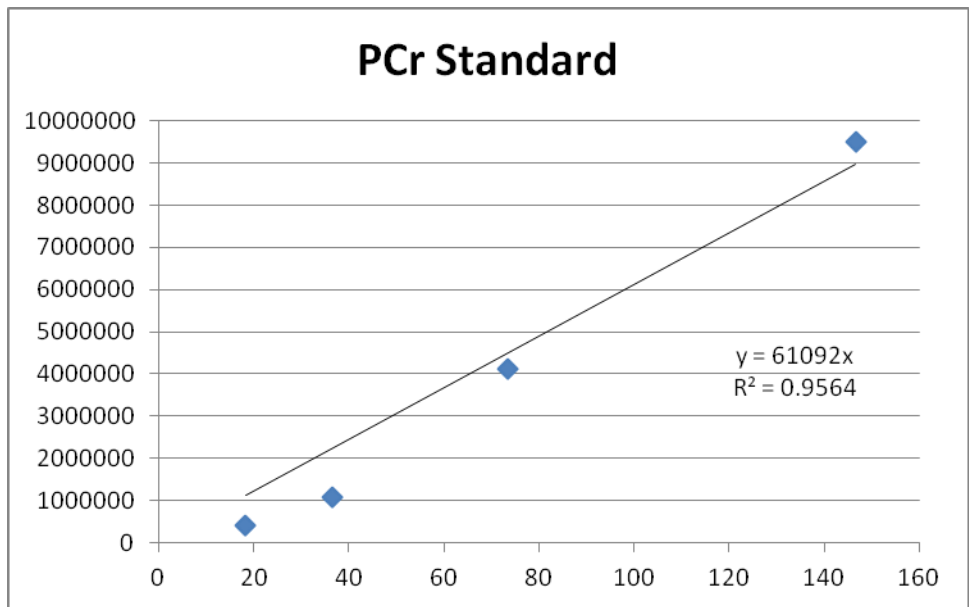
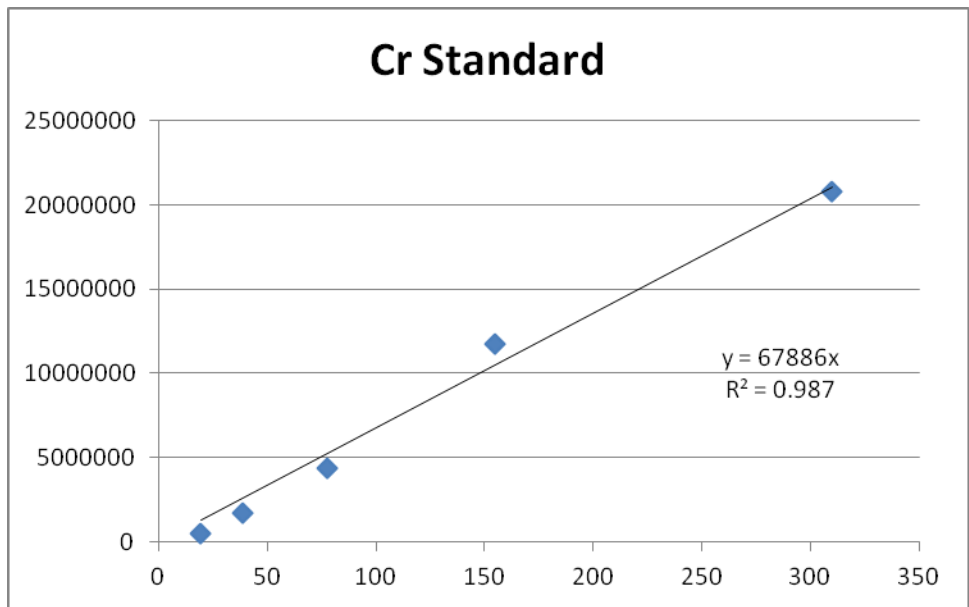
APPENDIX A
BLOOD GLUCOSE DATA

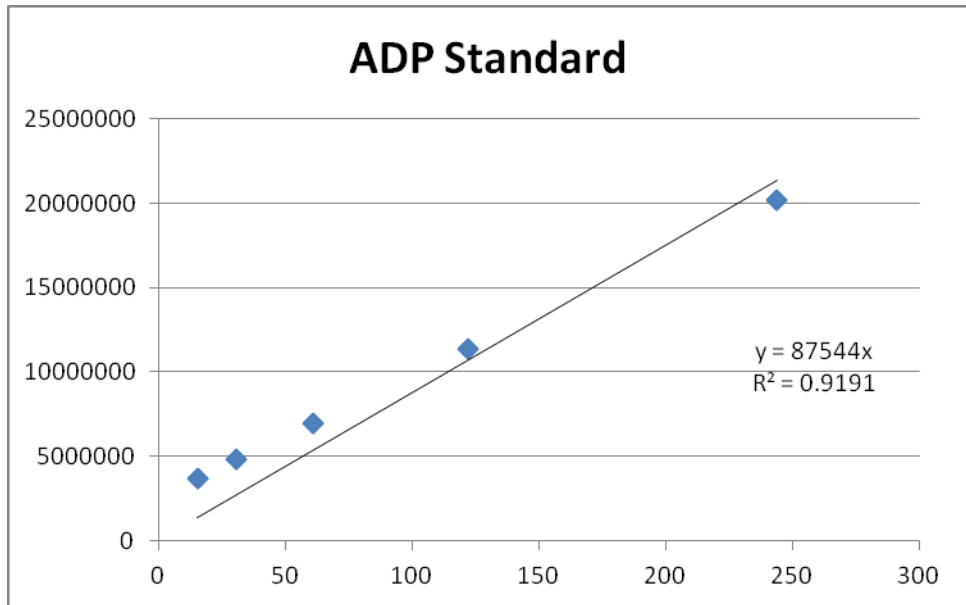
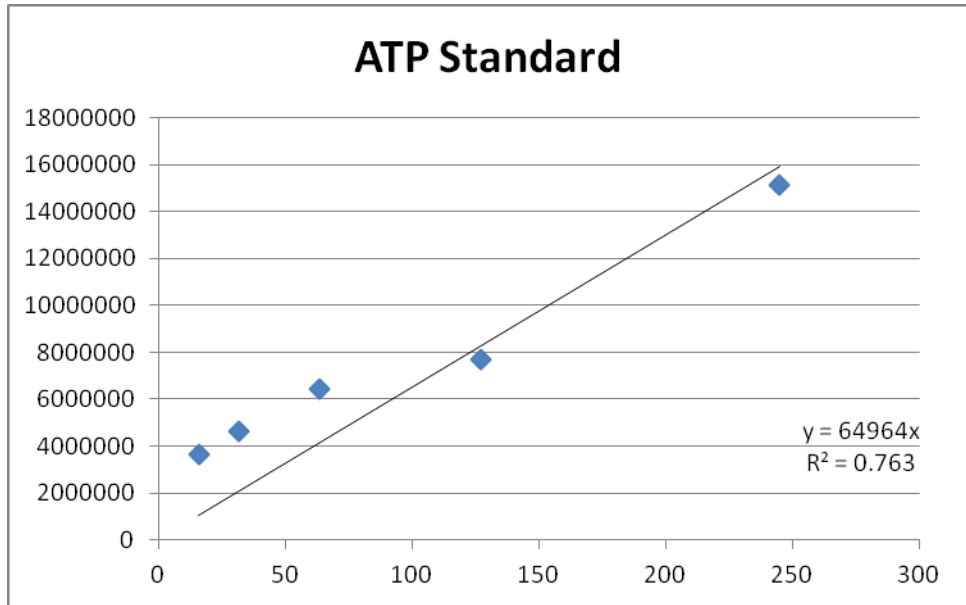
Blood Glucose Data

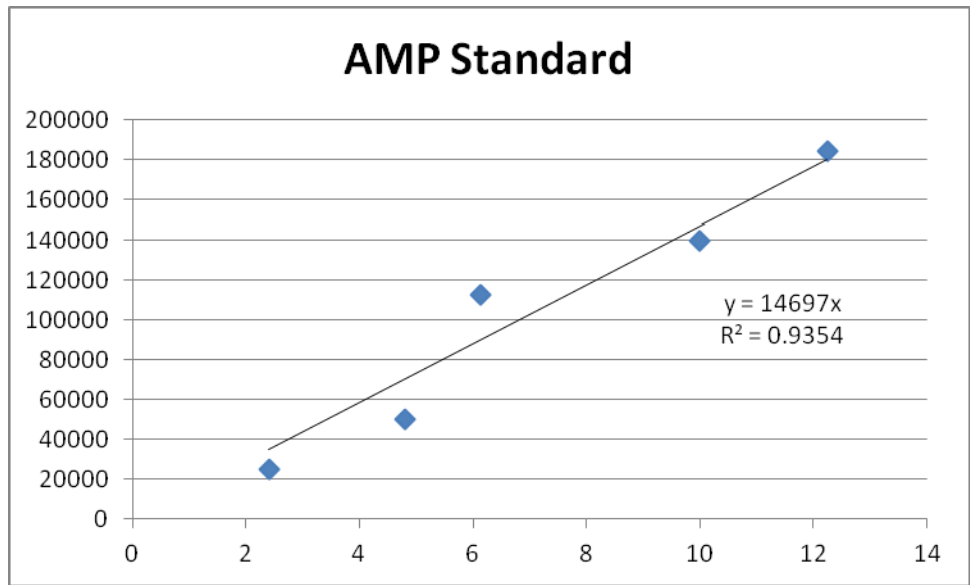
		Week	0	1	2	3	4	5	6	7	8
CIT.SED.SAL	M		102	105	103	105	109	106	101	90	96
	SD		11	14	14	14	12	10	8	10	10
CIT.SED.DOX	M		102	105	101	99	106	102	97	103	98
	SD		12	13	14	11	14	15	10	12	14
CIT.TM.SAL	M		97	117	125	114	111	144	123	113	106
	SD		8	18	33	18	14	49	37	12	9
CIT.TM.DOX	M		98	99	112	112	106	119	128	114	110
	SD		8	12	17	15	13	27	40	16	13
STZ.SED.SAL	M		102	347	516	557	581	584	578	587	589
	SD		11	195	130	77	49	55	71	30	20
STZ.SED.DOX	M		108	382	512	600	587	600	600	600	599
	SD		5	118	55	0	28	0	0	0	3
STZ.TM.SAL	M		98	208	564	587	600	586	600	600	597
	SD		11	164	67	18	0	35	0	0	7
STZ.TM.DOX	M		101	268	551	566	599	593	600	600	600
	SD		7	198	70	69	2	22	0	0	0



APPENDIX B
METABOLITE STANDARD CURVES
FOR HIGH PERFORMANCE
LIQUID CHROMATOGRAPHY







APPENDIX C
STATISTICAL ASSUMPTIONS

Prior to running hypothesis testing, assumptions were tested. Independence was not violated, as the occurrence of one event does not affect the probability of another event. Normal distribution was confirmed with a Shapiro-Wilk test ($p > .05$), normal probability plots were also used to assess the sample distribution. Residual box plots and Levene's test were used to assess the equality of variance; both concluded the data have equal variances ($p > .05$).

APPENDIX D
PILOT STUDY

A pilot study consisting of 25 animals, both males and females was conducted to determine feasibility and to assess the degree of cardiac dysfunction that might be expected with combined STZ-DOX treatment. A group (12 animals) received an STZ injection of 65 mg/kg. All animals in this group weighed between 190 and 215 grams at the time of injection. Eleven of the 12 animals had confirmed diabetes three days post injection. Those with confirmed diabetes maintained very high blood glucose levels around 600 mg/dL for 10 weeks. Four of the 12 animals died during the 10 week study. The average body weight at the end of the 10 weeks was 143 grams for diabetic animals treated with 65 mg/kg STZ. Cardiac function was measured with the isolated working heart model. A second group of 12 animals received an STZ injection of 45 mg/kg. These animals weighed between 200 and 280 grams at the time of STZ injection. Of the 12 injected with STZ, 10 had confirmed diabetes 3 days following, and no deaths occurred as a result of STZ exposure. Seven animals in this group were injected with 10 mg/kg of DOX after 10 weeks of being diabetic. One of the STZ + DOX animals died prior to analysis. *In vivo* and *ex vivo* measures were used to assess cardiac function. Table 3 and Figures 3- 6 summarized the findings of this pilot study. Significance was determined by one-way ANOVA analyses. These pilot studies indicated that in our laboratory, STZ at 45 mg/kg STZ showed elevated blood glucose, but blood glucose did not exceed the euthanization cutoff value of 600 mg/dL in any animal at any time. Furthermore, these studies clearly established that in sedentary animals STZ alone and DOX alone cause significant cardiac dysfunction, and the combination of STZ and DOX exacerbated cardiac dysfunction. From these pilot studies, it appears that this will be an appropriate model to answer the proposed research questions.

	65 mg/kg STZ	45 mg/kg STZ	10 mg/kg DOX
Success Rate	92%	83%	-----
Mortality Rate	33%	0%	14%

Table 3: Calculated success and mortality rates

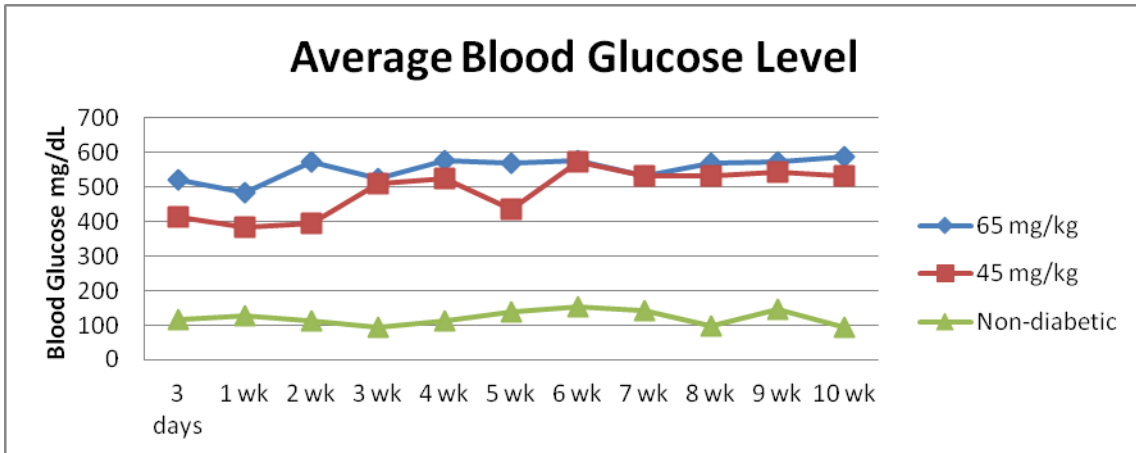


Figure 3: Average blood glucose levels measured throughout the 10 weeks, grouped by dose of STZ. Non-diabetic, animals given STZ resulting in non-confirmed diabetes.

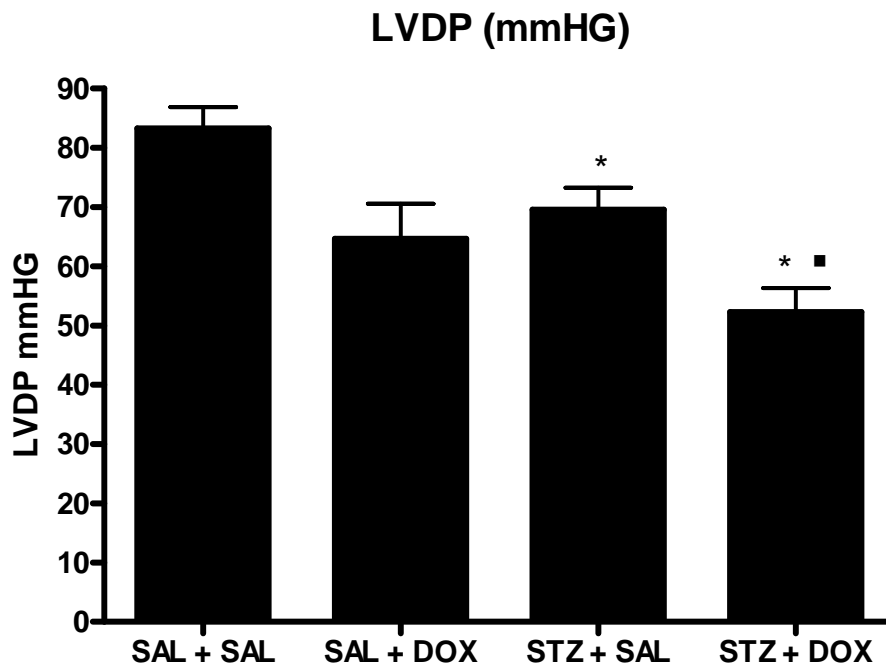


Figure 4: Left ventricular developed pressure. * significant difference compared to SAL + SAL; ■ significant difference compared to STZ + SAL

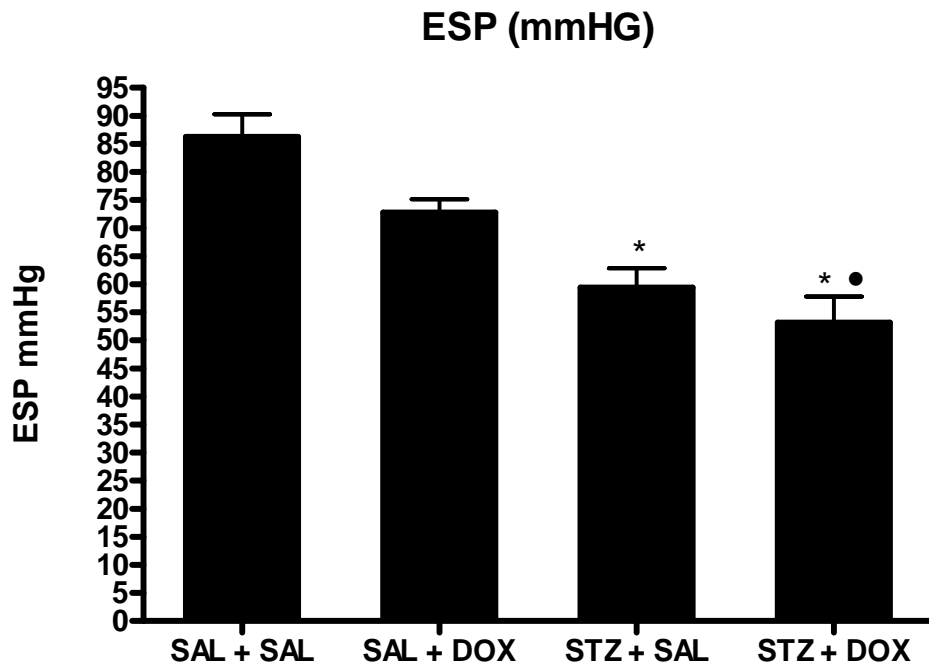


Figure 5: End systolic pressure. * significant difference compared to SAL + SAL; • significant difference compared to SAL+DOX

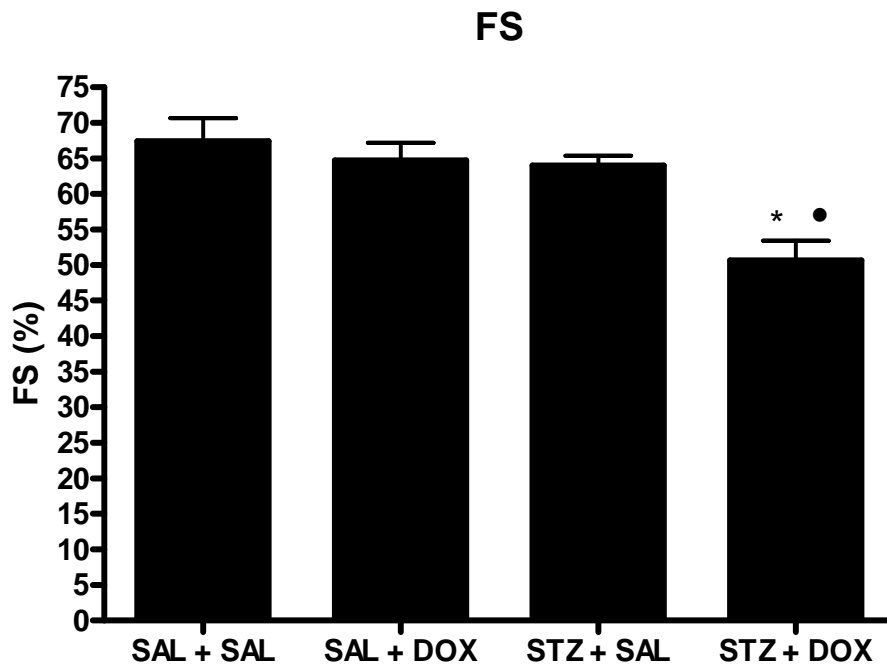


Figure 6: Fractional Shortening. * significant difference compared to SAL + SAL; • significant difference compared to SAL+DOX