8-1-2011

Effect of exercise on cardiac function and doxorubicin accumulation in left ventricular tissue of rats

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THE EFFECT OF EXERCISE ON CARDIAC FUNCTION AND DOXORUBICIN ACCUMULATION IN LEFT VENTRICULAR TISSUE OF RATS

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

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College of Natural and Health Sciences
School of Sport and Exercise Science
Program of Exercise Science

August 2011

This Dissertation by: Brock Thomas Jensen

Entitled: *The Effect of Exercise on Cardiac Function and Doxorubicin Accumulation in Left Ventricular Tissue of Rats*

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

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ABSTRACT

Jensen, Brock Thomas. *The Effect of Exercise on Cardiac Function and Doxorubicin Accumulation in Left Ventricular Tissue of Rats* Published Doctor of Philosophy Dissertation, University of Northern Colorado, 2011.

Doxorubicin (DOX) is an anthracycline antibiotic that has cytotoxic actions. The therapeutic use of DOX to treat a wide array of cancers is limited by a dose-dependent cardiotoxicity. Although DOX is known to have several adverse side-effects, acute and chronic cardiotoxicity have received the most attention as both may eventually lead to heart failure. While exercise has been shown to protect against DOX cardiotoxicity, a clear and consistent mechanism to explain its cardioprotective effects is lacking. High performance liquid chromatography (HPLC) is a valuable instrument that can be used to evaluate cardiac DOX accumulation. We hypothesized that a reduction in cardiac DOX accumulation may be a mechanism of exercise-induced cardioprotection. Therefore, the purpose of this study was to determine if exercise preconditioning reduces cardiac DOX accumulation, thereby providing a possible mechanism to explain the cardioprotective effects of exercise against DOX toxicity. Female Sprague-Dawley rats were randomly assigned to 1 of 3 primary experimental groups: sedentary (SED), voluntary wheel running (WR) or treadmill (TM). Animals in WR and TM groups completed 10 weeks of exercise prior to DOX treatment. DOX was administered 24 hours after the last training session as a bolus i.p. injection at 10 mg/kg. Subgroups of rats from each primary group were sacrificed at 1, 3, 5, 7, and 9 days post exposure and cardiac function was analyzed.

Twenty-four hours following sacrifice, cardiac DOX accumulation was analyzed using HPLC. DOX treatment resulted in both *in vivo* and *ex vivo* cardiac dysfunction. However, 10 weeks of either involuntary or voluntary exercise preconditioning preserved cardiac function. Additionally, significant differences were observed between sedentary and exercise groups for DOX accumulation. The greatest accumulation of DOX was observed in SED+DOX 1 day post injection. When compared to SED+DOX (day 1), DOX accumulation in TM+DOX (day 1) and WR+DOX (day 1) groups was significantly reduced ($p < 0.05$). Similarly, DOX accumulation in SED+DOX at both 3 and 5 days was significantly greater than both TM+DOX and WR+DOX at 3 and 5 days, respectively ($p < 0.05$). DOX accumulation in TM+DOX and WR+DOX groups was non-existent at both 7 and 9 days. Because DOX accumulation remained elevated in SED+DOX at 7 days, it was significantly greater than TM+DOX and WR+DOX groups ($p < 0.05$). Therefore, it is possible that the cardioprotective effects of exercise against acute DOX-induced injury may be due, in part, to a reduction in myocardial DOX accumulation.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my dissertation chair Dr. Reid Hayward for his generous and unconditional guidance, assistance and experience, and constant support and kindness throughout my graduate studies. His work ethic set a fine example of what it takes to be a successful researcher and mentor. You are an incredible man of many talents. I feel privileged and grateful to have had the chance to work with you. Next, I must thank my academic advisor Dr. Carole M. Schneider. If it was not for her, I would have never become a doctoral student at the University of Northern Colorado. Her counsel, encouragement, and commitment were unprecedented. I feel blessed to have personally witnessed your perseverance and have forever been inspired. With most sincere respect and appreciation, I would like to thank Dr. David S. Hydock, not only for his insight regarding science, but for insight regarding life in a much larger scope. I am grateful for all the time we spent together and for providing me a fine example of what a combined researcher and teacher should be. His never ending support, guidance and friendship have taught me to be a better person.

Dr. Patrick D. Burns earned my acknowledgment a long time ago for his support, willingness to help, and commitment to education. I would also like to thank Dr. Chia-Ying Lien for whom without his assistance and friendship, this project would not have been possible. Special thanks to Dr. Richard M. Hyslop and Mr. Peter Karnilaw for their guidance with HPLC. I must also thank Dr. Yen-Kuang Lin, Mr. Noah Gibson,

Ms. Stephanie Gruefe, Ms. Traci Parry and all the other graduate and undergraduate students for their assistance with the study and for making my time spent at the institute significant and enjoyable.

Finally, I would like to express my deepest love, admiration, and appreciation to my best friend and beautiful wife, Michaella. I am truly thankful for your vast encouragement, infinite patience, and inestimable support along the way. Additionally, I'd like to thank all of our family members. I could never have accomplished what I have without their endless love, support and encouragement. Even when I am unsure of myself, you have always been there to support me and my decisions. With all of you by my side, anything is possible and everything is worth striving to accomplish.

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CHAPTER I

INTRODUCTION

Epidemiological research over the last decade suggests that cancer incidence and death rates for all cancers combined are decreasing mainly as a result of advances in early detection and improved therapeutic strategies (National Cancer Institute, 2009). Chemotherapy is commonly used for the treatment of disease via chemicals that either have a specific toxic effect upon disease-producing microorganisms or selectively destroy cancer cells. The most common chemotherapeutic anthracycline used clinically is doxorubicin (DOX; trade name Adriamycin). In 1974, clinicians started prescribing DOX to cancer patients as a chemotherapy treatment (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004; Weiss, 1992). While the antineoplastic activity of DOX is evident, the exact mechanisms are unknown. It is hypothesized that DOX causes deoxyribonucleic acid (DNA) biosynthesis inhibition (Gewirtz, 1999), apoptosis (Clementi, Giardina, Di Stasio, Mordente, & Misiti, 2003), and free radical-mediated cellular injury (Minotti, Menna, et al., 2004). Despite its effective antineoplastic properties, the administration of DOX is limited as a result of its relatively high rate of dose-dependent cardiotoxicity (Jain, 2000).

DOX-induced cardiotoxicity is characterized by acute cardiac injury that may progress to irreversible cardiomyopathy and congestive heart failure months to years following treatment (Singal & Iliskovic, 1998; Singal, Li, Kumar, Danelisen, & Iliskovic, 2000). The exact mechanisms of DOX mediated cardiotoxicity are unknown; however,

oxidative stress appears to be the largest contributor. The C-ring of DOX undergoes heavy redox cycling in cardiomyocytes. The DOX quinone moiety oxidizes nicotinamide adenine dinucleotide dehydrogenase oxidoreductase (NADH) to form a semiquinone. In an attempt to reform the quinone, oxygen (O_2) is immediately reduced and reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are formed (Minotti, Menna, et al., 2004).

Several studies suggest that chronic exercise training prior to DOX treatment attenuates DOX-induced cardiotoxicity (Chicco, Schneider, & Hayward, 2005, 2006; Hydock, Lien, Schneider, & Hayward, 2008; Wonders, Hydock, Greufe, Schneider, & Hayward, 2009). Additionally, recent research indicates that acute and short-term exercise is cardioprotective against DOX-mediated cardiac dysfunction (Jensen, et al., 2009; Jensen, Hydock, Lien, Schneider, & Hayward, 2008; Lien, Hydock, Jensen, Schneider, & Hayward, 2009; Wonders, Hydock, Schneider, & Hayward, 2008). The mechanisms associated with exercise-induced cardioprotection against DOX cardiotoxicity are not fully understood. However, exercise may protect cardiac tissue from DOX-induced dysfunction by increasing antioxidative enzyme activity and expression (Ascensao, Magalhaes, Soares, et al., 2005; Chicco, Hydock, Schneider, & Hayward, 2006), decreasing lipid peroxidation (Ascensao, Magalhaes, et al., 2005a; Ascensao, Magalhaes, et al., 2005b; Chicco, Schneider, et al., 2006; Wonders, et al., 2009), upregulating heat shock protein 72 (HSP72) (Chicco, et al., 2005), preserving myosin heavy chain (MHC) isoform distribution (Hydock, Lien, Jensen, et al., 2009; Hydock, et al., 2008; Jensen, et al., 2009; Lien, et al., 2009), or downregulating proapoptotic enzymes (Wonders, et al., 2009). Additionally, a recent pilot study by our

laboratory using high performance liquid chromatography (HPLC) has shown that less DOX accumulates in cardiac tissue of exercised rats when compared to sedentary rats (Bradshaw, Karnilaw, Hayward, & Hyslop, 2009). Thus the cardioprotective effects of exercise may be due, at least in part, to a difference in the accumulation of DOX in the heart.

Since both short-term and chronic exercise preconditioning are cardioprotective in rats receiving DOX, it is hypothesized that DOX accumulation would be reduced in exercise preconditioned rats when compared to their sedentary counterparts. One possible explanation for a reduction in DOX accumulation could be an increased expression of multidrug resistance associated proteins (MRPs). Cancer cells express MRPs, which are glycoproteins capable of antitumor agent translocation (Krause, et al., 2007). DOX that is introduced to cancerous cells can initiate apoptosis or necrosis; however, when MRPs are expressed, DOX is extruded from the cell thereby preventing its accumulation inside the cell. The result is a decrease in DOX accumulation and an increased likelihood of tumor proliferation. If MRPs are upregulated in cardiomyocytes of exercised animals, these cardiac cells may accumulate less DOX (Krause, et al., 2007) thereby providing a theoretical basis for exercise-induced cardioprotection against DOX toxicity.

Statement of Purpose

The purpose of this study was two-fold: (a) to investigate the effects of chronic exercise preconditioning on cardiac DOX accumulation and to what extent it is related to DOX induced cardiac dysfunction, and (b) to apply an HPLC based method for a time-

course determination of DOX clearance in a complex biological sample matrix (rat left ventricular tissue).

Research Hypotheses

H1 Detectable quantities of DOX from left ventricular tissue will decrease as time elapses following DOX exposure.

H2 Chronic exercise trained animals will have less DOX accumulation in left ventricular tissue when compared to sedentary animals throughout a 9-day observation period.

H3 Chronic exercise before DOX treatment will preserve cardiac function, and this will be related to a decline in DOX accumulation in the hearts of exercised animals.

Need for the Study

Cancer survivorship has improved in recent decades, however, toxicity associated with some of the most effective chemotherapeutic antibiotics still exists. One of the most widely used antineoplastic agents is DOX (Jain, 2000). DOX is used in the treatment of several cancers, however, it is associated with a dose-dependent cardiotoxicity that can eventually progress to heart failure (Singal & Iliskovic, 1998). Doxorubicin cardiotoxicity is characterized by acute cardiac injury that may progress to irreversible cardiomyopathy and congestive heart failure months to years following treatment (Ferrans, Clark, Zhang, Yu, & Herman, 1997). However, exercise training can offer protection against a variety of acute and chronic myocardial injuries (Ascensao, Magalhaes, Soares, Ferreira, Neuparth, Appell, et al., 2005). It has been shown that exercise preconditions the myocardium so that it is protected against oxidative stress by increasing antioxidative enzyme activity and expression, increasing heat shock protein content, preserving MHC, and possibly reducing the accumulation of DOX in cardiomyocytes. Less DOX accumulation in rat myocardia may reduce the various forms

of injury to cardiac tissue, and this adaptation may protect the hearts of preconditioned rats against cardiac dysfunction and injury induced by DOX treatment. Currently, there are no studies that have examined the effectiveness of exercise training on cardiac DOX accumulation. Therefore, this investigation is focused on the use of exercise to attenuate cardiac DOX accumulation and cardiac dysfunction associated with DOX treatment.

Table 1

Abbreviations.

ABC - ATP binding cassette	LVDPd - LVDP during diastole
AFC - affinity chromatography	LVDs - LV end systolic diameter
AIF - apoptosis-inducing factor	LVEDV - LV end diastolic volume
ATP - adenosine triphosphate	LVESV - LV end systolic volume
Ca^{++} - calcium	MDR - multidrug resistance proteins
CAT - catalase	MHC - myosin heavy chain
CO - cardiac output	MPT - membrane permeability transition
DNA - deoxyribonucleic acid	MPTP - membrane permeability transitional pore
DAUN - daunorubicin	MRPs - multidrug resistance associated proteins
DOX - doxorubicin	mtDNA - mitochondrial DNA
DOX-ol - doxorubicinol	NADH - nicotinamide adenine dinucleotide dehydrogenase oxidoreductase
dP/dt_{max} - maximum rate of developed pressure	NO - nitric oxide
dP/dt_{min} - minimum rate of developed pressure	$ONOO^-$ - peroxynitrite
EDP - end diastolic pressure	O_2 - oxygen
EPI - epirubicin	$O_2^{\cdot -}$ - superoxide anion
EPIDAUN - epidaunorubicin	·OH - hydroxyl radical
ESP - end systolic pressure	PWd - posterior wall thickness during diastole
ET - ejection time of aortic blood flow	PWs - posterior wall thickness during systole
ERK - extracellular signal-regulated kinases	ROS - reactive oxygen species
Fe^{2+}/Fe^{3+} - iron	RONS - reactive oxygen and nitrogen species
FS - fractional shortening	RPLC - reversed phase liquid chromatography
GPx - glutathione peroxidase	RWT - relative wall thickness
HPLC - high performance liquid chromatography	SEC - size exclusion chromatography
HR - heart rate	SED - sedentary
HSPs - heat shock proteins	SOD - superoxide dismutase
HSP70 - heat shock protein 70	SV - stroke volume
HSP72 - heat shock protein 72	SWd - septal wall thickness during diastole
H_2O_2 - hydrogen peroxide	SWs - septal wall thickness during systole
IDA - idarubicin	TM - treadmill
IEC - ion exchange chromatography	A-V_{max} - maximal aortic flow velocity
i.p. - intraperitoneal	A-V_{mean} - mean aortic flow velocity
i.v. - intravenous	M-V_{max} - maximal mitral flow velocity
LV - left ventricle	M-V_{mean} - mean mitral flow velocity
LVDd - LV end diastolic diameter	VTI - velocity time integral
LVDP - LV developed pressure	WR - voluntary wheel running
LVDs - LV end systlic diameter	

Delimitation of the Study

Samples in this study included 10-11 week old female Sprague-Dawley rats (180-210 g). Cardiotoxicity was induced using a 10 mg/kg bolus intraperitoneal (i.p.) injection of DOX. The exercise training groups ran on a motorized treadmill at 30 m/min up an 18% grade for 60 minutes, 5 days a week for 10 weeks.

Definition of Terms

Apoptosis - programmed cell death.

Cardiolipin - obligatory phospholipids localized to the inner mitochondrial membrane, responsible for maintenance of the structure and metabolic function of the mitochondria membrane proteins.

Cardioprotection - adaptations in the heart that provide increased tolerance to ischemic or chemical induced injury.

DOX-induced cardiotoxicity - development of cardiac abnormalities following the exposure to DOX.

Echocardiogram - an ultrasonic technique used to image real-time cardiac tissue dimensions and blood flow velocities.

High performance liquid chromatography - a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify and quantify compounds. HPLC can utilize a column that holds chromatographic packing material, a pump that moves the mobile phase through the column, and a detector that shows the retention times of molecules such as DOX.

MHC - an abundant contractile protein. The ATPase associated with each MHC hydrolyzes ATP. In cardiomyocytes, there are two MHC isoforms (α and β). The α-MHC has faster ATPase activity in comparison to β-MHC.

M-mode - An imagining mode used to determine cardiac geometry during echocardiography.

Multidrug resistant proteins - proteins found in cellular membranes responsible for the efflux of xenobiotics.

Membrane permeable transitional pores - protein pore formed in the mitochondrial membrane under stress induced conditions. When induced, it contributes to mitochondrial swelling, dysfunction, and cellular apoptosis.

Mitochondrial dysfunction - the impairment of function or abnormalities observed exclusively in the mitochondria. The dysfunction is primarily due to the adverse effects of drugs, infections, or other environmental causes.

Necrosis - the premature death of living cells and tissue.

Xenobiotic - a chemical neither expected to be found in an organism nor naturally synthesized.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Anthracyclines are a specific group of antineoplastic drugs known to have the widest range of clinical use of any class of drugs in oncology (Minotti, Menna, et al., 2004). The anthracyclines are highly effective anticancer antibiotics used in the treatment of a variety of malignancies. Of the anthracyclines, DOX, is the most effective and has the greatest range of treatment, however, its use is limited clinically by a dose-dependent cardiotoxicity which may eventually lead to heart failure (Lipshultz, et al., 2005; Singal, et al., 2000). The acute form of cardiotoxicity often develops within a short period of time following DOX treatment (Ferrans, et al., 1997; Tokarska-Schlattner, Zaugg, Zuppinger, Wallimann, & Schlattner, 2006). Numerous molecular mechanisms for DOX cardiotoxicity have been hypothesized (Minotti, Menna, et al., 2004; Zucchi & Danesi, 2003), however, oxidative stress (Krause, et al., 2007; Schimmel, Richel, van den Brink, & Guchelaar, 2004), mitochondrial dysfunction (Zhou, Starkov, Froberg, Leino, & Wallace, 2001) and apoptosis (Minotti, Menna, et al., 2004) appear to be the largest contributors.

Results from animal studies indicate that chronic exercise training (Chicco, Hydock, et al., 2006; Chicco, et al., 2005; Chicco, Schneider, et al., 2006; Hydock, et al., 2008; Wonders, et al., 2009) and short-term exercise (Jensen, et al., 2009; Jensen, et al., 2008; Wonders, et al., 2008) can attenuate DOX-mediated cardiotoxicity. DOX

treatment leads to impairment in both *in vivo* (Hydock, Lien, & Hayward, 2009; Hydock, et al., 2008) and *ex vivo* (Hydock, Wonders, Schneider, & Hayward, 2009; Wonders, et al., 2009) cardiac function. It has been demonstrated that exercise protects against DOX-induced cardiac dysfunction by preserving end systolic pressure (ESP), end diastolic pressure (EDP), left ventricular developed pressure (LVDP), maximum rate of developed pressure (dP/dt_{max}), and minimum rate of developed pressure (dP/dt_{min}) (Hydock, et al., 2008; Jensen, et al., 2008; Wonders, et al., 2009; Wonders, et al., 2008). The mechanisms by which exercise offers protection from myocardial injury are not fully understood; however, an increase in antioxidative capacity (Ascensao, Magalhaes, Soares, et al., 2005; Chicco, Hydock, et al., 2006) appears to play an important role.

In order to directly study cardioprotective cellular mechanisms, many instruments and techniques have been used. One such method is chromatography, a physical separation of components in a mixture of compounds (Loadman & Calabrese, 2001; Wang, Mallette, & Parcher, 2008). Bradshaw et al. (2009) assert that HPLC is useful for quantifying DOX accumulation in experiments with sedentary and exercise preconditioned rats.

The purpose of this study was to investigate the effects of chronic exercise preconditioning on cardiac DOX accumulation and its relationship to DOX-induced cardiac dysfunction. An additional purpose of this investigation was to apply an HPLC-based method for a time-course determination of DOX accumulation in a complex biological sample matrix.

Doxorubicin

Daunorubicin (DAUN) and DOX are among the most effective and commonly prescribed antineoplastic chemotherapeutic agents (Weiss, 1992). In 1963, researchers' derived DAUN from *Streptomyces peucetius*. Shortly after its discovery, DAUN was used clinically to treat myeloblastic leukemias (Weiss, 1992). Over a decade later, DOX, a 14-hydroxy analog of DAUN, was clinically prescribed to cancer patients in the United States for the treatment of childhood tumors, lymphomas, and breast cancer (Minotti, Menna, et al., 2004). To date, there are over 2000 antrhacycline analogs; however DAUN, DOX, epirubicin (EPI), and idarubicin (IDA) are the most clinically relevant. Despite the common use of DOX, the antineoplastic mechanisms are not fully understood. The primary antitumor mechanism of DOX is hypothesized to involve DNA intercalation. DOX inhibits DNA biosynthesis by migrating into cells via simple diffusion and binding to proteosomes in the cytoplasm, thereby forming a DOX-proteasome complex that can cross the nuclear membrane. Upon dissociation of the proteasome, DOX binds to DNA. Consequently, DOX stimulates double-stranded DNA scission and inhibits topoisomerase activity which prevents DNA replication in the cell (Cummings, Anderson, Willmott, & Smyth, 1991; Gewirtz, 1999; Lothstein, et al., 2000). Furthermore, the antineoplastic effects of DOX may in part be attributed to cytotoxic free radicals induced by DOX (Minotti, Menna, et al., 2004). Free radicals are responsible for events leading to lipid peroxidation, DNA damage and apoptosis; however, their role is more predominantly involved in DOX-mediated cardiotoxicity.

Doxorubicin Cardiotoxicity

The utilization of DOX is limited due to acute and chronic cardiotoxicity. DOX cardiotoxicity is characterized by acute cardiac injury that may progress to irreversible cardiomyopathy and congestive heart failure months to years following treatment (Singal & Iliskovic, 1998; Singal, et al., 2000). The acute form of cardiotoxicity often develops within minutes, hours, or days following DOX treatment (Ferrans, et al., 1997; Tokarska-Schlattner, et al., 2006). Cardiac arrhythmias, pericarditis, hypotension, myofibrillar disorganization, cardiomyocyte vacuolization, and elevated serum enzyme markers are all signs of acute cardiotoxicity (Abdel-Wahab, et al., 2003; Ferrans, et al., 1997; Lefrak, Pitha, Rosenheim, & Gottlieb, 1973; Mihm, Yu, Weinstein, Reiser, & Bauer, 2002; Parry, et al., 2009; Schimmel, et al., 2004; Singal & Iliskovic, 1998). Even though the early signs of cardiac dysfunction are reversible, acute toxicities associated with DOX are highly predictive of chronic toxicities (Cardinale, et al., 2002; Nousiainen, Jantunen, Vanninen, & Hartikainen, 2002).

Chronic DOX cardiotoxicity may present months, years, or decades after DOX has been cleared from the body. DOX cardiotoxicity is responsible for structural changes of the myocardia which may lead to functional changes. Decreased cardiac function due to DOX cardiotoxicity (indicated by a reduction in left ventricular ejection fraction) (Ferrans, et al., 1997), is often caused by dilated cardiomyopathy (Singal & Iliskovic, 1998), which may eventually lead to congestive heart failure and dysrhythmias years following exposure to DOX (Steinherz, Steinherz, Tan, Heller, & Murphy, 1991). Approximately 5% of patients who receive a 550 mg/m^2 commutative dose of DOX will develop dilated cardiomyopathy within 4-20 years (Steinherz, et al., 1991). This

percentage increases when higher doses are administered (Singal, Iliskovic, Li, & Kumar, 1997).

Mechanisms of Doxorubicin Cardiotoxicity

Oxidative Stress

The exact mechanisms of DOX mediated cardiotoxicity are unknown; however, oxidative stress appears to be the largest contributor. The production of excessive quantities of reactive oxygen and nitrogen species (RONS) is an important mechanism of DOX-induced cardiotoxicity. The C-ring of DOX undergoes redox cycling in cardiomyocytes. The DOX quinone moiety oxidizes NADH to form a semiquinone. In an attempt to reform the quinone, O_2 is immediately reduced and ROS such as $O_2^{\cdot-}$ and H_2O_2 are formed (Minotti, Menna, et al., 2004). $O_2^{\cdot-}$ is a potent free radical that can injure cardiomyocytes. In the presence of the antioxidant enzyme, superoxide dismutase (SOD), $O_2^{\cdot-}$ can undergo dismutation in which $O_2^{\cdot-}$ is converted to hydrogen peroxide H_2O_2. If $O_2^{\cdot-}$ is not converted to H_2O_2, it may combine with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a powerful nitrogen oxygen species known to affect key proteins in myocardial tissue (McCord, 1985). Glutathione peroxidase (GPx) or catalase (CAT), two antioxidant enzymes located in cardiomyocytes, can convert H_2O_2 into water. However, if H_2O_2 is not converted to water, it may react with iron (Fe^{2+}/Fe^{3+}) to form a hydroxyl radical (·OH), which is known to be the most potent free radical produced and has a greater cellular toxicity than $O_2^{\cdot-}$ (Muindi, Sinha, Gianni, & Myers, 1984). Therefore, RONS induced by the redox cycling of DOX may further exacerbate cardiac dysfunction.

Mitochondrial Dysfunction

Mitochondria play a key role in cardiac cell survival and death. They are essential for adenosine triphosphate (ATP) production, regulation of intracellular calcium (Ca^{++}) homeostasis, and the main generators of intracellular ROS (Zhou, et al., 2001). Damage to the mitochondrial membranes may result in the excessive production of ROS, formation and dysfunction of membrane permeability transitional pores (MPTP), and a release of apoptotic proteins. Therefore, mitochondria control many of the pathways that lead to cellular death (Crompton, 1999; Singal, et al., 2000).

DOX exhibits a strong affinity for cardiolipin, an abundant and obligatory lipid that is localized to the inner mitochondrial membrane (Cheneval, Muller, Toni, Ruetz, & Carafoli, 1985; Goormaghtigh, Huart, Praet, Brasseur, & Ruysschaert, 1990; Yaroslavov, et al., 2003). Cardiolipin when combined with DOX causes cytochrome c to be released, decreasing both ATP production and inducing apoptosis. A recent report suggested that DOX is capable of migrating beyond the membrane and penetrating the mitochondrial matrix to directly interact with mitochondrial DNA (Ashley & Poulton, 2009b). This in part, may explain the cardioselective mitochondrial toxicity of DOX. Additionally, the large quantity of mitochondria in cardiomyocytes (approximately 30-35% of the total cell volume), the cardiomyocyte demand of a continuous supply of ATP for contractile purposes, and the abundance of ATP derived from oxidative metabolism all contribute to the mitochondrial specificity of DOX and the deleterious effects on mitochondrial bioenergetics (Carvalho, et al., 2010; Herman & Young, 1979; Tokarska-Schlattner, et al., 2006).

Several other mitochondrial structures and processes provide primary targets for drug-induced toxicity and cell death (Starkov & Wallace, 2002). Lemasters and colleagues (1998) reported that drug-induced oxidative injury caused pyridine nucleotide oxidation, mitochondrial generation of RONS, and increased mitochondrial Ca^{++} concentrations, leading to an induction of MPTP. The theory behind MPTP induction is that cytoxicity stimulates mitochondrial permeability transition (MPT). MPT is a phenomenon whereby the controlled permeability of the inner mitochondrial membrane is altered and the mitochondria are no longer selectively permeable to solutes such as Ca^{++}; thus leading to depolarization of the membrane and osmotic swelling (Crompton, 1999; Kroemer & Reed, 2000; Lemasters, et al., 1998). It is hypothesized that the disruption of the mitochondrial membrane due to the unnecessary depolarization of the membrane may lead to the release of cytochrome c and other apoptogenic factors in the intermembrane space, such as apoptosis-inducing factor (AIF), endonuclease G, and procaspase-9 (Crompton, 1999; Gillick & Crompton, 2008; Kroemer & Reed, 2000).

Mitochondrial dysfunction induced by MPT is linked to DOX cardiotoxicity. MPT is a direct consequence of mitochondrial Ca^{++} overload, known to cause the inhibition of ATP synthesis and the release of apoptotic factors (Gillick & Crompton, 2008; Lemasters, et al., 1998). Calcium enters the mitochondria electrophoretically and is controlled by a transport cycle driven by the proton pumps of the respiratory chain. Under physiological conditions mitochondrial Ca^{++} controls key metabolic regulatory enzymes such as pyruvate dehydrogenase, oxoglutarate dehydrogenase, and isocitrate dehydrogenase in the mitochondrial matrix. However, under conditions associated with ATP depletion and oxidative stress, mitochondrial Ca^{++} stimulates the opening of MPTP,

allowing more Ca^{++} to enter the matrix and inducing Ca^{++} overload which is known to be an important factor in cellular injury (Crompton, 1999; Gillick & Crompton, 2008).

In DOX treated rats, the stimulation of mitochondrial calcium cycling is marked by an increased sensitivity of cardiac mitochondria to calcium-induced membrane depolarization (Sokolove & Shinaberry, 1988). The significance of increased sensitivity to calcium is that the mitochondrial response to changing calcium concentrations is compromised (Solem, Heller, & Wallace, 1996; Solem, Henry, & Wallace, 1994). Thus, induction of MPTP by DOX results in an energy-consuming cyclical uptake and release of calcium across the inner membrane, depolarization, reduced ATP, and eventually, cell death. Mitochondrial dysfunction may also be the result of DOX's ability to intercalate mitochondrial DNA (mtDNA). Recently, Ashley et al., (2009a) reported that DOX and other DNA intercalators, intercalated mtDNA within living cells and caused mtDNA depletion. Therefore, DOX-mediated mitochondrial dysfunction does not appear to be limited to oxidative stress (Ascensao, Magalhaes, Soares, et al., 2005), calcium homeostasis (Solem, et al., 1994), or MPT induction (Crompton, 1999).

Exercise-Induced Cardioprotection

The benefits of chronic exercise training are well established. Endurance training can improve cardiac function (Vella & Robergs, 2008) and is known to play an essential role in the management of heart failure (Papathanasiou, Tsamis, Georgiadou, & Adamopoulos, 2008), hypertension (Hansen, Nielsen, Saltin, & Hellsten, 2010), hyperlipidemia (Wang, Hu, et al., 2009), hypercholesterolemia (Blake & Triplett, 1995), and atherosclerosis (Kadoglou, Iliadis, & Liapis, 2008). Other physiological adaptations that occur with endurance exercise include increased stroke volume (SV), decreased

resting heart rate (HR), and increased cardiac output (CO) which contributes to overall improved cardiac function (Wilmore, et al., 2001). Exercise is also recognized for its role in cardioprotection. Studies have been conducted using both chronic exercise training (Chicco, Hydock, et al., 2006; Chicco, et al., 2005; Chicco, Schneider, et al., 2006; French, et al., 2008; Hydock, et al., 2008; Quindry, et al., 2005; Wonders, et al., 2009) and short-term exercise (Demirel, et al., 2001; French, et al., 2008; Hamilton, et al., 2001; Wonders, et al., 2008) to limit the cardiac injury associated with ischemia and various pharmacological agents. Several cellular mechanisms have been attributed to the cardioprotective effects of exercise training. Many studies have reported that DOX-mediated acute cardiotoxicity can be attenuated by an increase in myocardial antioxidant capacity, HSP72 expression, α-MHC isoform, and DOX extrusion.

Increased Antioxidant Capacity

If RONS are not managed by myocardial antioxidants, enhanced production of RONS results in several destructive processes in cardiomyocytes. Overexpression of a free radical scavenger protein, metallothionein, is known to play a significant role in the reduction of DOX-induced cardiotoxicity in transgenic mice (Kang, Chen, Yu, Voss-McCowan, & Epstein, 1997; Naganuma, Satoh, & Imura, 1988; Satoh, Naganuma, & Imura, 1988). Antioxidant enzymes such as SOD, CAT, and GPx are important proteins found in cardiomyocytes, and when overexpressed in transgenic mice, the deleterious effects of DOX are attenuated (Gouaze, et al., 2001; Kang, Chen, & Epstein, 1996; Yen, Oberley, Vichitbandha, Ho, & St Clair, 1996). Additionally, it has been hypothesized that increases in myocardial antioxidant capacity can be induced by exercise, contributing to cardioprotection during periods of elevated oxidative stress (Ascensao, Magalhaes,

Soares, et al., 2005; Atalay & Sen, 1999). Numerous studies have reported that exercise increases the overall antioxidant capacity and it appears that increases in content (Siu, Bryner, Martyn, & Alway, 2004) and/or activity (Chicco, et al., 2005; Gunduz, Senturk, Kuru, Aktekin, & Aktekin, 2004; Husain, 2002, 2003; Husain & Somani, 1997; Kanter, Hamlin, Unverferth, Davis, & Merola, 1985; Powers, et al., 1998) of SOD, CAT and GPx in cardiac tissue are responsible for this adaptation.

In contrast to the aforementioned studies, a significant increase in myocardial SOD, CAT, and/or GPx activities are not a requisite for exercise-induced cardioprotection. Some studies have reported inconsistencies in the antioxidant adaptive response to exercise (Atalay & Sen, 1999; Ji & Mitchell, 1994; Rinaldi, et al., 2006). Ji and Mitchell (1994) reported that neither myocardial GPx nor CAT activities were altered by exercise. Jensen et al., (2008) reported no statistical increase or preservation of SOD and CAT activities in short-term preconditioned rats that received a 10 mg/kg bolus i.p. injection of DOX, however, cardioprotection was still observed. Therefore, the role that exercise plays in the regulation of the antioxidant system is complex in nature and currently not fully understood.

Myocardial Heat Shock Protein Expression

Heat shock proteins (HSPs) are known to play a key role in attenuating oxidative injury in cardiomyocytes (Hamilton, et al., 2003; Starnes & Taylor, 2007). In models of ischemia-reperfusion injury (Murlasits, Lee, & Powers, 2007; Quindry, et al., 2007) and acute DOX cardiotoxicity (Chicco, et al., 2005; Chicco, Schneider, et al., 2006), exercise induced an upregulation of HSP and offered cardioprotection in rats. HSPs are believed to have protective effects by reducing oxidative injury, preventing the denaturing of key

regulatory proteins, and reducing apoptosis and necrosis (Ascensao, Magalhaes, et al., 2006; Powers, Quindry, & Hamilton, 2004). Animals that trained on a treadmill 5 days/wk up a 6% grade at 20 m/min for 60 min expressed a 12.3-fold increase in myocardial HSP70 when compared to sedentary rats (Harris & Starnes, 2001). Similarly, Chicco et al. (2005) reported that 8 weeks of voluntary wheel running led to an upregulation of cardiac HSP72 expression and prevented cardiac dysfunction induced in rats by the perfusion of 10 μM of DOX for 60 minutes. In another study by Chicco et al. (2006), results suggested that DOX-induced cardiac dysfunction was attenuated by increased cardiac expression of HSP72 in rats chronically trained for 12 weeks, when compared to the SED+DOX animals. Furthermore, Murlasits et al. (2007) reported that short-term exercise upregulated HSP72 and offered cardioprotection. Despite evidence that suggests exercise induced HSP upregulation reduces cardiac injury, other reports suggested cardioprotection is not necessarily due to an increase in myocardial HSP expression (Starnes, Choilawala, Taylor, Nelson, & Delp, 2005), especially with short-term exercise (Hamilton, et al., 2001; Jensen, et al., 2009; Taylor, Harris, & Starnes, 1999).

Myosin Heavy Chain Isoform Distribution

MHC isoform distribution is known to influence cardiac function, and the shifting of MHC isoforms can be significantly affected by DOX treatment (Hydock, et al., 2008; Hydock, Wonders, et al., 2009). It was reported that 5 days post DOX treatment, sedentary animals displayed a significant increase in the percentage of β-MHC isoform, which contains the slower ATPase of the two isoforms. Exercise preconditioning, however, attenuates this isoform shift (Hydock, et al., 2008).

Additionally, rats that performed voluntary wheel running exercise while receiving DOX expressed significantly higher levels of α-MHC isoform, the faster ATPase isoform, when compared to their sedentary counterparts (Hydock, Wonders, et al., 2009). Although a preservation of the MHC isoform distribution may not completely explain the protective effects of exercise, it may be one of several adaptations that contribute to its cardioprotective effects.

Doxorubicin Accumulation

Following the administration of anthracyclines such as DOX and EPI, accumulation in cardiac tissue can occur very quickly (Buja, Ferrans, Mayer, Roberts, & Henderson, 1973; Salvatorelli, Menna, Lusini, Covino, & Minotti, 2009; Urva, Shin, Yang, & Balthasar, 2009). Timour et al. (1988) reported that intracellular concentrations of DOX (4,000 ng/g) in canine myocardial cells, far exceeded plasma concentrations 3 hours after administration. Additionally, it was reported, following a 1.5 mg/kg intravenous (i.v.) infusion of DOX, no detectable concentration was observed in plasma, however, 200 ng/g was still detected in the myocardial tissue 1 week post-infusion. Little is known regarding the accumulation of DOX in rat cardiac tissue. Recently, many methods have been developed to quantify DOX in plasma, serum, and tissues (Ahmed, et al., 2009; Maudens, Stove, Cocquyt, Denys, & Lambert, 2009; Urva, et al., 2009). However, none of the reported methods have validated their findings with cardiac function. Based on the proposed mechanisms of DOX cardiotoxicity, it may be possible that a reduction in cardiac DOX accumulation could result in a reduced state of oxidative stress, mtDNA interference, mitochondrial dysfunction and apoptosis. Furthermore, a recent pilot study by our laboratory using HPLC has shown that less DOX accumulates in

cardiac tissue of exercised rats when compared to sedentary controls (Bradshaw, et al., 2009). Thus a cardioprotective effect of exercise may be the result of a difference in the accumulation of DOX in the cardiac tissue of exercise trained rats.

Multidrug Resistance Proteins

A reduction in DOX accumulation may be the result of increased expression of MRPs. MRPs are glycoproteins embedded in the cellular membrane which are responsible for pumping xenobiotics out of the cell (Krause, et al., 2007; Patel, Dunn, & Sorokin, 2002). These proteins belong to a superfamily of proteins known as ATP binding cassette (ABC) transporters (Sorokin, 2004). Depending on location and tissue type, MRPs can be further categorized into subgroups including: MRP-1, MRP-2, MRP-3, MRP-4, MRP-5, MRP-6 and MRP-7. Studies have reported the expression of MRP-1, MRP-2 (Rosati, et al., 2003) and MRP-5 (Dazert, et al., 2003; Meissner, et al., 2007) in cardiac tissue. Additionally, the MRP-1/GS-X pumps have received attention due to their potential role in DOX extrusion (Krause, et al., 2007). A theory was proposed by Krause et al. (2007) which considered the possibility that DOX may be extruded via MRP-1/GS-X pumps known to be expressed in cell membranes (de Bittencourt Junior, Curi, & Williams, 1998) and suggested less DOX would reside in cardiomyocytes if MRP-1/GS-X pumps were upregulated in cardiac cells. Furthermore, Krause et al. (2007) reported rats that performed 60 minutes of swimming exercise for 1 week expressed a 2.4-fold increase in myocardial MRP-1, when compared to their sedentary counter-parts; however, DOX accumulation was not determined. Therefore, if MRPs are upregulated in cardiomyocytes of exercised animals, cardiac cells may accumulate less DOX thereby providing a theoretical basis for exercise-induced cardioprotection against DOX toxicity.

High Performance Liquid Chromatography

One purpose of this study was to determine the accumulation of DOX in rat cardiac tissue as time elapses following DOX exposure using HPLC. Chromatography is a method of physically separating the components in a mixture of compounds. This technique provides information about the compounds general structures (polar characteristics) (Korecka & Shaw, 2009; Zacharis, 2009). Specifically, liquid chromatography can separate liquid samples by passing the sample over a solid (stationary phase) with constant polarity. The stationary phase is composed of hydrated silica gel (hydrocarbons). The hydrocarbons are polar and adhere to a steel column. A liquid sample, when introduced to a liquid solvent, may be dissolved (mobile phase). The mobile phase is responsible for wicking (wetting) the stationary phase. As the solvent wicks the stationary phase, it interacts with the sample. The solvent dissolves the sample due to the intermolecular attractions between the sample and the solvent. The sample will also be attracted to the stationary phase. As the sample is dissolved in the solvent, it moves down the column in a "zigzag path" (i.e. dissolved in the solvent, absorbed on the stationary phase, dissolved in the solvent, absorbed on the stationary phase, and so on). Different compounds will have different attractions for the solvent and for the stationary phase, thus they will move down the stationary phase at different rates. It is in this process that separation occurs. The effectiveness of separating a complex sample mixture depends on the "zigzag" frequency (Gooding & Regnier, 2002).

The extent of sample separation is proportional to the total surface area, therefore, if the stationary phase has a greater surface area, the components of a mixture have more opportunity to interact with the stationary phase. In HPLC, the stationary phase is ground

to a fine powder (to increase surface area) and particles are packed tightly together in a steel column making it more difficult for the solvent to elute through the column. The flow rate is increased by using high pressures to force the solvent through the column (Schiel, Joseph, & Hage, 2010). Both increased surface area and high pressure allows for a more efficient separation of compounds, hence the name *high performance liquid chromatography* (Korecka & Shaw, 2009). HPLC is a highly precise technique capable of separating out different compounds that possess even the slightest differences. Therefore, HPLC may be used to identify the concentration of various compounds in biological samples (e.g., concentration levels of DOX in cardiac tissue).

Several extensive reviews regarding the investigation of anthracyclines with the use of HPLC have been published (Chen, Thoen, & Uckun, 2001; Loadman & Calabrese, 2001; Tjaden & de Bruijn, 1990). However; the analysis, isolation, and purification of biomolecules can be accomplished by a number of different chromatographic modes (DiFrancesco, Griggs, Donnelly, & DiCenzo, 2007; Dodde, et al., 2003; Kummerle, et al., 2003; Li & Huang, 2004; Wall, McMahon, Crown, Clynes, & O'Connor, 2007). Each method is based on interactions between the sample biomolecules and the packing material of the column. The various methods of liquid chromatography are based on separations due to the biomolecule's charge, size, and hydrophobicity during elution through porous packing material. Therefore, it is important to identify the inherent differences between the modalities used for quantification purposes. The primary methods used for quantification of biological samples, including methods specific to DOX, will be reviewed in this section.

Size Exclusion Chromatography

The most commonly used modes are size exclusion chromatography, ion exchange chromatography, affinity chromatography, and adsorption chromatography. Size exclusion chromatography (SEC) is a versatile chromatographic method in which components of a biological sample are separated according to their molecular size with respect to the porous packing material (Barth, Boyes, & Jackson, 1994; Yao & Lenhoff, 2004). Larger molecules elute from the column first due to their inability to penetrate the pores of the packing material (Yao & Lenhoff, 2004), while some smaller molecules can enter into the packing particles thus delaying their elution (Barth, et al., 1994; Stadalius, Ghrist, & Snyder, 1987). SEC is an uncomplicated method for separating biomolecules, because it is not necessary to keep the composition of the mobile phase constant during elution (Barth, et al., 1994; Stulik, Pacakova, & Ticha, 2003). However, in comparison to other methods such as reversed-phase chromatography, this method is limited by its capacity for separation of biomolecules (Neue, 2008; Stulik, et al., 2003).

Affinity Chromatography

Affinity chromatography (AFC) is based on the properties of a molecule's ability to specifically bind to a ligand (Schiel & Hage, 2009). AFC is commonly used in basic science research and pharmaceutical drug development for the purification of chemicals or biomolecules using specific interactions between biological pairings (e.g., enzyme and enzyme substrate) via ligand binding (Ohtsu, et al., 2005). Ligands are used in the column packing material to adsorb specific biomolecules. Upon ligand binding, a conformational change occurs and the elution of the biomolecule is altered. In

comparison to other methods, AFC is an efficient and very selective technique used for separation of large volumes of biomolecules (Neue, 2008; Schiel & Hage, 2009).

Ion Exchange Chromatography

The basis for ion exchange chromatography (IEC) is simple; charged biomolecules will bind to oppositely charged molecules in column packing materials (Jungbauer & Hahn, 2009). Proteins have charged molecules on their surfaces, which change with the pH of the solution (Jungbauer & Hahn, 2009; Yigzaw, Hinckley, Hewig, & Vedantham, 2009). Biomolecules with a greater charge bind more strongly to the packing material regardless of pH, however, weaker charges are easily influenced by slight changes in pH. IEC is a very selective separation technique and has a high binding capacity that can be used on a wide variety of proteins over a short period of time (Jungbauer & Hahn, 2009; Langford, Xu, Yao, Maloney, & Lenhoff, 2007) .

Reversed Phase Chromatography

Reversed phase liquid chromatography (RPLC) is a specific type of adsorption chromatography that allows for separation based on hydrophobic interactions between the biomolecule and the ligand on the chromatographic column (Roses, Subirats, & Bosch, 2009; Wang, Tian, Bi, & Row, 2009). Typically, the RPLC's stationary phase contains a large volume of hydrophobic ligands, while the mobile phase additives, such as acetic acid, increase protein hydrophobicity by forming ion pairs that strongly adsorb to the stationary phase (Nikitas & Pappa-Louisi, 2009; Wang, Tian, et al., 2009). The main use for RPLC is for the separation peptides, however, less frequently, it can also be used to separate proteins (Li & Huang, 2004; Neue, 2008; Wang, et al., 2008). The main draw to RPLC is that the method is the most efficient of all the biopolymer separation techniques

(Sandra, et al., 2008). RPLC is particularly effective for separating chemical compounds in biological samples, which makes RPLC the most coveted mode for the determination of anthracyclines, such as DOX, in plasma, serum, and tissues (Dodde, et al., 2003; Kummerle, et al., 2003; Li & Huang, 2004; Urva, et al., 2009; Wall, et al., 2007).

High Performance Liquid Chromatography and Doxorubicin

Several reverse phase methods for determination of anthracyclines in plasma, serum, or tissues have recently been published (Ahmed, et al., 2009; Maudens, et al., 2009; Salvatorelli, et al., 2009; Urva, et al., 2009). The most recent recommended methods for anthracycline analysis are summarized in Table 2. Urva et al. (2009) reported a less labor intensive method that was successfully used to quantify DOX accumulation in plasma and cardiac samples following administration of an i.v. bolus dose at 10 mg/kg in Swiss–Webster mice. A Zorbax 300SB C_{18} (250 mm × 4.6 mm, 5 μm) column was used for separation. The mobile phase was comprised of 25% acetonitrile and 75% water in the presence of 0.1% triethylamine (pH 3). A constant flow rate of 1.2 mL/min was used for the separation. Plasma and tissue sample treatment involved a single protein precipitation step with perchloric acid. DOX and DAUN retention times were reported to be 5.9 and 13.1 minutes, respectively. The limit of detection for doxorubicin in the heart tissue was: 1.54 ng/mL. The method was validated for precision and accuracy over a concentration range of 5-1000 ng/mL and DOX accumulation was measured up to 3 days post DOX exposure. A significant decline in DOX accumulation in measured parameters reduced quickly over a 12 hour period. However, DOX was still detected in cardiac tissue 72 hours post treatment. The aforementioned study's methodology is most comparable to the method used in the study

by Bradshaw et al. (2009) to quantify DOX in rat cardiac tissue. Bradshaw et al. (2009) reported an HPLC method that successfully determined the accumulation of DOX in LV tissue of sedentary and exercise trained rats. Similarly, other methods have reported the use of HPLC in the determination of anthracyclines (Ahmed, et al., 2009; Arnold, Slack, & Straubinger, 2004; DiFrancesco, et al., 2007; Gilbert, McGeary, Filippich, Norris, & Charles, 2005; Kummerle, et al., 2003; Li & Huang, 2004; Urva, et al., 2009). Specifically, DOX can be determined in various biological matrices (i.e. plasma, serum, effluent, and tissues) using reverse phase HPLC (Kummerle, et al., 2003; Urva, et al., 2009). EPI (Li & Huang, 2004), and doxorubicinol (DOX-ol) (Ahmed, et al., 2009; Arnold, et al., 2004; DiFrancesco, et al., 2007; Gilbert, et al., 2005), a secondary alcohol metabolite of DOX, were also quantified using various HPLC methods. Methods specifics can vary based on the internal standards used for accumulation comparison, sample preparation, column type, and detection. In the following section, various methods' specifics for the quantification of DOX will be reviewed.

Table 2

Recommended methods for quantifying Doxorubicin accumulation.

Reference	Tissue Analyzed	Anthracycline(s)	Column	Detection	Extraction	Separation Time	Excitation	Emission
(Maudens, et al., 2009)	Human Plasma Saliva	Doxorubicin, Epirubicin, Daunorubicin, Idarubicin, Doxorubicinol, Epirubicinol, Daunorubicinol Idarubicinol	Zorbax C-18	Fluorescence	Liquid–liquid	14 min	480 nm	555 nm
(Urva, et al., 2009)	Mouse plasma, kidney, liver, spleen, gut, adipose, muscle, testis, lung, heart and brain	Doxorubicin	Zorbax C-18	Fluorescence	Single protein precipitation	16 min	480 nm	560 nm
(Ahmed, et al., 2009)	Mouse, plasma, tumor, heart, spleen, liver, gastrointestinal tract, brain, lung and kidney	Doxorubicin	Luna C-8	Chem-iluminescence	Single protein precipitation (acetone/ ZnSO4)	14 min	482 nm	550 nm
(Salvatorelli, et al., 2009)	Human myocardial strips, plasma	Doxorubicin, Doxorubicinol, Epirubicin, Epirubicinol, Doxorubicinone, Doxorubicinolon	Macrosphere C-18	Fluorescence	Single protein precipitation	15 min	477 nm	560 nm
(Bradshaw, et al., 2009)	Rat Heart	Doxorubicin	Zorbax C-18	Fluorescence	Single protein precipitation (acetone/ ZnSO4)	20 min	470 nm	550 nm

Note: Abbreviations: HPLC- High performance liquid chromatography; C- Carbon; min- minutes.

Internal Standards

An internal standard is a known concentration of a known compound. It differs from an analyte, in which the concentration of the compound is unknown. Typically, the signal from an analyte is compared to the internal standard's signal in order to calculate the concentration of the analyte. DAUN is the most commonly used internal standard for the quantification of DOX (DiFrancesco, et al., 2007; Kummerle, et al., 2003; Urva, et al., 2009). However, with success, epidaunorubicin (EPIDAUN) was reportedly used as an internal standard (Li & Huang, 2004). In a study by Bradshaw et al. (2009), DAUN was used as an internal standard for the quantification of DOX accumulation in rat cardiac tissue and eluted approximately 2 minutes after the elution of DOX.

Sample Preparation

In order for a biological sample to be analyzed using HPLC, it must first be prepared for separation. With samples containing DOX, a purification step must be completed. The most commonly used processes include deproteinization (Ahmed, et al., 2009; Arnold, et al., 2004; Kummerle, et al., 2003; Urva, et al., 2009), liquid-liquid extraction (Maudens, et al., 2009), solid phase extraction (DiFrancesco, et al., 2007; Li & Huang, 2004), and back extraction (Loadman & Calabrese, 2001). Deproteinization (a process of protein removal from a substance) occurs with the use of acids and it is an essential step in the HPLC analysis of DOX. Acids used for deproteinization in previous studies included perchloric acid, trichloroacetic acid and picric acid for protein precipitation (Ahmed, et al., 2009; Bradshaw, et al., 2009; Kummerle, et al., 2003; Urva, et al., 2009). Solid phase extraction (process by which compounds are dissolved or suspended in a liquid) was successfully used to purify samples for the analysis of both

DOX (DiFrancesco, et al., 2007) and EPI (Li & Huang, 2004). A very time intensive technique was used by Gilbert et al. (2005) to purify plasma samples from parrots in which deproteinization was followed by a liquid–liquid extraction and then a back-extraction (a sequential extraction of organic material) for the analysis of DOX and DOX-ol accumulation. Based on the reports from the studies mentioned afore, it may be concluded that the purification of DOX, in biological samples, is not limited to technique.

Separation Column and Detection

The specificity of HPLC analyses depends largely on the separation column and the detection method. For adequate separation and elution, the column must incorporate the analyte molecule size. Many studies have used C-18 columns (18-carbon chain packing material) (Arnold, et al., 2004; Bradshaw, et al., 2009; DiFrancesco, et al., 2007; Kummerle, et al., 2003; Li & Huang, 2004; Urva, et al., 2009) and reported high accuracy and precision when quantifying DOX, however, only two studies reported the use of a less hydrophobic column, C-8 phenylhexyl (column containing a shorter carbon chain packing material) (Ahmed, et al., 2009; Gilbert, et al., 2005) to quantify DOX. Results from these studies suggest that DOX, due to its size, may be more easily eluted through a C-18 column with greater separation than a C-8 column. Following the elution of DOX through the column, it must be detected. Fluorescence (Bradshaw, et al., 2009; Gilbert, et al., 2005; Kummerle, et al., 2003; Urva, et al., 2009), chemiluminescence (Ahmed, et al., 2009), and tandem-mass spectrometry (Arnold, et al., 2004; DiFrancesco, et al., 2007; Li & Huang, 2004) are the most commonly used methods in the detection of DOX in biological samples. Thus, the detection of DOX is not limited to one type of detector; however, some reports suggest that tandem-mass spectrometry may have a greater

specificity over fluorescence detectors (Freisleben, Schieberle, & Rychlik, 2003; Sai, Kaniwa, Ozawa, & Sawada, 2002).

Summary

DOX is an anthracycline antibiotic that has cytotoxic actions. The therapeutic use of DOX to treat a wide array of cancers is limited by a dose-dependent cardiotoxicity. DOX is known to have several adverse side-effects, however, acute and chronic cardiotoxicity have received the most attention as both may eventually lead to heart failure. It is well accepted that cardiotoxicity of DOX is associated with generation of oxidative stress and an interference with mitochondrial function. While exercise has been shown to be cardioprotective against DOX cardiotoxicity, a clear and consistent mechanism to explain its cardioprotective effects is lacking. HPLC is a valuable instrument that can be used to evaluate cardiac DOX accumulation. It was hypothesized that exercise will reduce the accumulation of DOX in cardiac tissue, which may contribute to the preservation of cardiac function and protect the heart against the acute injury induced by DOX treatment. Therefore, it is possible that the cardioprotective effect of chronic exercise preconditioning may be due to a reduction of DOX accumulation in the cardiac tissue of rats receiving DOX treatment.

CHAPTER III

METHODOLOGY

Experimental Design

The purpose of this study was to determine the effects of chronic exercise preconditioning on DOX accumulation and cardiac function. This was achieved by training rats using two different exercise protocols prior to DOX exposure (Figure 1). DOX was administered 24 hours after the last training session as a bolus i.p. injection in doses that are known to result in acute cardiac injury. The cumulative dose of DOX was 10 mg/kg. Control animals received a bolus i.p. injection of 0.9% saline. Rats were sacrificed 1, 3, 5, 7, and 9 days post exposure and cardiac function was analyzed. One day following sacrifice, DOX accumulation was analyzed via HPLC.

Subjects

Female Sprague-Dawley rats (10-11 week old, 190-210 g) were housed two per cage in a temperature-controlled facility with a 12:12-hr light-dark cycle. Rats were provided standard rat chow and water *ad libitum*. All protocols used for the study 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. All rats were randomly assigned to sedentary (SED), treadmill (TM), or wheel running (WR) exercise groups.

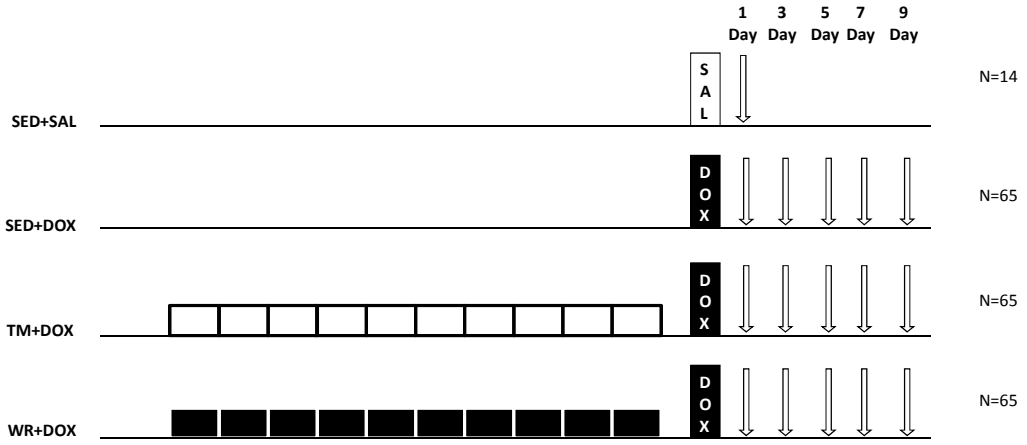

Figure 1. Exercise Timeline and Experimental Design. Each open horizontal rectangle (▭) represents 1 week of involuntary treadmill running exercise. Each closed horizontal rectangle (▬) represents 1 week of voluntary wheel running exercise. Open arrows (⇩) represent sacrifice times. SED = sedentary TM = 10 weeks of treadmill exercise; WR = 10 weeks of wheel running exercise; SAL = saline injection; DOX = doxorubicin injection.

Exercise Training

Animals randomly assigned to TM groups participated in a progressive treadmill training protocol for ten consecutive weeks. Animals assigned to participate in 10 consecutive weeks of TM exercise followed a progressive training protocol that has previously been shown to be cardioprotective against DOX-induced cardiotoxicity (Wonders, et al., 2009) (Table 3). Animals trained five days per week for 10 weeks during their dark cycle. Rats initially ran at 13 m/min up a 5% grade for 20 minutes per day. Exercise intensity and duration gradually increased during weeks 1-4, until reaching 30 m/min and 18% grade for 60 minutes. This workload was maintained for the remainder of the study. When necessary, rats were motivated to run by manual prodding and tapping on the equipment. Animals selected to participate in voluntary wheel running exercise were housed one per cage and had free access to commercially available running wheels (MiniMitter, Bend, OR) 24 hours per day for 10 consecutive weeks.

Wheel running distances were monitored using Vital View data acquisition systems (MiniMitter, Bend, OR).

Table 3

Chronic exercise treadmill training protocol.

Variable	Week									
	1	2	3	4	5	6	7	8	9	10
Speed (m/min)	13	21	26	30	30	30	30	30	30	30
Grade (%)	5	10	15	18	18	18	18	18	18	18
Duration (min)	20	30	30	60	60	60	60	60	60	60

Drug Treatment

After the completion of the sedentary or activity period, all animals remained sedentary 24 hours prior to treatment with DOX. Following the 24 hour sedentary period, each animal was randomly assigned to one of four experimental groups as illustrated in Figure 1: SED+SAL, SED+DOX, TM+DOX, WR+DOX. Animals undergoing saline treatment received a 1.0 mL i.p. bolus injection of 0.9% saline. Animals treated with DOX received a 10 mg/kg bolus injection of DOX (Bedford Labs, Bedford, OH). Subgroups of rats from each primary group were sacrificed at 1, 3, 5, 7, and 9 days following injections.

Cardiac Function

Left ventricle (LV) function was assessed *in vivo* and *ex vivo*. *In vivo* cardiac function was assessed using transthoracic 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. Animals were placed in the left lateral decubitus position and the probe was positioned to obtain short-axis, long-axis, and four-chamber views. 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 using pulsed wave Doppler, with the smallest possible sample volume placed at the level of the aortic annulus. Using a four-chamber apical view, mitral flow was assessed using pulsed wave Doppler with the smallest possible sample volume placed at the tips of the mitral valve. LV mass was calculated as $1.04[(LVDd + PWd + SWd)^3 – LVDd^3]$, and fractional shortening (FS) was calculated as (LVDd – LVDs)/LVDd.

From pulsed Doppler mitral and aortic flow images, the velocity time integral (VTI), maximal flow velocity (V_{max}), mean flow velocity (V_{mean}), acceleration time (AT), and deceleration time (DT) were measured. Measures of filling time (FT) and ejection time (ET) were obtained from mitral and aortic Doppler flow images, respectively. For all measures, data from three consecutive cardiac cycles, when possible, was obtained and averaged.

Ex vivo cardiac function was analyzed using an isolated working heart model (ADInstruments, Colorado Springs, CO). This analysis was conducted immediately following echocardiography procedures. After each animal received an i.p. injection of heparinized (100U) sodium pentobarbital (50 mg/kg), the heart was quickly excised. The aorta was cannulated and the heart was subjected to retrograde perfusion using Krebs buffer (120 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 17 mM glucose, and 0.5 mM EDTA) until the coronary vasculature was cleared of blood. The pulmonary vein was cannulated and flow was re-directed to enter the left atrium.

Preload was set at 10 cm H_2O and afterload set at 100 cm H_2O above the cannula. A microtip catheter pressure transducer (Scisense, Ontario, Canada) was inserted into the LV cavity via the apex for measurement of LVDP, maximal rate of pressure development (dP/dt_{max}) and the maximal rate of pressure decline (dP/dt_{min}). Hearts were paced at 240 beats/min using electrodes attached to the cannulae, and LV performance data were collected using a PowerLab/8e data acquisition system (ADInstruments, Colorado Springs, CO) following a 5 minute equilibration period. LVDP was calculated by subtracting left ventricular ESP from EDP.

Biochemical Analysis

High Performance Liquid Chromatography

Left Ventricular Tissue Preparation. Immediately after the perfusion period, hearts were trimmed free of surrounding connective tissue and fat, blotted dry, and weighed. The LV was then isolated, flash frozen in liquid nitrogen, and stored at –80°C for 24 hours. Tissue samples were then subjected to a drug extraction procedure taken from previously validated methods (Alvarez-Cedron, Sayalero, & Lanao, 1999; Bradshaw, et al., 2009). Approximately fifty milligrams of LV tissue was diluted with a 0.067 M phosphate buffer (pH 7.4) and homogenized at 8000 RPM for 20 seconds using a Virtishear homogenizer (Virtis, Gardner, NJ). The concentration of heart tissue was approximately 25 mg/mL. Homogenates were then subjected to protein precipitation by adding 200 µL of a 50:50 (v/v) mixture of HPLC grade methanol and 40% $ZnSO_4$ to 150 µL of homogenized heart tissue. Fifty microliters of DAUN (Sigma, St. Louis, MO) at an initial concentration of 500 ng/mL was added to the sample as an internal standard. The sample was vigorously vortexed for one minute before centrifugation at 1500*g* for 10

minutes. The supernatant fluid was filtered through a 0.2 micron syringe filter and injected directly onto the column to initiate the analytical method.

Analytical Method

The HPLC system consisted of two LC-10AT LC pumps for high-pressure gradient elution (Shimadzu Co., Japan). A reverse-phase Zorbax Rx-C8 4.6mm x 15 cm column (Agilent Technologies, Santa Clara, CA) was used for separation and operated at 40°C. The following mobile phase (solvent) line configuration was held for all analyzes: solvent line A - HPLC-grade water, solvent line B - HPLC-grade acetonitrile, solvent line C - phosphate buffer, and solvent line D - HPLC-grade methanol. At the end of each day's HPLC analyses, solvent line C was purged of phosphate buffer and stored in water and solvent line B was purged of acetonitrile and stored in HPLC-grade methanol.

The initial phase conditioned the system in the following manner: 65:35 (v/v) methanol/water at 0.1 mL/min flow rate through the column over-night and through the next morning by the conditioning method displayed in Table 4. Figure 2 illustrates the mobile phase composition profile used for the conditioning method.

Table 4

HPLC mobile phase composition and pump parameters used for the system conditioning program (conditioning method). The pump mobile phase program configuration was: solvent line A - HPLC-grade water, solvent line B - HPLC-grade water, solvent line C - HPLC-grade methanol, and solvent line D - HPLC-grade methanol. The initial mobile phase composition was 65:35 (v/v) methanol (from solvent line D)/water (from solvent line A) at a 0.1mL/min flow rate. Conc. = concentration of designated solvent.

Time	Module	Action	Value
0.01	Pumps	Total Pump A Flow	1.00 mL/min
30.00	Pumps	Solvent D Conc.	100.0%
180.00	Pumps	Solvent D Conc.	100.0%
240.00	Pumps	Solvent D Conc.	10.0%
260.00	Pumps	Solvent D Conc.	10.0%
360.00	Pumps	Solvent D Conc.	65.0%
400.00	Controller	Stop	

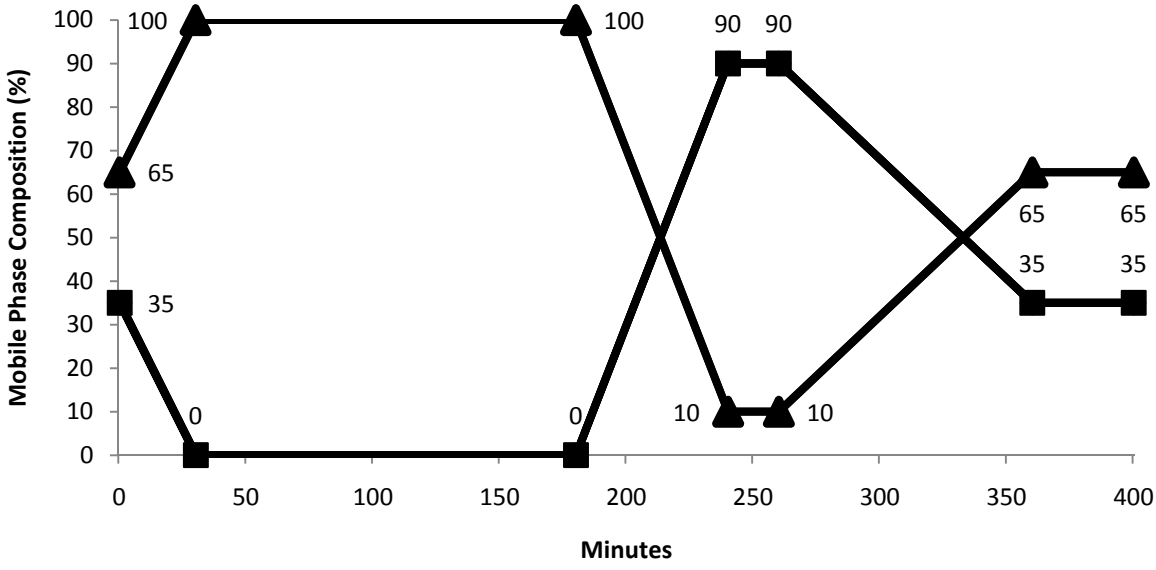

Figure 2. Mobile phase composition profile for the startup conditioning method (▲- methanol, ■ - water). For this method, solvent lines B and C were held at 0% for the duration of the run. The initial mobile phase composition was 65:35 (v/v) methanol/water (from solvent line A). After the 400 minute run, the system returned to the initial mobile phase composition.

The system was then stored in 65:35 (v/v) methanol/water until use. Before any analysis, the system was conditioned by pumping 65:35 methanol/water, flowing at 0.1

mL/min through the column overnight. To prepare the system for analyses, the analysis setup method parameters were set as displayed in Table 5. Figure 3 illustrates the mobile phase composition profile used for the setup method. Just prior to the end of the 60 minute run, the pumps were shut off, the setup method was stopped, and the run method described in Table 6 was initiated. Therefore, the system was allowed to equilibrate for at least 60 minutes with a mobile phase composition of (A) 0%, (B) 25%, (C) 75%, and (D) 0% flowing at 1.0 mL/min before the analysis were performed. Before each use, the mobile phase solvents (water, phosphate buffer, and methanol) were passed through a MAGNA-R® nylon-supported plain 0.22 µm filter using a vacuum filtration apparatus and were degassed by holding the filtered solvent under a vacuum for at least 20 minutes. Acetonitrile was passed through a Teflon-laminated 0.22 µm filter using a vacuum filtration apparatus and was degassed by holding the filtered solvent under vacuum for at least 20 minutes.

Table 5

HPLC mobile phase composition and pump parameters used for the system analysis setup program (setup method). The pump mobile phase configuration was: solvent line A - HPLC-grade water, solvent line B - HPLC- acetonitrile, solvent line C - phosphate buffer, and solvent line D - HPLC-grade methanol. The initial mobile phase composition was 65:35 (v/v) methanol (from solvent line D)/water (from solvent line A) at a 0.1mL/min flow rate. Conc. = concentration of designated solvent.

Time	Module	Action	Value
0.01	Pumps	Total Pump A Flow	1.00 mL/min
45.00	Pumps	Solvent C Conc.	75.0%
45.00	Pumps	Solvent B Conc.	25.0%
60.00	Controller	Stop	

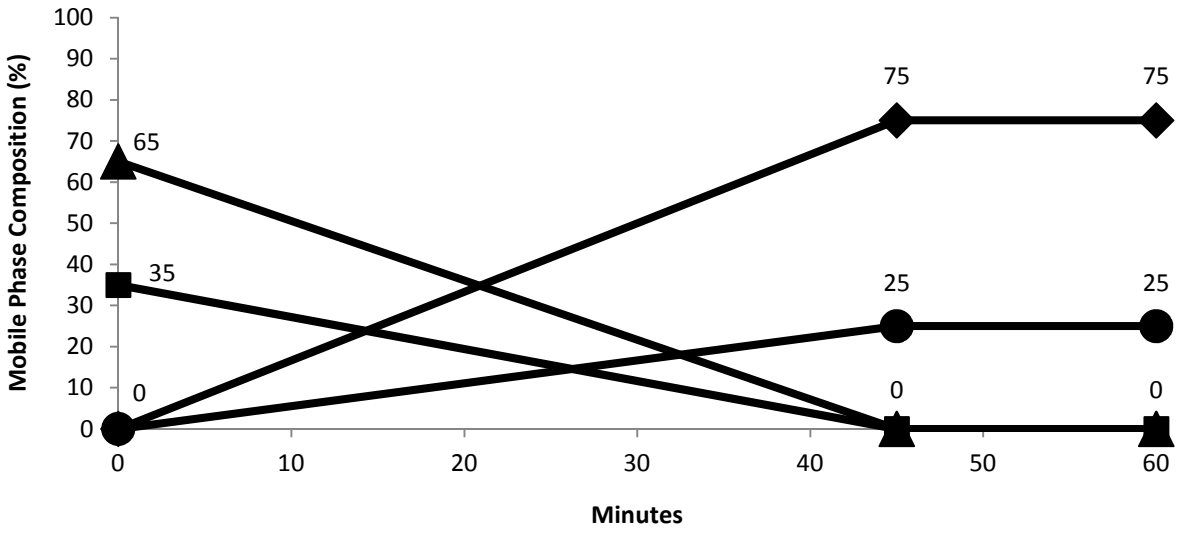

Figure 3. Mobile phase composition profile for the setup method (▲- methanol, ■ - water, ♦ - phosphate buffer, ● - acetonitrile). For this method the initial mobile phase composition was 65:35 (v/v) methanol (from solvent line D)/water (from solvent line A). The final mobile phase composition was (A) 0%, (B) 25%, (C) 75%, and (D) 0% flowing at 1.0 mL/min.

The system was prepared for sample analysis by initiating the run method given in Table 6. Figure 4 illustrates the mobile phase composition profile used for the run method. The following mobile phase solvent configuration was held for the analyses: solvent line A - HPLC-grade water, solvent line B - HPLC-grade acetonitrile, solvent line C - phosphate buffer, and solvent line D - HPLC-grade methanol. The system initial composition of (A) 0%, (B) 25%, (C) 75%, and (D) 0% flowing at 1.0 mL/min was held for four minutes, a linear gradient was then used to change the mobile phase to phosphate:acetonitrile (50:50) over the next four minutes (8 minute mark).

Table 6

HPLC mobile phase composition and pump parameters used for the system analysis (run method). The pump mobile phase configuration was: solvent line A - HPLC-grade water, solvent line B - HPLC- acetonitrile, solvent line C - phosphate buffer, and solvent line D - HPLC-grade methanol. The initial solvent composition was (A) 0%, (B) 25%, (C) 75%, and (D) 0% flowing at 1.0 mL/min. Conc. = concentration of designated solvent.

Time	Module	Action	Value
0.01	Pumps	Total Pump A Flow	1.00 mL/min
4.00	Pumps	Solvent C Conc.	75.0%
4.00	Pumps	Solvent B Conc.	25.0%
14.00	Pumps	Solvent C Conc.	50.0%
14.00	Pumps	Solvent B Conc.	50.0%
16.00	Pumps	Solvent C Conc.	5.0%
16.00	Pumps	Solvent B Conc.	95.0%
18.00	Pumps	Solvent C Conc.	75.0%
18.00	Pumps	Solvent B Conc.	25.0%
20.00	Controller	Stop	

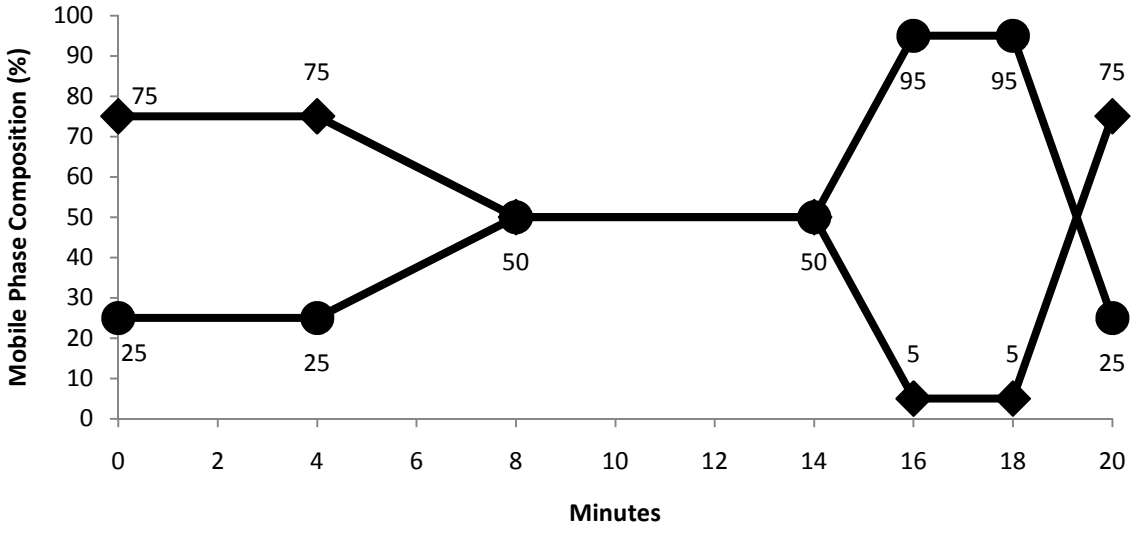

Figure 4. Mobile phase composition profile used for the run method (♦ - phosphate buffer, ● - acetonitrile). For this method the initial mobile phase composition was (A) 0%, (B) 25%, (C) 75%, and (D) 0% flowing at 1.0 mL/min.

The secondary mobile phase was maintained ten minutes into the run (14 minute mark), at which time a linear gradient was used to change the mobile phase of phosphate:acetonitrile from (50:50) to (5:95) over the course of the next two minutes (16 minute mark). This phase was maintained for two minutes (18 minute mark) and then the mobile phase returned via a linear gradient to the initial conditions (phosphate:acetonitrile (75:25), 20 minute mark). The photo diode-array detector absorbance wavelength range was set to 328-342 nm, all other settings were set to program defaults. The fluorescence detector SPD-10Avp UV (Shimadzu Co., Japan) used for excitation/emission wavelengths was maintained at 470/550 nm, the gain was raised to 16X, and all other settings were set to program defaults. The detector lamps were turned on a minimum of one hour prior to analysis to allow for proper stabilization. Data analysis was performed using Shimadzu CLASS-VP 5.0 data analysis software (Shimadzu Co., Japan). Quantification of DOX in LV samples was determined from the peak area of each component relative to calibration standard curve displayed in Figure 5.

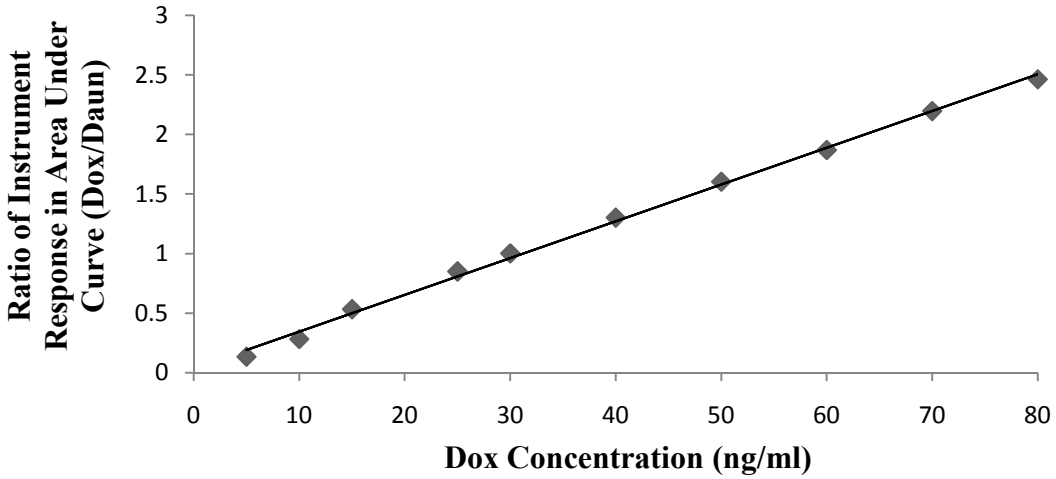

Figure 5. Doxorubicin standard curve with daunorubicin. The y-intercept equals 0.309x + 0.344 and the $R^2 = 0.9974$.

Preparations of Solutions

HPLC mobile phase stock phosphate buffer **(**100 mM (0.1 M) NaH_2PO_4**).** To make a total volume of 0.5 L, 6.9 g of solid sodium phosphate monobasic (NaH_2PO_4) was dissolved in approximately 400 mL of HPLC-grade water. The solution was then placed in a 500 mL volumetric flask and diluted to volume with HPLC-grade water. The buffer solution was passed through a 0.22 nylon filter and stored in an amber bottle at 4 °C.

Running phosphate buffer (10 mM (0.01 M) NaH_2PO_4). To make a total volume of 1.0 L, 100.0 mL of stock phosphate buffer (0.1 M NaH_2PO_4) was diluted in approximately 600 mL of HPLC-grade water and the pH was adjusted to 3.0 using 0.01 M phosphoric acid (0.01 M phosphoric acid was made by adding 0.17 mL of 14.7 M phosphoric acid to 250.0 mL of HPLC grade water). The solution was then placed in a 1.0 L volumetric flask and diluted to volume with HPLC-grade water. The buffer solution was passed through a 0.22 nylon filter and stored in a clear bottle at 4 °C.

LV tissue-preparation phosphate buffer (0.067 M NaH_2PO_4 at pH 7.4). To make a total volume of 0.2 L, 134.0 mL of stock phosphate buffer (0.1 M NaH_2PO_4) was diluted in approximately 150 mL of HPLC-grade water and the pH will be adjusted to 7.4 using 5 M NaOH. The solution was then placed in a 200 mL volumetric flask and diluted to volume with HPLC-grade water. The buffer solution was passed through a 0.22 nylon filter and stored in a clear bottle at 4 °C.

Statistical Analysis

Group data are reported as mean (M) ± standard error of mean (SEM). To determine significant differences in *ex vivo* and *in vivo* cardiac function, a three-way (drug X exercise X time) multiple analysis of variance (MANOVA) was used. The

dependent variables of *in vivo* function are M-VTI, M-V_{max}, M-V_{mean}, A-VTI, A-V_{max}, A-V_{mean}, and FS. The ESP, EDP, LVDP, dP/dt_{max}, and dP/dt_{min} were used as dependent variables of *ex vivo* function. To determine significant differences in DOX accumulation, a two-way (exercise X time) analysis of variance (ANOVA) was used. Individual means of all DOX groups were then compared to the SED+SAL control mean using independent sample t-tests. A significant level of $p < 0.05$ was used for all statistical analyses. Statistical Analysis System (SAS; SAS Institute Inc., Cary, North Carolina) software was used to analyze all data.

CHAPTER IV

RESULTS

Introduction

The purpose of this study was to determine the effects of exercise preconditioning on cardiac DOX accumulation and to what extent it is related to DOX-mediated cardiac dysfunction. In addition, an HPLC based method was used for a time-course determination of DOX clearance in rat left ventricular tissue.

General Observations

Nineteen animals died during the experiment (SED+DOX, n = 6; TM+DOX, n = 7; WR+DOX, n = 7). Therefore, the mortality rate in the SED+DOX group was approximately 9% (6 of 67) and the mortality rate was approximately 11% in both the TM+DOX and WR+DOX groups (7 of 65). Running distances for WR+DOX groups were recorded weekly (Figure 6) and peaked at 49718 ± 17925 m during week 5. Using the previously described HPLC method, mean DOX retention time for all samples was 6.47 minutes and mean DAUN retention time was 8.78 minutes. Typical chromatograms of sedentary and exercise groups 1 day post DOX exposure are shown in Figure 7. Figure 8 displays typical chromatograms of sedentary and exercise groups 7 days following DOX exposure.

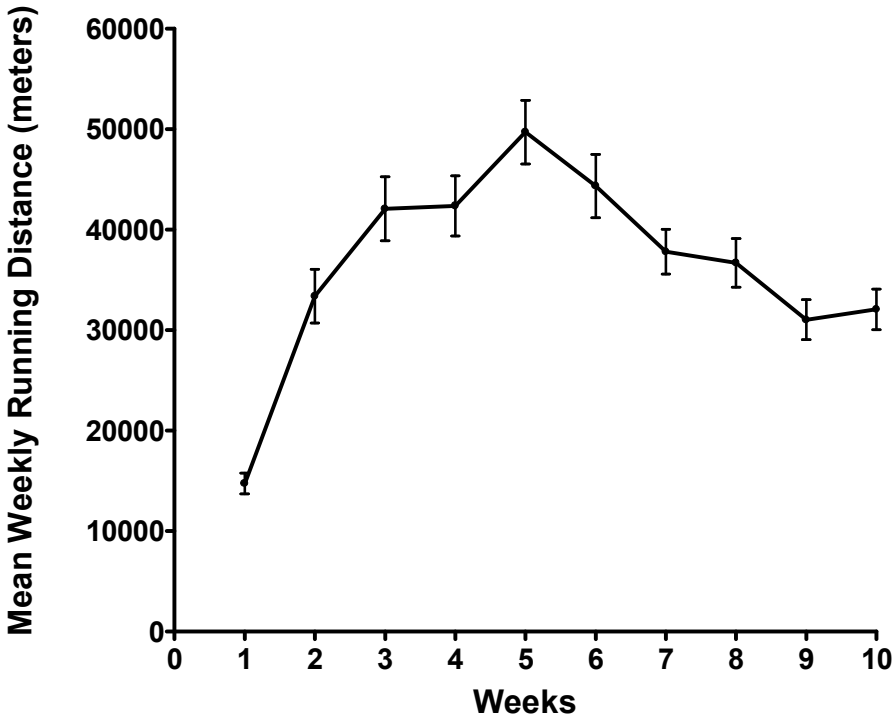

Figure 6. Weekly running distance for WR+DOX groups

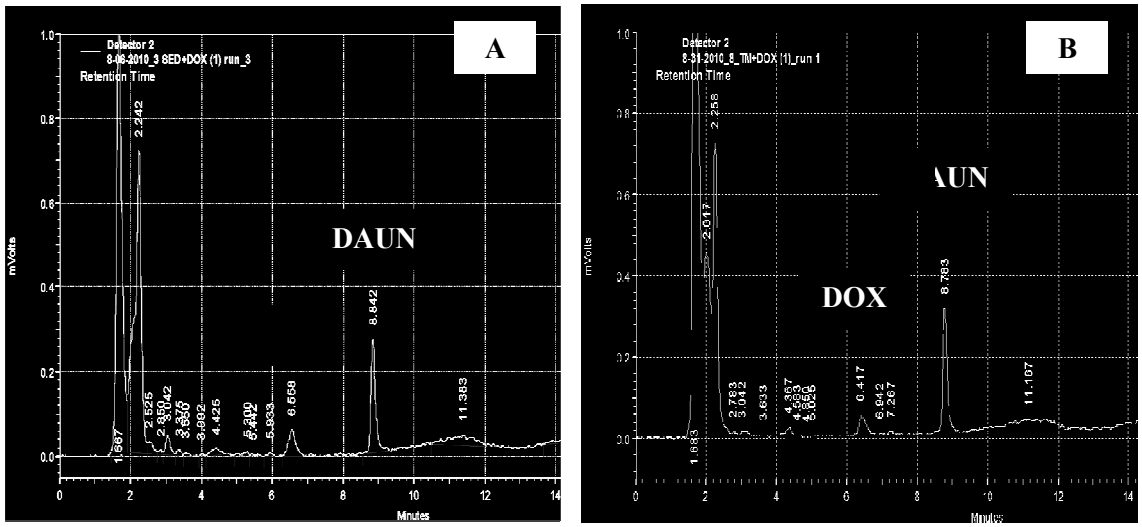

Figure 7. Chromatogram of SED+DOX 1 day post (A) and TM+DOX 1 day post (B) DOX exposure analyzed by the proposed HPLC method

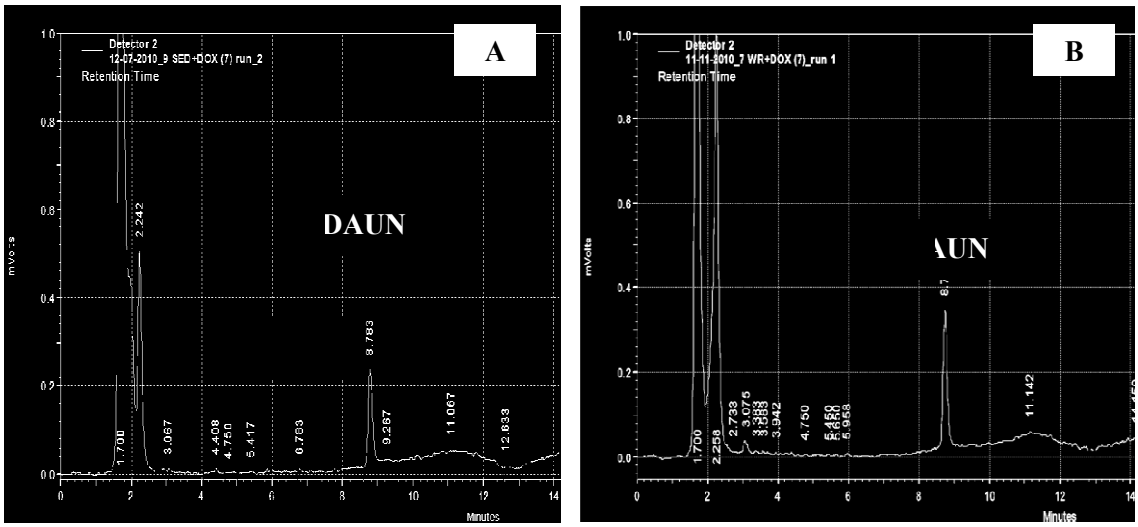

Figure 8. Chromatogram of SED+DOX 7 day (A) and WR+DOX 7 day (B) post DOX exposure analyzed by the proposed HPLC method

Cardiac Function

In Vivo Cardiac Function

The selected *in vivo* variables M-VTI, M-V_{max}, M-V_{mean}, A-VTI, A-V_{max}, A-V_{mean} and FS are displayed in Figures 9-15. The main effect of DOX treatment on *in vivo* cardiac function was significantly different across all groups, F (9, 110) = 2.81, p = 0.0052. A post hoc test was used to determine which group's *in vivo* function was different. A significant difference for *in vivo* function was observed between SED+SAL and SED+DOX, F (9, 110) = 2.81, $p < 0.0052$. Likewise, a significant difference for *in vivo* function was observed between SED+SAL and TM+DOX, F (9, 110) = 13.03, $p <$ 0.0001 and between SED+SAL and WR+DOX, F (9, 110) = 9.09, $p < 0.0001$.

For *in vivo* cardiac function there was a significant exercise effect, F (18, 220) = 6.46, p = 0.0001. However, post hoc testing revealed no significant differences existed between SED+DOX and TM+DOX, SED+DOX and WR+DOX, or TM+DOX and WR+DOX, $p > 0.05$. Additionally, a significant effect of time following DOX treatment on *in vivo* cardiac function was observed for all groups F (9, 110) = 3.14, p = 0.0021.

Doppler blood flow measurements were obtained from the mitral valve to assess the LV diastolic functional changes for all experimental groups and individual t-tests for *in vivo* variables were performed for comparisons to SED+SAL. DOX treatment significantly impaired M-VTI in SED+DOX groups at 3, 5, 7 and 9 days when compared to SED+SAL (Figure 9, $p < 0.05$). However, the impairment was less obvious in TM+DOX and WR+DOX groups. When compared to SED+SAL, M-VTI was only reduced 5 days post DOX treatment in WR+DOX while TM+DOX groups were impaired at 7 and 9 days post exposure. Similar to M-VTI, mitral maximal and mean blood flow

velocities were significantly reduced in the SED+DOX groups when compared to SED+SAL (Figures 10-11, $p < 0.05$); however, M-V_{max} and M-V_{mean} in TM+DOX and WR+DOX groups were not statistically different from the SED+SAL group at any time point ($p > 0.05$).

Doppler measurements obtained at the aortic valve revealed that DOX treatment significantly impaired A-VTI in SED+DOX groups at 3 and 7 days when compared to SED+SAL (Figure 12, $p < 0.05$). With the exception of TM+DOX 1 day post DOX treatment, A-VTI in TM+DOX and WR+DOX groups was not significantly reduced when compared to SED+SAL ($p > 0.05$). Aortic blood flow velocities (A-V_{max} and A-V_{mean}) were significantly reduced in the SED+DOX group when compared to SED+SAL (Figures 13-14, $p < 0.05$). However, TM+DOX and WR+DOX groups were not statistically different from the SED+SAL group at any time ($p > 0.05$). In addition to impaired Doppler measurements, DOX significantly impaired FS in the sedentary groups. A significant reduction in FS was observed in SED+DOX at all time points when compared to SED+SAL (Figure 15, $p < 0.05$), while FS was not significantly different than SED+SAL at any time point for TM+DOX or WR+DOX.

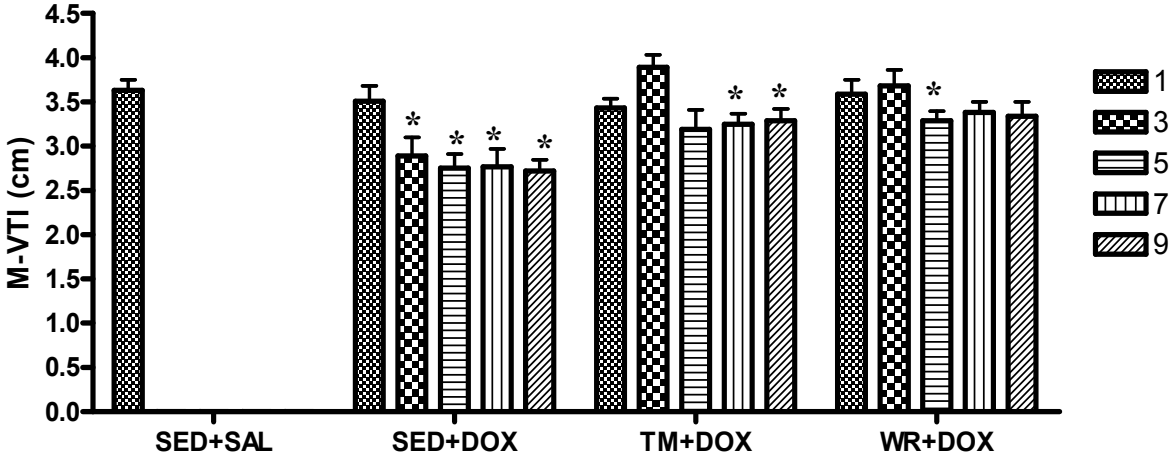

Figure 9. In vivo mitral velocity time integral
* significant difference from SED+SAL

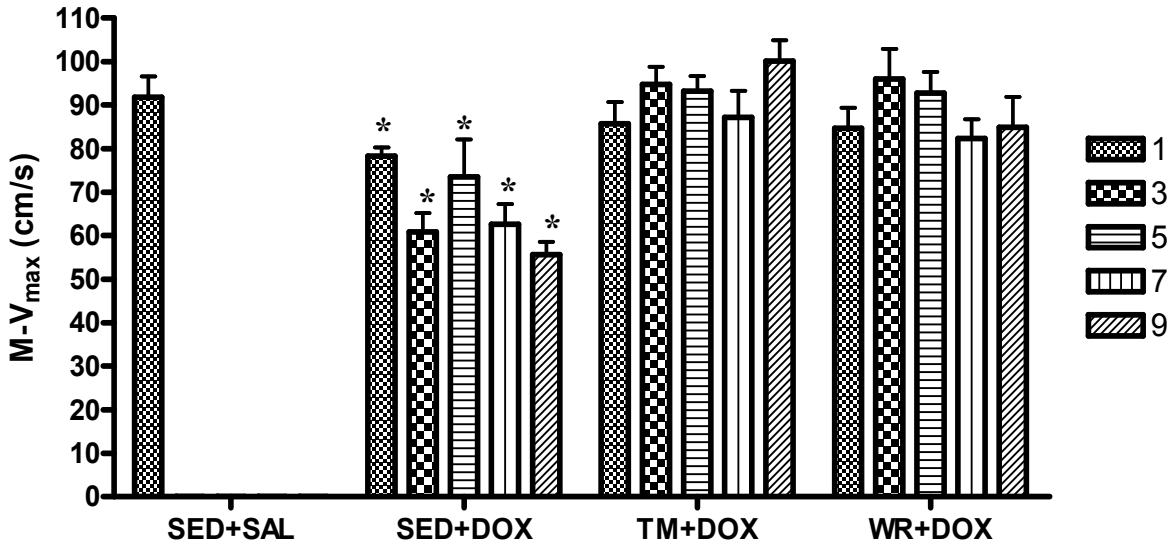

Figure 10. In vivo mitral maximal flow velocity
* significant difference from SED+SAL

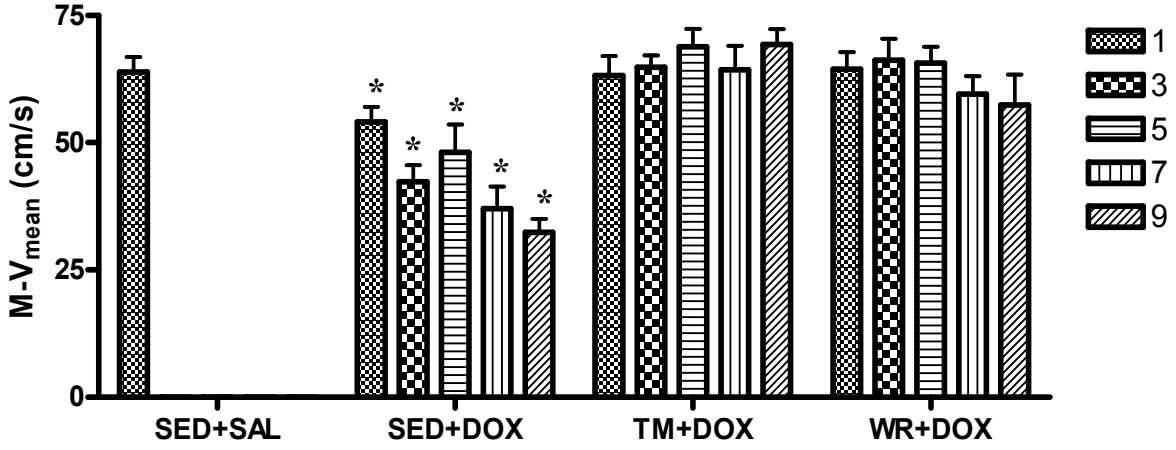

Figure 11. In vivo mitral mean flow velocity
* significant difference from SED+SAL

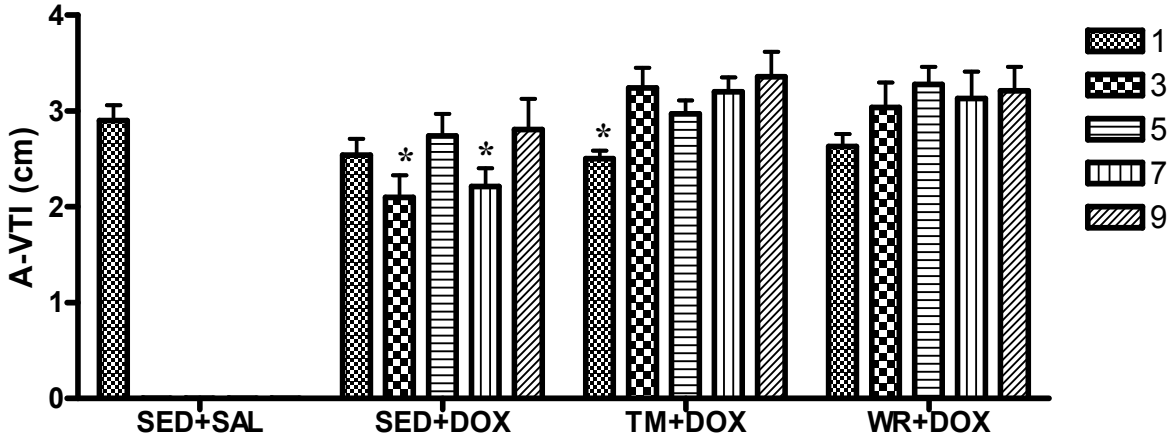

Figure 12. In vivo aortic velocity time integral
* significant difference from SED+SAL

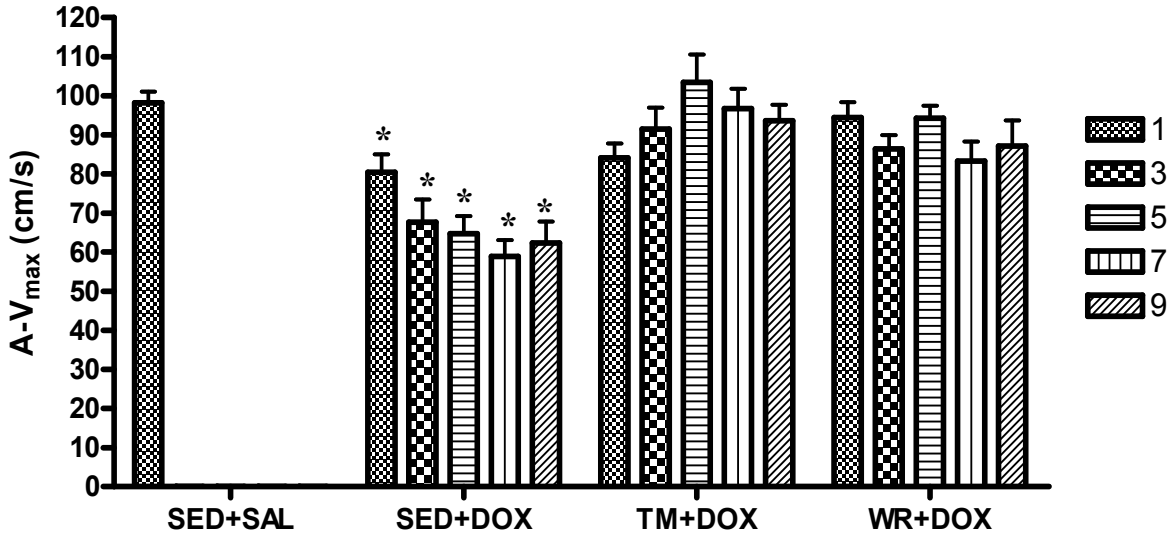

Figure 13. In vivo aortic maximal flow velocity
* significant difference from SED+SAL

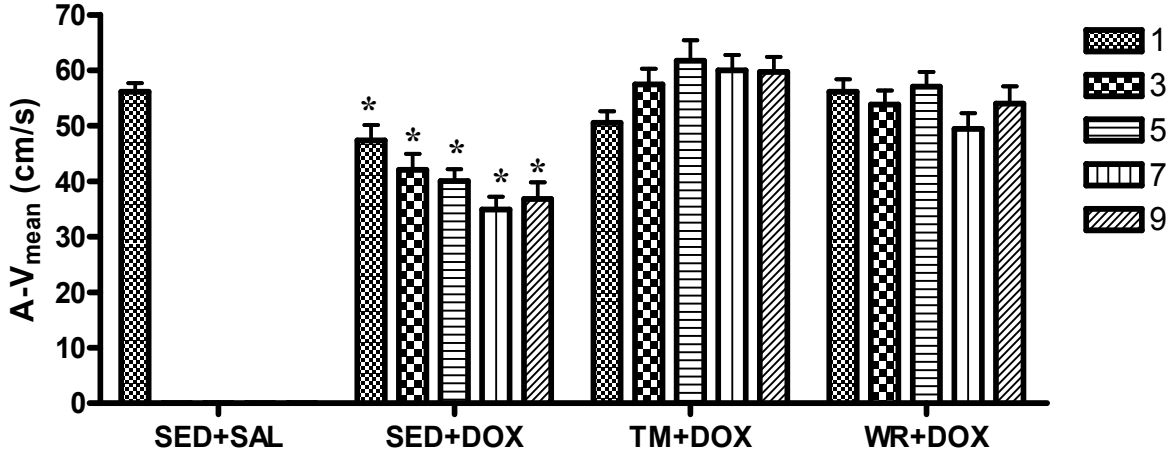

Figure 14. In vivo aortic mean flow velocity
* significant difference from SED+SAL

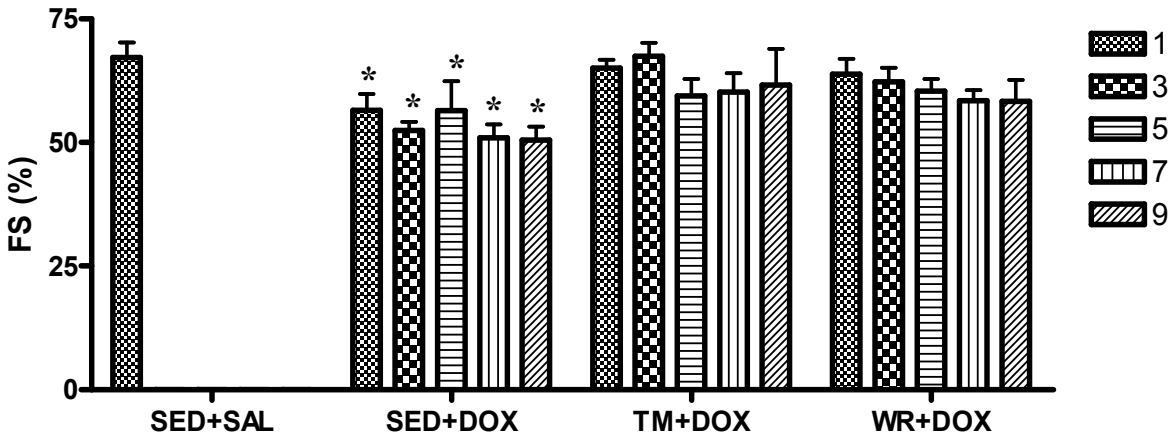

Figure 15. In vivo fractional shortening
* significant difference from SED+SAL

Ex Vivo Cardiac Function

Following echocardiographic measurements, LV function was analyzed using an isolated working heart apparatus. This model allows for *ex vivo* LV function analysis without intrinsic influence from the nervous and endocrine systems. The selected *ex vivo* variables ESP, EDP, LVDP, dP/dt_{max} and dP/dt_{min} are displayed in Figures 16-20 (unpaced) and Figures 21-25 (paced). The main effect of DOX treatment on *ex vivo* cardiac function was significantly different across all groups, F (30, 479.11) = 2.68, $p <$ 0.0001. A post hoc test was used to determine which group's *ex vivo* function was different. A significant difference for *ex vivo* function was observed between SED+SAL and SED+DOX, F (10, 163) = 2.17, p = 0.0222. Additionally, a significant difference for *ex vivo* function was observed between SED+SAL and TM+DOX, F (10, 163) = 4.77, p < 0.0001 and between SED+SAL and WR+DOX, F (10, 163) = 3.35, p = 0.0005.

For *ex vivo* cardiac function there was a significant exercise effect, F (20, 326) = 3.06, $p <$ 0.0001. Post hoc testing revealed a significant difference existed between

SED+DOX and TM+DOX groups, F (10, 163) = 2.23, p = 0.0186. However, no significant differences were observed between SED+DOX and WR+DOX groups, F (10, 163) = 1.66, p = 0.0944; or TM+DOX and WR+DOX groups, F (10, 163) = 1.25, p = 0.2625. Additionally, a significant effect of time following DOX treatment on *ex vivo* cardiac function was observed for all groups F (10, 163) = 11.45, $p < 0.0001$.

Unpaced Data

Unpaced pressure measurements were obtained to assess the LV functional changes for all experimental groups and individual t-tests for *ex vivo* variables were performed for comparisons to SED+SAL. DOX treatment alone significantly impaired ESP in SED+DOX groups at 5, 7 and 9 days when compared to SED+SAL (Figure 16, $p < 0.05$). However, the decrement was less pronounced in TM+DOX and WR+DOX groups. When compared to SED+SAL, ESP was significantly higher 1 day post DOX treatment ($p < 0.05$). The reduction at days 7 and 9 in TM+DOX and day 9 in the WR+DOX was significant when compared to SED+SAL (Figure 16, $p < 0.05$). Interestingly, EDP was not significantly different between SED+SAL and SED+DOX (Figure 17, $p > 0.05$). However, when compared to SED+SAL, EDP was significantly lower at day 1 in TM+DOX and significantly higher at day 3, while WR+DOX was significantly higher 1 day post DOX treatment.

Similar to ESP, DOX treatment alone significantly impaired LVDP in SED+DOX groups at 5, 7 and 9 days when compared to SED+SAL (Figure 18, $p < 0.05$). However, the decrement was less pronounced in TM+DOX and WR+DOX groups. When compared to SED+SAL, LVDP was significantly higher 1 day post DOX treatment ($p <$

0.05). The reduction at 7 and 9 days in the TM+DOX groups and day 9 in the WR+DOX was significant when compared to SED+SAL (Figure 18, $p < 0.05$).

In SED+DOX groups, at 5 and 9 days, a significant impairment of dP/dt_{max} and dP/dt_{min} was observed when compared to SED+SAL (Figure 19 and Figure 20, respectively, $p < 0.05$). However, dP/dt_{max} in TM+DOX at day 1 was significantly greater than SED+SAL ($p < 0.05$). The WR+DOX groups at 5 and 9 days displayed a significant impairment of dP/dt_{max} when compared to SED+SAL (Figure 19, $p < 0.05$), while the impairment was not evident in TM+DOX until 9 days post DOX treatment. At the 1 day time point, TM+DOX and WR+DOX displayed improved dP/dt_{min} when compared to the SED+SAL group (Figure 20, $p < 0.05$). When compared to SED+SAL, impairment was evident at the 7 day time point in TM+DOX and day 9 in TM+DOX and WR+DOX (Figure 20, $p < 0.05$).

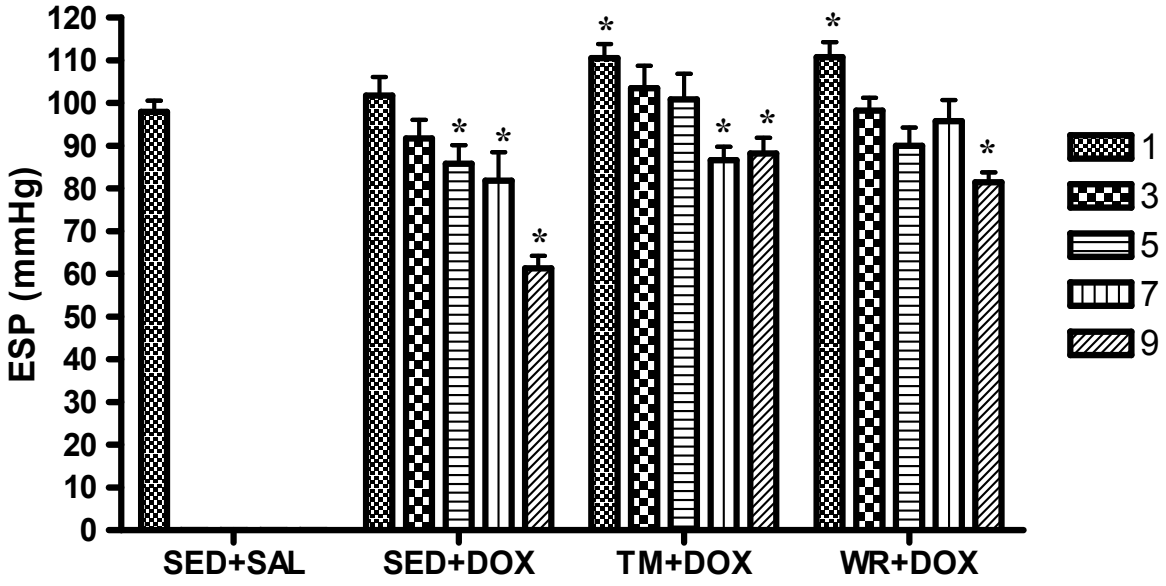

Figure 16. Unpaced *ex vivo* end systolic pressure
* significant difference from SED+SAL

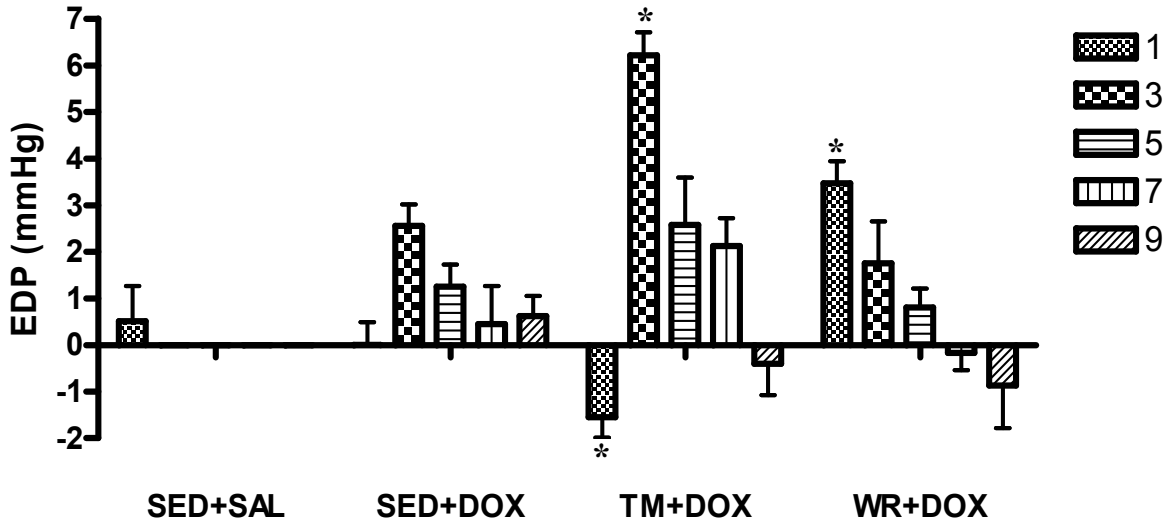

Figure 17. Unpaced *ex vivo* end diastolic pressure
* significant difference from SED+SAL

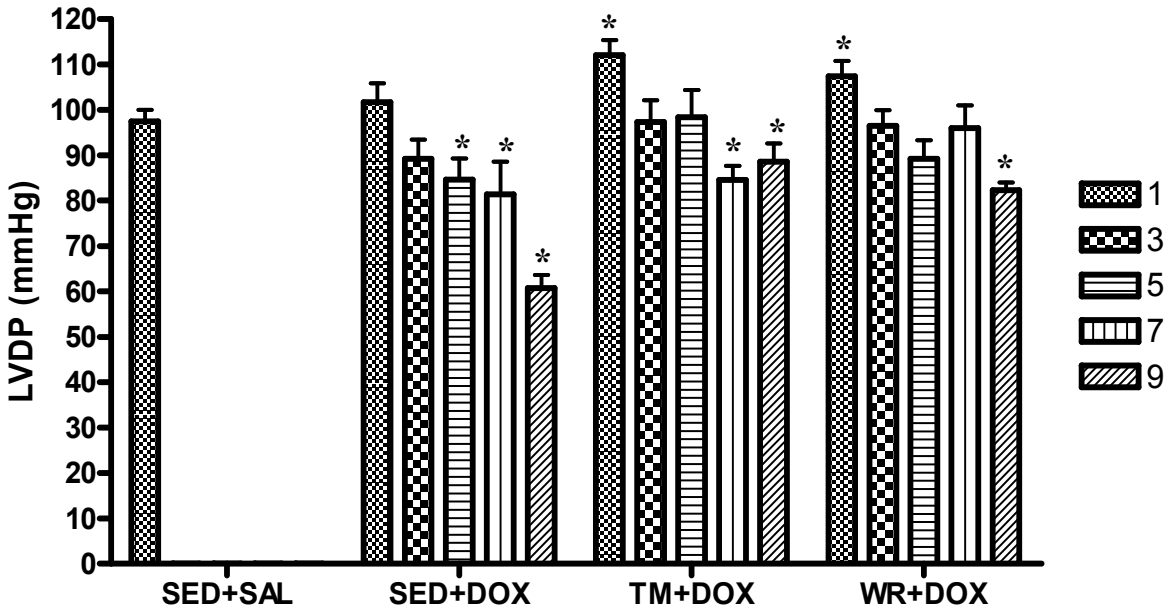

Figure 18. Unpaced *ex vivo* left ventricular pressure development
* significant difference from SED+SAL

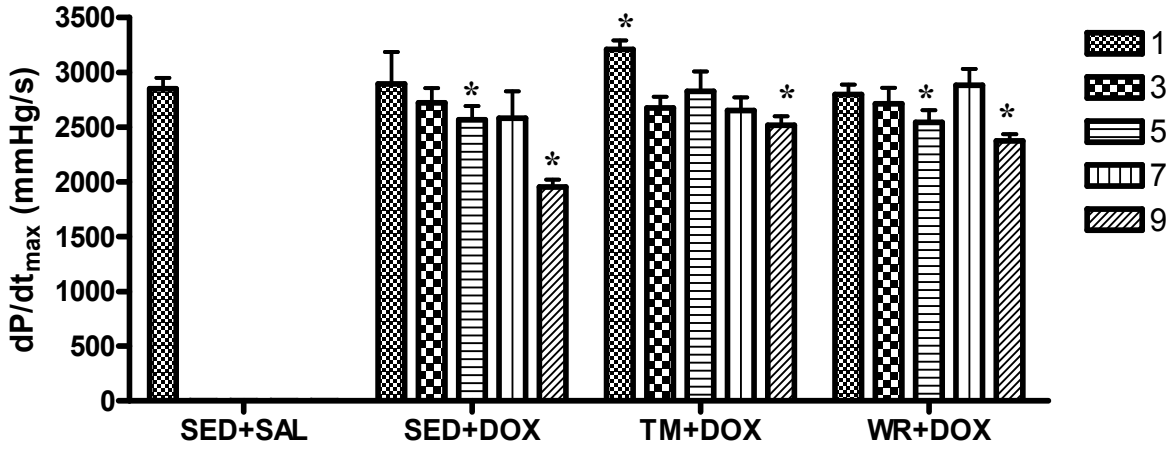

Figure 19. Unpaced *ex vivo* maximal rate of pressure development
* significant difference from SED+SAL

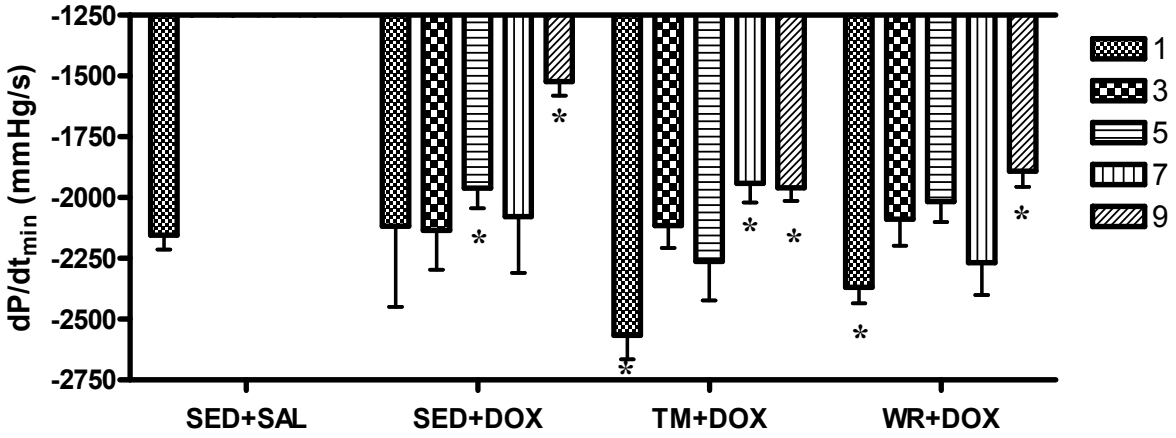

Figure 20. Unpaced *ex vivo* maximum rate of pressure decline
* significant difference from SED+SAL

Paced 240 Data

To obtain standardized LV pressure measurements, hearts were paced at 240 beats/min following the collection of unpaced data. DOX treatment alone significantly impaired ESP in SED+DOX groups at all the measured time points when compared to SED+SAL (Figure 21, $p < 0.05$). However, the decrement did not occur in TM+DOX

until days 7 and 9. In contrast to TM+DOX, WR+DOX displayed significant impairment when compared to SED+SAL at 3, 5, 7, and 9 days (Figure 21, $p < 0.05$). Comparable to unpaced EDP, paced EDP was not significantly different between SED+SAL and SED+DOX (Figure 22, $p > 0.05$). However, when compared to SED+SAL, EDP was significantly lower at day 1 in TM+DOX and significantly higher at day 3, while WR+DOX was significantly higher 1 day post DOX treatment.

Similar to ESP, DOX treatment alone significantly impaired LVDP in SED+DOX groups at 1, 3, 5, 7 and 9 days (Figure 23, $p < 0.05$ vs. SED+SAL). When compared to SED+SAL, LVDP was significantly lower 3, 7, and 9 day post DOX treatment in TM+DOX and at 3, 5, and 9 days post exposure in WR+DOX ($p < 0.05$). At 3, 5, 7 and 9 days, SED+DOX, displayed a significant impairment of dP/dt_{max} when compared to SED+SAL (Figure 24, $p < 0.05$). However, TM+DOX displayed significant differences in dP/dt_{max} at 3, 7 and 9 days ($p < 0.05$ vs. SED+SAL). Significant impairment of dP/dt_{max} in WR+DOX was observed at 3, 5 and 9 days ($p < 0.05$ vs. SED+SAL). Impairment of dP/dt_{min} occurred in SED+DOX at 5, 7 and 9 days (Figure 25, $p < 0.05$ vs. SED+SAL). However, dP/dt_{min} impairment in TM+DOX and WR+DOX occurred at 3 and 9 days post DOX treatment (Figure 25, $p < 0.05$ vs. SED+SAL).

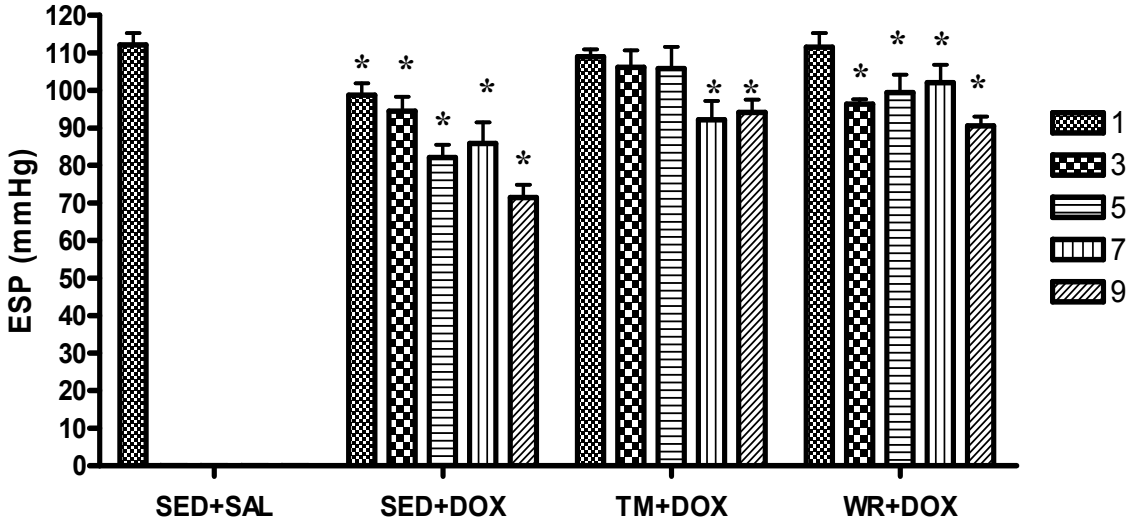

Figure 21. Paced (240) *ex vivo* end systolic pressure
* significant difference from SED+SAL

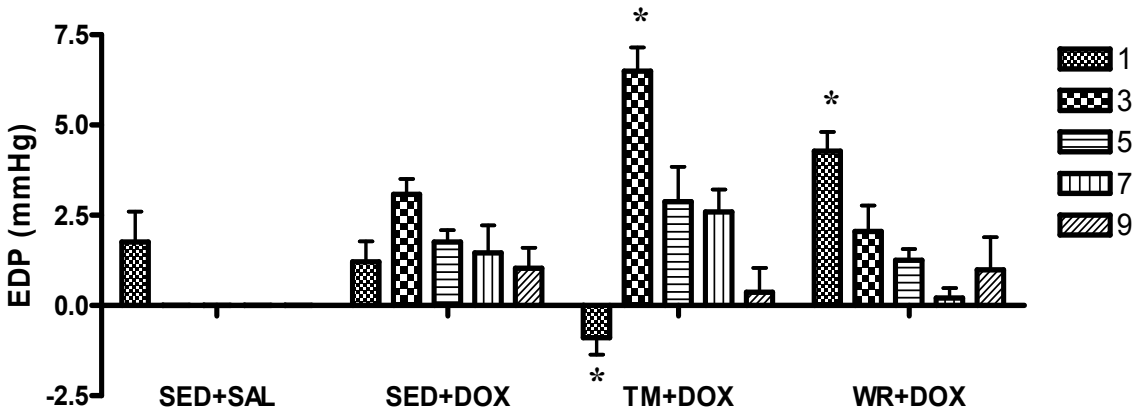

Figure 22. Paced (240) *ex vivo* end diastolic pressure
* significant difference from SED+SAL

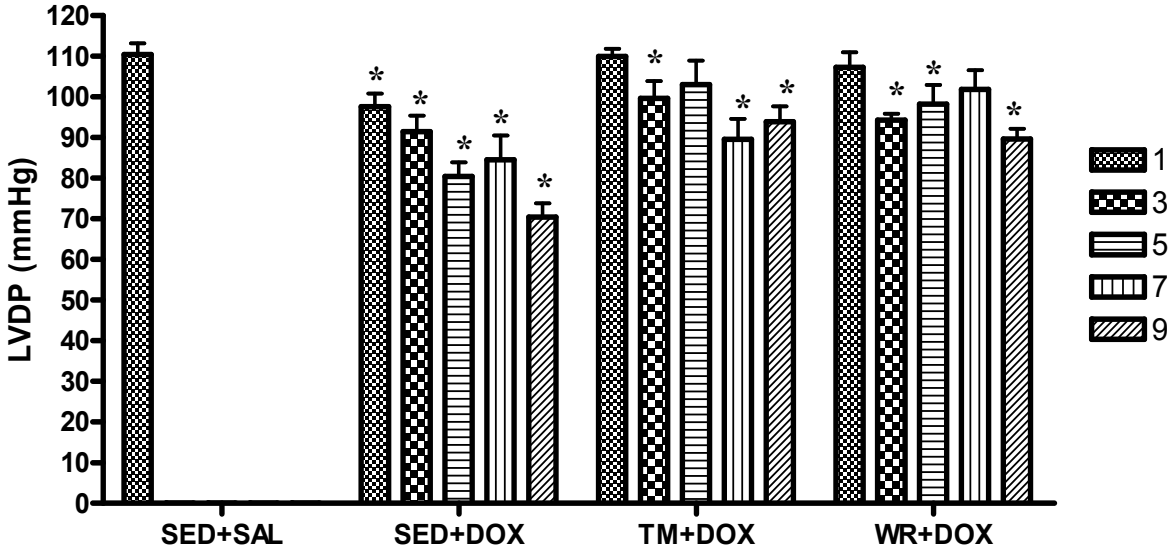

Figure 23. Paced (240) *ex vivo* left ventricular pressure development
* significant difference from SED+SAL

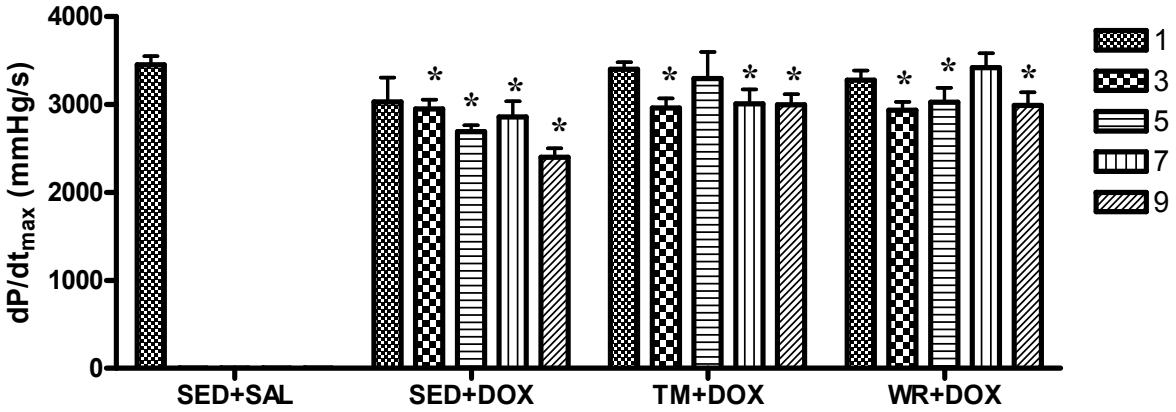

Figure 24. Paced (240) *ex vivo* maximum rate of pressure development
* significant difference from SED+SAL

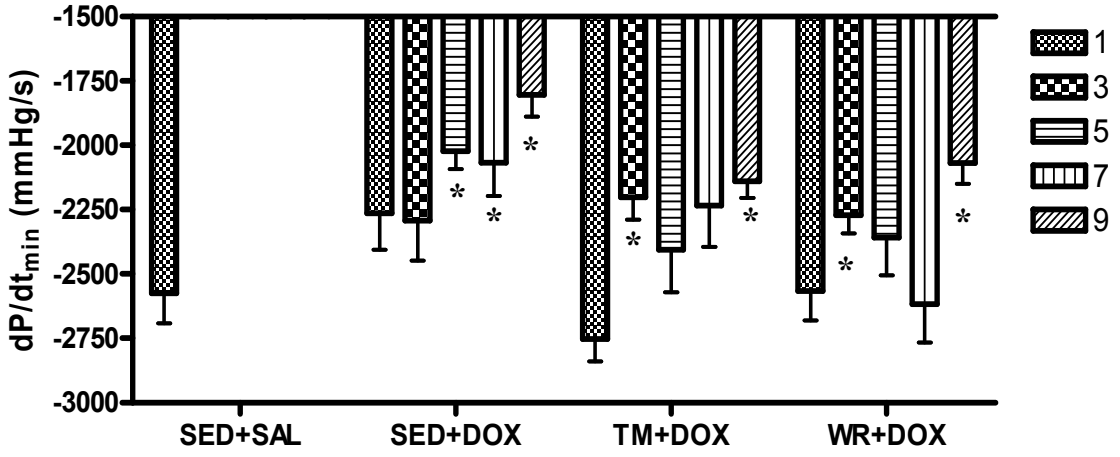

Figure 25. Paced (240) *ex vivo* maximum rate of pressure decline
* significant difference from SED+SAL

Biochemical Analysis

Doxorubicin Accumulation

After all functional analyses were performed, LV samples were isolated and flash frozen in liquid nitrogen. Twenty-four hours following sacrifice, cardiac DOX accumulation was analyzed using HPLC. All data are presented in Figure 26. DOX accumulation was significantly different between groups, F (2,145) = 40.38, $p < 0.001$. A post hoc test was used to determine which group's DOX accumulation was different. The greatest accumulation of DOX was observed in SED+DOX 1 day post injection. When compared to SED+DOX (day 1), DOX accumulation in TM+DOX (day 1) and WR+DOX (day 1) groups was significantly reduced (Figure 26, $p < 0.05$).

Similarly, DOX accumulation in SED+DOX at both 3 and 5 days was significantly greater than both TM+DOX and WR+DOX at 3 and 5 days, respectively (p <0.05). DOX accumulation in TM+DOX and WR+DOX groups was non-existent at both

7 and 9 days. Because DOX accumulation remained elevated in SED+DOX at 7 days, it was significantly greater than TM+DOX and WR+DOX groups ($p < 0.05$). Unlike the exercised groups DOX was not completely cleared from the LV until 9 days after treatment. DOX accumulation was not significantly different between TM+DOX and WR+DOX at any time point ($p > 0.05$).

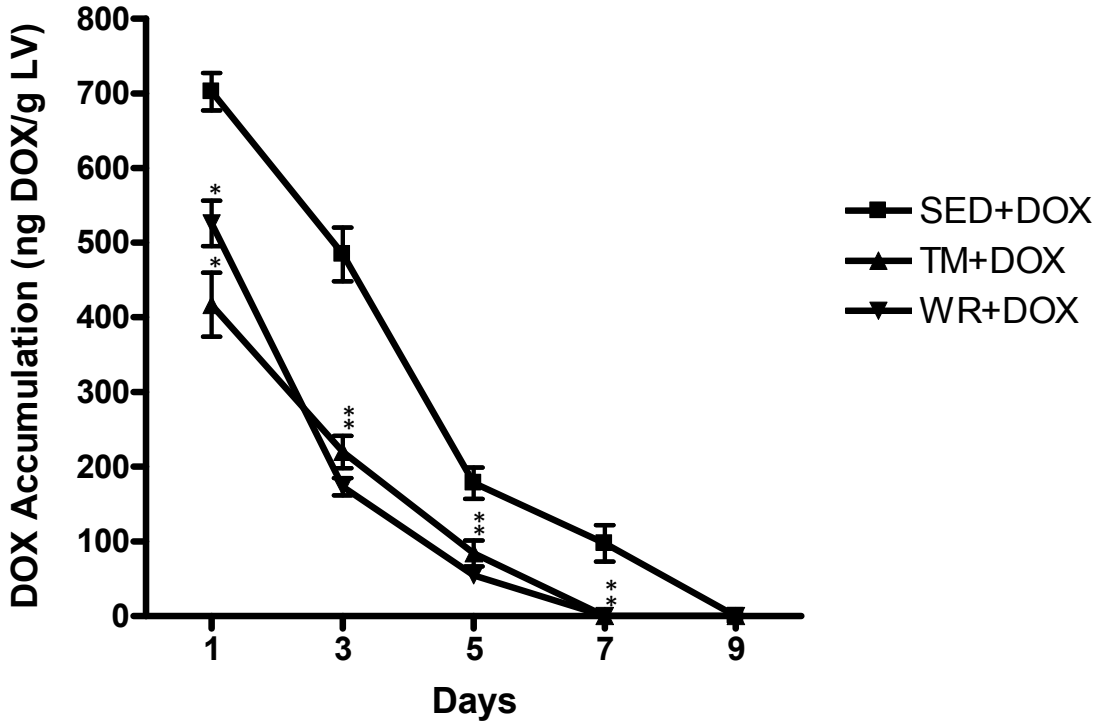

Figure 26. Accumulation of DOX in rat LV tissue.
* Significant difference from SED+DOX

Summary

Using the described HPLC based method, DOX accumulation was successfully quantified in the LV of female rats. In this study, DOX-induced chronic cardiotoxicity resulted in both *in vivo* and *ex vivo* cardiac dysfunction. However, 10 weeks of either involuntary or voluntary exercise preconditioning preserved cardiac function. Significant

differences were observed between sedentary and exercise groups for DOX accumulation, suggesting that reduced DOX accumulation may be one of the underlying mechanisms responsible for the cardioprotective effects of exercise.

CHAPTER V

DISSCUSSION

Introduction

The purpose of this study was to determine if exercise preconditioning reduces cardiac DOX accumulation, thereby providing a possible mechanism to explain the cardioprotective effects of exercise against DOX cardiotoxicity. The results indicate that exercise had a profound effect on DOX accumulation as evidenced by significant decreases in LV DOX accumulation 1, 3, 5, and 7 days after injection. Additionally, DOX treatment resulted in significant cardiac function impairment at 1, 3, 5, 7, and 9 day time points when compared to SED+SAL. Ten weeks of exercise preconditioning prior to DOX treatment attenuated these responses, suggesting that exercise-mediated cardioprotection is afforded by decreases in cardiac DOX accumulation.

Time-course of Doxorubicin Cardiotoxicity

DOX is a highly effective antineoplastic agent and used to treat a variety of cancers including breast, bladder, Hodgkin's and non-Hodgkin's lymphomas. However, its use clinically is limited by a dose-dependent cardiotoxicity that can subsequently lead to heart failure (Lipshultz, et al., 2005; Singal, et al., 2000). The acute form of cardiotoxicity often develops within hours or days following DOX treatment (Ferrans, et al., 1997; Tokarska-Schlattner, et al., 2006). Patients receiving DOX may display signs of acute cardiotoxicity including EKG changes, hypotension, myocarditis and pericarditis (Schimmel, et al., 2004). In experiments using a rat model, indices of DOX-mediated

cardiotoxicity are commonly reported as impairments to *in vivo* and *ex vivo* cardiac function variables (Hydock, Lien, & Hayward, 2009; Hydock, et al., 2008; Hydock, et al., 2010). This investigation examined the effects of DOX on *in vivo* and *ex vivo* cardiac function over time. The selected variables measured in the current investigation are indicative of systolic (FS, A-Vmax, A-Vmean, ESP, LVDP and dP/dt_{max}) and diastolic (M-Vmax, M-Vmean, EDP and dP/dt_{min}) cardiac function. Cardiac dysfunction induced by DOX treatment resulted in reduced Doppler blood flow measurements, decreased FS, ESP, LVDP, and dP/dt_{max}, and increased dP/dt_{min} at 1, 3, 5, 7 and 9 days post treatment with a greater tendency of impaired function as time progressed.

Cardiac dysfunction has been reported to occur 10 days (Hydock, et al., 2008), 7 days (Hayward & Hydock, 2007), 5 days (Chicco, Hydock, et al., 2006; Chicco, Schneider, et al., 2006; Hydock, Lien, & Hayward, 2009; Wonders, et al., 2008), and even 1 day (Neilan, et al., 2006) post DOX treatment with cumulative doses varying from 10-20 mg/kg. However, a cumulative dose of 9 mg/kg (Monti, Prosperi, Supino, & Bottiroli, 1995) has been shown to decrease LVDP 10 days following DOX exposure and 7.5 mg/kg (Sacco, et al., 2003) was responsible for a decline in dP/dt_{max} 3 days post DOX treatment. It is understood that both the cumulative dose and time course of DOX treatment play an important role in the onset and degree of cardiac dysfunction (Minotti, Menna, et al., 2004). Hayward and Hydock (2007) assessed the effects of different DOX dosing schedules on *in vivo* cardiac function in male rats. The time course of cardiac dysfunction was clearly affected by the DOX treatment regimen. In the current study, female rats received the same DOX regimen (10 mg/kg bolus i.p. injection of DOX) which resulted in a similar reduction in FS (-24%) 7 days post DOX treatment when

compared to the previously reported reduction of FS (-22%) in male rats (Hayward & Hydock, 2007). Furthermore, previous studies have reported comparable declines in Doppler blood flow measurements in rats receiving a 10-15 mg/kg cumulative dose of DOX evidenced by a significant decrease in M-V_{max} and M-V_{mean} (Hydock, et al., 2010), A-TVI (Hayward & Hydock, 2007), A-V_{max} and A-V_{mean} (Hayward & Hydock, 2007; Hydock, Lien, & Hayward, 2009).

In this investigation, 10 mg/kg of DOX significantly impaired not only *in vivo* cardiac function but also *ex vivo* cardiac function in female rats 3, 5, 7 and 9 days after treatment. Similarly, *in vivo* and *ex vivo* measurements of cardiac function in rats receiving a 10 mg/kg bolus of DOX, showed that at 5 and 10 days post treatment, there were significant decreases in Doppler measurements, dP/dt_{max} and LVDP (Hydock, et al., 2008). The SED+DOX group displayed classic signs of DOX cardiotoxicity including reductions in ESP, LVDP, dP/dt_{max} and impaired dP/dt_{min}. These general observations corroborate with findings reported in several other studies of DOX-mediated cardiotoxicity (Chicco, Hydock, et al., 2006; Chicco, et al., 2005; Chicco, Schneider, et al., 2006; Hydock, Lien, Jensen, Schneider, & Hayward, 2011; Hydock, et al., 2008; Hydock, et al., 2010; Wonders, et al., 2009; Wonders, et al., 2008). Numerous mechanisms are likely to contribute to the dysfunction observed with DOX treatment in the sedentary rats. Previous studies suggest DOX treatment results in the formation of RONS (Sarvazyan, 1996), apoptosis (Kumar, Kirshenbaum, Li, Danelisen, & Singal, 1999), mitochondrial dysfunction (Oliveira, Santos, & Wallace, 2006) and impaired Ca^{2+} handling in the cardiomyocyte (Hirano, Agata, Iguchi, & Tone, 1995; Kim, et al., 2006).

Collectively, these mechanisms are thought to have the greatest contribution to DOX-mediated cardiotoxicity.

Effects of Exercise on Doxorubicin Cardiotoxicity

In the present study, a 10 mg/kg bolus dose of DOX resulted in significant *in vivo* and *ex vivo* cardiac dysfunction in sedentary animals 1, 3, 5, 7 and 9 days post DOX treatment. This dysfunction was associated with DOX accumulation in cardiomyocytes. Conversely, rats that participated in either voluntary wheel running or treadmill exercise for 10 weeks did not exhibit the same degree of cardiac dysfunction. Furthermore, they displayed significantly less DOX accumulation at 1, 3, 5 and 7 days following DOX administration. Previous studies have determined that exercise preconditioning can attenuate DOX-induced cardiotoxicity (Chicco, Schneider, et al., 2006; Hydock, et al., 2011; Hydock, et al., 2008; Hydock, et al., 2010; Wonders, et al., 2009). As expected, general observations from the current investigation coincide with previous studies that used exercise preconditioning to mitigate the cardiotoxic effects of DOX.

A novel investigation conducted by Chicco et al., (2005) was the first to provide evidence supporting chronic voluntary exercise prior to DOX exposure in the attenuation of cardiac dysfunction. Until that time, experiments had shown promise for exercise-induced cardioprotection against DOX cardiotoxicity; however, cardiac function was not addressed (Ascensao, Magalhaes, et al., 2005b; Combs, Hudman, & Bonner, 1979; Ji & Mitchell, 1994). A follow up study was conducted by Chicco et al., (2006) in which rats trained on a treadmill for 12 weeks. Five days following a 15 mg/kg bolus i.p. injection of DOX, cardiac function was assessed. Results indicated that LVDP, dP/dt_{max}, and dP/dt_{min} were significantly impaired in sedentary DOX treated rats compared to exercise

trained rats. Collectively, results from these studies suggest that cardioprotection from DOX-induced dysfunction is achieved regardless of exercise modality. Therefore, results from the current investigation were as expected and voluntary wheel running afforded similar cardioprotection as treadmill exercise. Additionally, it was hypothesized that both treadmill and wheel running exercise prior to DOX treatment would result in less DOX accumulation when compared to sedentary rats. Thus, exercise preconditioning would not only improve cardiac function but also offer cardiomyocytes direct protection from DOX cardiotoxicity by reducing the degree of DOX exposure.

The use of voluntary exercise as a training regimen has been shown to increase the sarcomere length-tension relationship, cardiac contractility (Lambert & Noakes, 1990; Natali, Turner, Harrison, & White, 2001; Natali, et al., 2002) and oxygen consumption (Lambert & Noakes, 1990; Yano, Yano, Kinoshita, & Tsuji, 1997). Similar findings have been reported with treadmill exercise, evidenced by increased cardiomyocyte contractility and oxygen consumption (Wisloff, Helgerud, Kemi, & Ellingsen, 2001; Wisloff, Loennechen, et al., 2001). Furthermore, the cardiovascular adjustments between the two modalities appear to be similar (Yancey & Overton, 1993). In the current study, both preconditioning modalities resulted in cardioprotection from DOX-mediated dysfunction and less DOX accumulation compared to SED+DOX. Additionally, DOX accumulation was not affected by modality evidenced by similar observations of LV DOX in TM+DOX and WR+DOX groups. Thus, exercise preconditioning, independent of modality, protected against cardiotoxicity as a result of reduced DOX accumulation.

It has previously been suggested that subtle differences in exercise-mediated stress exist between voluntary and involuntary exercise (Moraska, Deak, Spencer, Roth, & Fleshner, 2000; Moraska & Fleshner, 2001). Moraska et al., (2001) reported that voluntary wheel running can reduce the deleterious effect of stress on immune function and involuntary treadmill training (Moraska, et al., 2000) elevated lymphocyte nitrite concentrations and suppressed lymphocyte proliferation, common immune responses, as the result of chronic stress. Thus, the exercise intensity from the aforementioned modalities may result in different chronic stress.

In the current study, exercise preconditioning prevented the *in vivo* and *ex vivo* cardiac dysfunction observed with DOX treatment alone. Several mechanisms may be responsible for the observed cardioprotective effects of exercise preconditioning. Most noteworthy, evidence that cardiac DOX accumulation was significantly lower in TM+DOX and WR+DOX hearts at 1, 3, 5, and 7 day time points suggest that a reduction in cardiac DOX exposure may be responsible for the preservation in cardiac function.

Mechanisms of Cardioprotection

This study demonstrated, for the first time, that exercise mitigated DOX accumulation and afforded cardioprotection. Determining the underlying mechanism behind exercise-induced cardioprotection from DOX has been of great interest. The observed reduction in LV DOX accumulation, as the result of exercise preconditioning, offers the greatest contribution to the explanation of exercise-mediated cardioprotection against DOX-induced cardiotoxicity. While these results confirm an exercise effect on DOX accumulation in the heart, the exact mechanisms responsible for the exercise-mediated reduction of LV DOX are still unknown. Therefore, the following discussion

will focus on the theoretical foundation for an exercise-induced reduction in DOX accumulation in cardiomyocytes.

Doxorubicin Accumulation

One potential explanation for less DOX accumulation in exercise preconditioned rats may be due to increased DOX extrusion as the result of an upregulation of ABC transporters. It is commonly accepted that cancer cells have the ability to develop a drug resistance phenotype (Shen, et al., 2008; Sorokin, 2004). Several mechanisms are thought to contribute to the drug resistance phenotype including increased drug extrusion, decreased permeability, altered drug metabolism and deactivation (Davin-Regli, et al., 2008). Additionally, non-cancerous cells, such as cardiomyocytes, display drug resistant mechanisms as a means for inherent cytoprotection (Jungsuwadee, et al., 2009). For example, cardiomyocytes normally express ABC transporters to allow or deny access of nutrients and xenobiotics. Recently, numerous reports involving experimental enhancement of ABC transporters have been shown to effectively pump xenobiotics out of the cell (Dazert, et al., 2003; Dong, et al., 2009; Gradilone, et al., 2008; Krause, et al., 2007; Meissner, et al., 2007; Shen, et al., 2008; Zhang, et al., 2009). The subfamily proteins that are most involved in the transport of xenobiotics, including DOX, are multidrug resistance proteins (MDR), MRPs and ABCA. Specific to location and tissue type, MDR and MRPs are sub-categorized (i.e. MDR-1, MRP-1, MRP-2, MRP-3, MRP-4, MRP-5, and ABCA-1).

Sub-family ABC transporters including a p-glycoprotein MDR-1 (Beaulieu, Demeule, Ghitescu, & Beliveau, 1997; Meissner, et al., 2004), MRP-1, MRP-2 (Rosati, et al., 2003), MRP-5 (Dazert, et al., 2003; Meissner, et al., 2007) and ABCA-1 (Nishimura,

Naito, & Yokoi, 2004) expression occurs in cardiac tissue. The aforementioned protein transporters may offer some insight to the transport of DOX and the regulation of its anticipated toxicities. Bellamy et al, (1995) reported cardiac DOX accumulation increased two-fold in non-tumor bearing mice when treated with a combination of DOX and Cyclosporin A, a potent MDR-1 inhibitor. In addition, a greater degree of myocardial insult was observed in mice exposed to the combination of DOX and Cyclosporin A than in those mice not receiving the combined treatment. A similar finding was reported in MDR-1 knock-out mice (*mdr1a*$^{-/-}$) 24 hours post DOX treatment. The accumulation of DOX and DOX-ol were greater than two-fold higher in the hearts of (*mdr1a*$^{-/-}$) mice when compared to control mice (van Asperen, van Tellingen, Tijssen, Schinkel, & Beijnen, 1999). *In vitro* inhibition of ABCA-1 genes via extracellular signal-regulated kinases (ERK) resulted in increased DOX accumulation determined by fluorescence microscopy (Shukla, et al.). Furthermore, isolated cardiomyocytes from Sprague-Dawley rats that were treated with calcium channel blockers *in vitro* to reverse the multidrug resistant phenotype showed an increased concentration of DOX and cardiotoxicity as indicated by increased heart levels of lactate dehydrogenase (Santostasi, Kutty, & Krishna, 1991). Similarly, it has been demonstrated that an enhancement of expression and/or activity of ABC transporters in the heart results in a desirable drug resistance phenotype (Dell'Acqua, Polishchuck, Fallon, & Gordon, 1999; Granzotto, et al., 2004; Magnarin, et al., 2004). Dell'Acqua et al., (1999) reported transgenic mice that overexpressed the MDR-1 gene displayed greater cardioprotection from a 30 mg/kg cumulative dose of DOX when compared to control mice. Collectively, these studies have demonstrated that a blockade or enhancement of ABC transporters significantly

influences extrusion of DOX and/or DOX-ol, and the subsequent drug exposure is related to heart damage. This notion confirms the important role of ABC transporters in cardioprotection from DOX-mediated cardiotoxicity.

Previous work suggests that exercise training is capable of increasing expression of ABC transporters including ABCA-1 (Ghanbari-Niaki, Khabazian, Hossaini-Kakhak, Rahbarizadeh, & Hedayati, 2007; Ghanbari-Niaki, Saghebjoo, & Hedayati, 2010; Khabazian, et al., 2009) and MRP-1(Krause, et al., 2007). Khabazian, et al., (2009) reported that exercise-induced increases in ABCA-1 expression were present in adult male Wistar rats 2 days following a 12 week treadmill training protocol. In addition, Ghanbari-Niaki, et al., (2007) reported significantly increased ABCA-1 expression in rats 24 hours post the completion of a 6 week treadmill training protocol when compared to sedentary controls. This suggests that upregulation of ABCA-1 may be an adaptation to increased endurance exercise. However, Ghanbari-Niaki, et al., (2010) recently reported increased ABCA-1 mRNA expression in female students who completed a single session of circuit-resistance training but the exercise-induced expression was observed in lymphocytes not cardiomyocytes. Furthermore, Krause et al. (2007) reported rats that performed 60 minutes of swimming exercise for 1 week displayed a significant increase in MRP-1 expression (2.4-fold increase) which translated into a significant increase in pumping activity, possibly suggesting that less DOX would reside in cardiomyocytes if MRP-1/GS-X pumps were upregulated. The current investigation did not measure ABCA-1 or MRP-1 expression; therefore, the relationship between the aforementioned ABC transporters and DOX accumulation in myocardial cells remains unclear. However, it is conceivable to speculate that exercise caused an upregulation of ABC transporters in

TM+DOX and WR+DOX rat hearts which resulted in greater DOX extrusion and less accumulation in the cardiac cell.

It has also been hypothesized that the accumulation of xenobiotics in target or non-target tissues is primarily dependent on a mechanical barrier which influences the intracellular concentration of the drug (Davin-Regli, et al., 2008). In a study by Versantvoort (1992), multidrug resistant cells were altered to examine DAUN accumulation and membrane permeability. A 5-fold efflux of DAUN was observed in multidrug resistant cells lacking p-glycoproteins compared to controls suggesting other mechanisms influenced DAUN accumulation in the MDR cells. Thus, it is probable that the accumulation of DOX, in cardiomyocytes, depends not only on the drug efflux, but also DOX influx across the cellular membrane (Han & Zhang, 2004). Further study is necessary to examine the effects of exercise on ABCA-1 and MRP-1 expression and determine if induction translates into less DOX accumulation, and a preservation of cardiac function from DOX-mediated cardiotoxicity.

Due to the large number of samples per experimental subgroup, DOX concentrations measured during the course of this experiment allowed for greater statistical confidence in the obtained results. DOX accumulation was reduced in all experimental groups over the course of 9 days. This finding is consistent with recent DOX pharmacokinetic studies (Ahmed, et al., 2009; Staples, et al., 2010; Urva, et al., 2009). DOX accumulation was not detected beyond 7 days of DOX exposure. Recently, Staples, et al., (2010) reported that DOX was still present in rat heart 7 days post treatment but could not be detected 8 days post using HPLC.

In the present study, understanding the distribution of DOX in cardiac tissue over time was also of interest. It is conceivable that greater DOX exposure (time or concentration) to cardiomyocytes would result in greater cardiac insult. While a reduction over time was as hypothesized, the degree of decline was unknown. The SED+DOX (day 3) group displayed a 31% reduction in accumulation from SED+DOX (day 1), a 63% reduction was observed in SED+DOX (day 5) compared to SED+DOX (day 3), and a 45% reduction was observed in SED+DOX (day 7) when compared to SED+DOX (day 5). The TM+DOX (day 3) group displayed a 47% reduction compared to TM+DOX (day 1), a 62% reduction was observed in TM+DOX (day 5) compared to TM+DOX (day 3), and no DOX was detected after 7 days post treatment. Similarly, WR+DOX displayed a 47% clearance from day 1 to day 3, a 62% clearance from day 3 to day 5, and 100% clearance from day 5 to day 7. At the 9 day time point, DOX was not detected in any of the exercised groups. It appears that the degree of decline, indicated by percent change from one time point to another, was not drastically influenced by the modality of exercise training. DOX metabolism was not measured in the current investigation. However, it is plausible that exercise training resulted in elevated drug metabolism immediately following exposure. After i.v. injection in rats and humans (Rousseau & Marquet, 2002), DOX has a distributive half-life of about 5 minutes and a terminal half-life of 20 to 48 hours (Danesi, Fogli, Gennari, Conte, & Del Tacca, 2002). This suggests that DOX uptake by the tissues happens rapidly, while tissue elimination is much slower. DOX is rapidly cleared from the plasma and extensively excreted through the biliary pathway following injection (Danesi, et al., 2002; Salvatorelli, et al., 2009). It has been reported that approximately 40% of DOX is excreted in bile and around 5-12%

in the urine over the course of 5 days (Danesi, et al., 2002). It is plausible that the initial uptake of DOX in LV tissue of exercise trained rats is reduced due to the upregulation of MRP-1, resulting in a greater concentration of DOX in the plasma and reduced concentration in the tissue.

Doxorubicin Metabolism

Another possible explanation for the reduction of DOX accumulation observed in exercise trained rats is altered drug metabolism. DOX-induced cardiotoxicity occurs following exposure of the parent drug and its metabolites (Sacco, et al., 2003). Cytoplasmic aldo/keto or carbonyl reductases convert DOX to DOX-ol (Minotti, et al., 2001). Doxorubicin is composed of an aglycone (doxorubicinone) and a sugar (daunosamine). Doxorubicinone is a tetracyclic ring system with quinone-hydroquinone moieties and a side chain with a carbonyl group at C-13. One-electron reduction of the DOX quinone moiety is followed by redox cycling of oxygen and formation of ROS, while a 2-electron reduction of the side-chain carbonyl group results in DOX-ol formation (Minotti, et al., 2000; Sacco, et al., 2003). It has been suggested that the secondary alcohol metabolite, DOX-ol, is associated with increased cardiotoxicity. In a study by Olson et al., (1988) DOX-ol exhibited greater acute cardiotoxicity than the parent drug DOX evidenced by reduced systolic function and impaired Ca^{++} handling. Additionally, an investigation with overexpression of carbonyl reductases in transgenic mice resulted in an increased conversion of DOX to DOX-ol and advanced the development of cardiomyopathy (Forrest, Gonzalez, Tseng, Li, & Mann, 2000). Furthermore, levels of DOX-ol in the heart have a greater correlation to cardiac dysfunction than DOX (Sacco, et al., 2003). Investigators have suggested that the

relationship between the secondary metabolites and cardiotoxicity may be due, in part, to the fact that DOX-ol is more polar than DOX and subsequently more likely to accumulate in the heart (Forrest, et al., 2000; Minotti, Recalcati, et al., 2004; Sacco, et al., 2003). Collectively, these studies have demonstrated that increased DOX metabolism and/or DOX-ol formation is related to heart damage suggesting the important role of cytoplasmic reductases.

Evidence, though minimal, has linked exercise training to a reduction in reductase enzyme expression which may explain its cardioprotective effects. Exercise has been reported to alter the expression of cytoplasmic reductases. Following 30 minutes of exercise, Aldo-keto reductase family 1, member-3, gene expression was down-regulated 5 fold (Connolly, et al., 2004). Similarly, in a recent study by Lee et al., (2011) it was reported that exercise training down-regulates Nrf-2, a gene primarily responsible for the coding of NAD(P)H quinone oxidoreductase. NAD(P)H quinone oxidoreductase is a sub-family protein to oxidoreductases which are included in the carbonyl reductase family. Furthermore, it has been reported that lower levels of carbonyl reductases results in less conversion of DOX to DOX-ol in carbonyl reductase family 1 (*cbr1* $^{+/-}$) knockout mice. Knockout mice (*cbr1* $^{+/-}$) were protected from DOX-cardiotoxicity compared to *cbr1* $^{+/+}$ suggesting that a reduction of carbonyl reductase resulted in less DOX-ol formation (Olson, et al., 2003).

The current investigation did not measure the expression of cytoplasmic reductase enzymes; therefore, the relationship between exercise and DOX metabolism in cardiomyocytes is unknown. However, reductases play a significant role in metabolizing DOX and increasing the cardiotoxic properties of the drug. Thus, it is conceivable to

speculate that TM+DOX and WR+DOX groups produced less secondary alcohol metabolites than SED+DOX.

Secondary Cardioprotective Mechanisms

Other reports have shown that the protective effects of exercise against DOX cardiotoxicity are associated with increased antioxidant enzyme upregulation (Ascensao, Magalhaes, et al., 2005b; Chicco, Hydock, et al., 2006), a preservation of SERCA2a expression (Lien, et al., 2009), and maintenance of cardiomyocyte ultrastructure evidenced by decreased serum cardiac troponin I (Ascensao, Ferreira, Oliveira, & Magalhaes, 2006). Other reports have shown exercise is associated with a preservation of myosin heavy chain isoform expression (Hydock, Wonders, et al., 2009), decreased lipid peroxidation (Wonders, et al., 2009), upregulation of HSP (Chicco, et al., 2005; Chicco, Schneider, et al., 2006), and the attenuation of DOX-mediated increases in caspase activity (Wonders, et al., 2009). It is likely that the aforementioned exercise-mediated cardioprotecive mechanisms may be explained, in part, by the underlying mechanism of reduced DOX accumulation in the left ventricular tissue of the exercised animals.

A large amount of evidence indicates that ischemia-reperfusion and DOX-mediated cardiac dysfunction are primarily mediated by oxidative stress (Abdel-Wahab, et al., 2003; Kalyanaraman, et al., 2002; Kumar, Kirshenbaum, Li, Danelisen, & Singal, 2001; Patel, Joseph, Corcoran, & Ray, 2010; Sarvazyan, 1996). In the DOX-induced cardiac dysfunction model, it is believed that the elevated myocardial oxidative stress is mainly due to increased production of RONS as a result of DOX redox cycling (Minotti, Recalcati, et al., 2004). The enhancement of SOD (Yen, et al., 1996) and endothelial

nitric oxide synthase (Kalyanaraman, et al., 2002) status has been shown to lessen the oxidative state, thus protecting the heart from DOX toxicity. In addition to DOX-mediated cardiotoxicity, oxidative stress appears to play a key role in another cardiac dysfunction model, ischemia reperfusion injury (Lennon, et al., 2004; Starnes, Taylor, & Park, 2003). Prior studies have reported that antioxidant supplementation resulted in cardioprotection from ischemia reperfusion injury (Coombes, et al., 2000; Hamilton, et al., 2003; Powers, DeRuisseau, Quindry, & Hamilton, 2004). Therefore, in both models, a reduced oxidant production appears to provide the myocardium protection and contributes to the attenuation of cardiac dysfunction.

Researchers commonly suggest that the cardioprotective effect of exercise is due, in part, to reduced production of ROS and increased antioxidant capacity (Ascensao, Ferreira, & Magalhaes, 2007; Powers, Quindry, et al., 2004). Analysis of the oxidative status was not conducted in the present study; thus, the degree of myocardial oxidative stress due to DOX treatment is not known. However, it is plausible that a reduction in DOX accumulation mediated by exercise training reduced the amount of DOX-mediated RONS. Gunduz, et al., (2004) suggested that exercise is capable of inducing the enhancement of other antioxidant defenses including GPx and CAT. In a study by Kanter et al., (1985) mice were exercise preconditioned for 9 weeks prior to DOX treatment. Interestingly, GPx and CAT activities were significantly increased in the trained rats and offered protection from DOX-induced toxicity evidenced by histological evaluation of the heart.

In contrast to primary antioxidant enzymes, it has been suggested that the myocardium intrinsically protects itself from oxidative stress via antioxidant enzymes

including thioredoxin (Berndt, Lillig, & Holmgren, 2007; Holmgren & Lu, 2010) and peroxiredoxin (Kumar, Kitaeff, Hampton, Cannell, & Winterbourn, 2009). A review by Berndt, et al., (2007) noted the potential cardioprotective role of thioredoxin and thioredoxin reductase and suggested that these enzymes protect against oxidative stress and inhibit apoptotic signaling. Similarly, peroxiredoxin has been reported to reduce oxidative stress in the hearts of ischemically injured mice (Kumar, et al., 2009). Therefore, in addition to the primary antioxidant enzymes associated with cardioprotection it appears the heart may have other oxidative defenses. Currently, the effect of exercise training on thioredoxin and peroxiredoxin in the heart is unknown. However, Lappalainen, et al., (2009) reported that 8 weeks of exercise training increased brain thioredoxin in male rats. Thus, it is plausible that exercise-induced changes in one or all of these antioxidant systems may contribute to the preservation of cardiac function. In addition to antioxidant defense, another explanation for the observed preservation of cardiac function in exercise trained rats could be due to an attenuation of mitochondrial dysfunction.

Mitochondria play a vital role in the synthesis of ATP. From a bioenergetic standpoint, it is imperative that the mitochondria synthesize enough ATP to match the cellular demand. When ATP synthesis is insufficient, metabolic demand is not met and cellular death may occur (Baines, 2009b; Crompton, 1999). Under normal physiological conditions, oxidative phosphorylation of acetyl coenzyme A (CoA), derived from the beta oxidation of fatty acyl-CoA found inside the mitochondrial matrix, is primarily responsible for meeting the cellular demand for ATP. The rate of ATP synthesis is controlled largely by oxidative enzymes. Heart failure is associated with a reduction in

key regulatory oxidative enzymes including acyl-CoA dehydrogenase and carnitine translocase (Baines, 2009a; Kelly & Strauss, 1994; Roschinger, et al., 2000). De Sousa, et al., (2002), reported that a downregulation in oxidative enzymes exacerbated the shift from beta oxidation to glycolysis, suggesting an association between heart failure and reduced fatty acid metabolism. Pellieux et al., (2006) overexpressed angiotensinogen in transgenic mice to induce congestive heart failure and reported that fatty acid oxidation was compromised. Recently, evidence has shown that *in vitro*, rat cardiomyocytes exposed to angiotensin II, a modulator in heart failure, reduced fatty acid oxidation (Pellieux, Montessuit, Papageorgiou, & Lerch, 2009). A reduction in fatty acid metabolism may lead to mitochondrial dysfunction (van Bilsen, van Nieuwenhoven, & van der Vusse, 2009). However, it was reported that 8 weeks of voluntary wheel running exercise attenuated the observed metabolic shift in aortic banded rats (De Sousa, et al., 2002). Furthermore, free carnitine, was reported to be increased in exercised trained rats, thus contributing to increased fatty acid metabolism (Cha, Kim, & Daily, 2003). In aortic banded pigs, low-intensity interval exercise training inhibited mitochondrial dysfunction and preserved LV function (Emter & Baines, 2010). The aforementioned effects may play an important role in the maintenance of mitochondrial biogenetics, improving its resistance to ischemic or chemical induced dysfunction.

In addition to ATP deprivation, the production of ROS in mitochondria can lead to mitochondrial dysfunction (Carvalho, et al., 2010; Lumini-Oliveira, et al., 2009; Zhou, et al., 2001). Mitochondrial impairment following either ischemia reperfusion injury (Baines, 2009a) or DOX treatment contributes to cardiac dysfunction (Ascensao, et al., 2007; Ascensao, Lumini-Oliveira, Oliveira, & Magalhaes, 2011). An increased oxidative

state can impair mitochondrial calcium homeostasis (Solem, et al., 1994) and ATP synthesis which contributes to the opening of MPTP (Baines, 2009b). Starnes et al., (2007) reported that ROS production was elevated in myocardial mitochondria and resulted in less calcium influx following ischemia reperfusion. Additionally, Marcil et al. (2006) reported that mitochondria isolated from rat hearts exposed to anoxia-reoxygenation injury *in vitro* resulted in calcium-induced MPTP opening. Similar to reperfusion injury models, DOX treatment results in increased mitochondrial oxidative stress due to heavy redox cycling which occurs at complex I (Tokarska-Schlattner, et al., 2006; Wallace, 2003). It is believed that mitochondrial calcium homeostasis and MPTP impairment is the result of an increased oxidative state (Baines, 2009b; Crompton, 1999). Additionally, the intercalation of DOX into mtDNA may also contribute to mitochondrial dysfunction. DOX and other DNA intercalators were reported to intercalate mtDNA and caused mtDNA depletion in myocardial cells (Ashley & Poulton, 2009a). An *in vitro* study by Sarvazyan (1996) indicated that a time-dependent accumulation of DOX occurred in the mitochondria of cardiomyocytes. Thus, it appears that DOX has a high affinity for mitochondria and DOX-induced mitochondrial dysfunction facilitates destructive events in cardiomyocytes that may contribute to cardiac dysfunction.

Interestingly, numerous studies have reported that exercise preconditioning results in cardiac mitochondrial adaptations that result in reduced ROS production (Kavazis, Smuder, Min, Tumer, & Powers, 2010; Starnes, et al., 2007) and MPTP formation (Emter & Baines, 2010; Kavazis, et al., 2010; Lumini-Oliveira, et al., 2010; Marcil, Bourduas, Ascah, & Burelle, 2006). In the current investigation, cardioprotection was evident; however, mitochondrial function was not determined. It is plausible, though, that the

reduced DOX accumulation observed in the exercise trained groups resulted in less DOX-mediated mitochondrial dysfunction, thereby providing a theoretical basis for the observed preservation of cardiac function. Further study is needed to explore the physiological significance of any interaction between physical activity and DOX accumulation on mitochondrial dependent ROS, ATP synthesis, calcium homeostasis, and MPTP opening in cardiomyocytes.

Summary and Conclusions

In the current investigation, DOX treatment resulted in acute cardiotoxicity. Impaired systolic and diastolic cardiac function was observed both *in vivo* and *ex vivo*. However, 10 weeks of exercise preconditioning resulted in a preservation of cardiac function. Additionally, HPLC was successfully used to quantify DOX accumulation in LV tissue of female rats at multiple time points. Voluntary wheel running activity or involuntary treadmill exercise performed prior to DOX treatment resulted in less LV DOX accumulation. This is believed to be the first study to investigate the cardioprotective mechanism of exercise-mediated DOX accumulation. These findings suggest that regular physical activity may be a valuable complementary therapy to offset cardiotoxicities that often occur in cancer patients receiving DOX treatment. Furthermore, cancer survivors participating in exercise training programs may be able to better tolerate drug exposure, resulting in greater survival and an improvement in their quality of life.

Limitations and Future Study

Based on the results of the current investigation, the following are limitations of this study and recommendations for future research.

1) This study employed the chemotherapeutic agent DOX to cancer-free rats to investigate the effects of exercise, DOX, and time on cardiac function and DOX accumulation. This investigation confirmed that exercise can reduce DOX accumulation in the LV. Using a tumor-bearing animal model would be of interest to determine the effect of exercise on DOX accumulation in tumor cells. Furthermore, future studies could use a tumor-bearing model to confirm the results of this investigation and additionally determine whether exercise affects the efficacy of this antineoplastic agent.

2) In this study, 10 weeks of either involuntary or voluntary exercise preconditioning reduced LV DOX accumulation and preserved cardiac function in female rats. In addition to chronic exercise preconditioning, short term exercise has also been reported to be cardioprotective against DOX cardiotoxicity (Jensen, et al., 2009; Kavazis, et al., 2010). Thus, the possibility exists that short term exercise may also result in decreased DOX accumulation and preservation of cardiac function. Future study is needed to determine the effect of short term exercise on DOX accumulation and cardiac function.

3) Using the described HPLC based method, DOX accumulation was successfully quantified in the LV of female rats. However, DOX metabolites such as DOX-ol, aglycone-DOX, and aglycone-DOXol were not quantified. Evidence suggests DOX metabolites are cardiotoxic and may have an even greater contribution to DOX-mediated cardiotoxicity (Minotti, Menna, et al., 2004). Therefore, it would be of great interest to investigate the effect of exercise on the accumulation of DOX metabolites.

4) In this study, the protective effects of exercise were associated with a reduction in DOX accumulation. Recent evidence shows that ABC transporters such as MRP-1 are

responsible for the cellular efflux of DOX (Dong, et al., 2009; Krause, et al., 2007; Shen, et al., 2008; Zhang, et al., 2009). In future studies, it may be of interest to determine if treadmill or wheel running exercise preconditioning effects the expression and/or activity of MRP-1, thereby contributing to the exercise-induced extrusion of DOX from cardiac cells.

5) In the current investigation, measurement of DOX accumulation was limited to the heart to determine a cardiotoxic relationship. Exercise resulted in reduction of DOX accumulation and preservation of cardiac function. However, DOX is known to cause toxicity in other tissues including kidney (Ray, Patel, Wong, & Bagchi, 2000), lung (Baciewicz, et al., 1991; Ray, et al., 2000) and liver (Mostafa, Mima, Ohnishi, & Mori, 2000). In future studies, it may be of interest to determine if exercise effects accumulation in other tissues including the liver, kidney, lung and skeletal muscle.

6) Using HPLC, DOX accumulation was measured at 5 different time points over the course of 9 days in sedentary and exercise preconditioned rats. However, pharmacokinetic parameters such as the area under the concentration-time curve, plasma clearance, distributive half-life, and volume of distribution were not included. The aforementioned pharmacokinetic parameters can be used to describe the movement of DOX throughout the body (Ahmed, et al., 2009; Staples, et al., 2010; Urva, et al., 2009). Future study is needed to determine the effect of exercise on these parameters thus allowing for the acquired data to then be compared to other pharmacokinetic models of typical behavior.

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APPENDIX A

Raw Data

Doppler mitral blood flow.

Group	Day	*N*	M-VTI (cm)	M-V_{max} (cm/s)	M-V_{mean} (cm/s)
SED+SAL	1	9	3.63 ± 0.12	91.85 ± 4.78	63.93 ± 2.94
SED+DOX	1	10	3.51 ± 0.17	78.30 ± 1.99*	54.07 ± 2.94*
	3	8	2.89 ± 0.21*	60.83 ± 4.37*	42.33 ± 3.24*
	5	7	2.75 ± 0.16*	73.48 ± 8.62*	48.10 ± 5.49*
	7	11	2.77 ± 0.20*	62.64 ± 4.68*	37.00 ± 4.36*
	9	13	2.72 ± 0.13*	55.62 ± 2.90*	32.38 ± 2.63*
TM+DOX	1	12	3.43 ± 0.11	85.69 ± 5.01	63.14 ± 3.88
	3	13	3.89 ± 0.14	94.77 ± 4.04	64.82 ± 2.36
	5	10	3.19 ± 0.22	93.23 ± 3.44	68.87 ± 3.46
	7	10	3.25 ± 0.12*	87.17 ± 6.15	64.37 ± 4.66
	9	6	3.29 ± 0.13*	100.11 ± 4.77	69.28 ± 3.03
WR+DOX	1	10	3.59 ± 0.16	84.70 ± 4.66	64.47 ± 3.32
	3	6	3.68 ± 0.18	96.00 ± 6.93	66.22 ± 4.21
	5	10	3.29 ± 0.11*	92.77 ± 4.91	65.67 ± 3.17
	7	8	3.38 ± 0.12	82.33 ± 4.37	59.54 ± 3.51
	9	7	3.34 ± 0.16	84.90 ± 7.03	57.43 ± 5.96

Values are M ± SEM
SED+SAL, n = 9; SED+DOX, n = 49; TM+DOX, n = 51; WR+DOX, n = 41
M-VTI = Mitral velocity time integral
M-V_{max} = Mitral maximal flow velocity
M-V_{mean} = Mitral mean flow velocity
* significant difference from SED+SAL

Doppler aortic blood flow.

Group	Day	*N*	A-VTI (cm)	A-V_{max} (cm/s)	A-V_{mean} (cm/s)
SED+SAL	1	9	2.90 ± 0.16	98.15 ± 2.95	56.07 ± 1.59
SED+DOX	1	10	2.54 ± 0.17	80.47 ± 4.52*	47.33 ± 2.78*
	3	8	2.10 ± 0.23*	67.71 ± 5.80*	42.00 ± 2.95*
	5	6	2.74 ± 0.23	64.67 ± 4.57*	40.06 ± 2.07*
	7	10	2.21 ± 0.19*	58.90 ± 4.15*	34.93 ± 2.23*
	9	12	2.81 ± 0.32	62.33 ± 5.52*	36.78 ± 3.01*
TM+DOX	1	12	2.50 ± 0.09*	84.11 ± 3.65	50.53 ± 2.08
	3	13	3.24 ± 0.21	91.49 ± 5.46	57.44 ± 2.84
	5	10	2.97 ± 0.14	103.50 ± 7.06	61.70 ± 3.74
	7	10	3.20 ± 0.15	96.67 ± 5.19	60.00 ± 2.74
	9	6	3.36 ± 0.26	93.56 ± 4.12	59.72 ± 2.68
WR+DOX	1	10	2.63 ± 0.13	94.37 ± 3.96	56.07 ± 2.30
	3	6	3.04 ± 0.26	86.39 ± 3.54	53.83 ± 2.49
	5	10	3.28 ± 0.14	94.27 ± 3.14	57.00 ± 2.71
	7	8	3.13 ± 0.28	83.29 ± 5.02	49.42 ± 2.79
	9	6	3.21 ± 0.25	87.17 ± 6.48	54.00 ± 3.10

Values are M ± SEM
SED+SAL, n = 9; SED+DOX, n = 46; TM+DOX, n = 51; WR+DOX, n = 40
A-VTI = Aortic velocity time integral
A-V_{max} = Aortic maximal flow velocity
A-V_{mean} = Aortic mean flow velocity
* significant difference from SED+SAL

M-mode echocardiography.

Group	Day	*N*	FS (%)
SED+SAL	1	5	67.13 ± 3.11
SED+DOX	1	11	56.50 ± 3.25*
	3	6	52.39 ± 1.79*
	5	5	56.48 ± 5.91*
	7	8	50.95 ± 2.72*
	9	8	50.47 ± 2.77*
TM+DOX	1	13	65.01 ± 1.73
	3	13	67.45 ± 2.69
	5	9	59.43 ± 3.39
	7	8	60.20 ± 3.86
	9	4	61.60 ± 7.28
WR+DOX	1	10	63.87 ± 3.03
	3	9	62.25 ± 2.83
	5	9	60.41 ± 2.46
	7	7	58.49 ± 2.09
	9	7	58.32 ± 4.33

Values are M ± SEM
SED+SAL, n = 5; SED+DOX, n = 38; TM+DOX, n = 47; WR+DOX, n = 42
FS = Fractional shortening
* significant difference from SED+SAL

Unpaced *ex vivo* cardiac function.

Group	Day	*N*	ESP	EDP	LVDP	dP/dt_{max}	dP/dt_{min}
SED+SAL	1	9	97.99 ± 2.61	0.51 ± 0.76	97.48 ± 2.53	2851.24 ± 99.46	-2154.38 ± 60.62
SED+DOX	1	13	101.76 ± 4.34	0.01 ± 0.48	101.74 ± 4.14	2894.12 ± 291.5	-2118.03 ± 331.6
	3	12	91.75 ± 4.41	2.56 ± 0.45	89.18 ± 4.30	2723.99 ± 131.6	-2134.89 ± 161.8
	5	11	85.85 ± 4.37*	1.25 ± 0.47	84.6 ± 4.65*	2567.01 ± 125.6*	-1961.95 ± 82.03*
	7	11	81.88 ± 6.66*	0.45 ± 0.82	81.43 ± 7.16*	2583.38 ± 245.7	-2077.90 ± 231.8
	9	14	61.35 ± 2.95*	0.62 ± 0.44	60.73 ± 2.93*	1954.08 ± 66.98*	-1523.75 ± 57.23*
TM+DOX	1	13	110.52 ± 3.32*	-1.55 ± 0.44*	112.06 ± 3.3*	3209.14 ± 82.37*	-2565.22 ± 98.99*
	3	12	103.56 ± 5.25	6.22 ± 0.49*	97.34 ± 4.83	2676.29 ± 100.6	-2115.69 ± 91.31
	5	10	100.91 ± 5.94	2.58 ± 1.01	98.33 ± 6.05	2826.56 ± 181.6	-2262.07 ± 160.8
	7	13	86.66 ± 3.15*	2.12 ± 0.60	84.54 ± 3.15*	2652.29 ± 120.0	-1942.06 ± 78.17*
	9	10	88.21 ± 3.63*	-0.40 ± 0.67	88.61 ± 3.98*	2517.73 ± 83.00*	-1958.84 ± 55.03*
WR+DOX	1	10	110.82 ± 3.49*	3.47 ± 0.47*	107.35 ± 3.46*	2799.00 ± 90.02	-2368.80 ± 65.85*
	3	9	98.25 ± 3.01	1.75 ± 0.90	96.50 ± 3.46	2713.57 ± 146.8	-2089.58 ± 108.2
	5	12	90.00 ± 4.26	0.81 ± 0.40	89.19 ± 4.19	2543.63 ± 110.9*	-2017.32 ± 83.82
	7	10	95.81 ± 4.90	-0.17 ± 0.37	95.98 ± 5.00	2882.18 ± 150.4	-2268.48 ± 131.9
	9	10	81.45 ± 2.35*	-0.87 ± 0.91	82.32 ± 1.69*	2376.79 ± 58.70*	-1891.85 ± 64.20*

Values are M ± SEM
SED+SAL, n = 9; SED+DOX, n = 61; TM+DOX, n = 58; WR+DOX, n = 51
ESP = End systolic pressure
EDP = End diastolic pressure
LVDP = Left ventricular developed pressure
dP/dt_{max} = Maximal rate of pressure developed
dP/dt_{min} = Maximal rate of pressure decline
* significant difference from SED+SAL

Paced *ex vivo* cardiac function.

Group	Day	*N*	ESP	EDP	LVDP	dP/dt_{max}	dP/dt_{min}
SED+SAL	1	9	112.13 ± 3.15	1.75 ± 0.85	110.38 ± 2.81	3450.68 ± 101.1	-2574.30 ± 118.2
SED+DOX	1	13	98.74 ± 3.15*	1.21 ± 0.57	97.52 ± 3.24*	3033.15 ± 276.1	-2264.74 ± 140.6
	3	12	94.49 ± 3.86*	3.08 ± 0.42	91.42 ± 3.93*	2952.32 ± 101.4*	-2292.44 ± 155.4
	5	11	82.18 ± 3.32*	1.76 ± 0.33	80.42 ± 3.44*	2691.47 ± 74.36*	-2021.69 ± 69.69*
	7	11	85.91 ± 5.60*	1.45 ± 0.77	84.46 ± 5.97*	2861.01 ± 176.5*	-2066.68 ± 130.5*
	9	14	71.49 ± 3.37*	1.03 ± 0.57	70.46 ± 3.28*	2401.42 ± 99.03*	-1803.16 ± 85.10*
TM+DOX	1	13	109.04 ± 1.91	-0.89 ± 0.47*	109.93 ± 1.87	3402.00 ± 78.50	-2751.75 ± 87.07
	3	12	106.18 ± 4.52	6.49 ± 0.66*	99.69 ± 4.17*	2960.59 ± 111.7*	-2202.45 ± 85.87*
	5	10	105.84 ± 5.79	2.88 ± 0.96	102.96 ± 5.98	3298.98 ± 300.9	-2405.33 ± 165.5
	7	13	92.17 ± 5.05*	2.59 ± 0.62	89.58 ± 5.00*	3009.52 ± 160.8*	-2233.95 ± 160.7
	9	10	94.20 ± 3.42*	0.37 ± 0.67	93.83 ± 3.76*	2997.16 ± 121.1*	-2139.66 ± 64.65*
WR+DOX	1	10	111.56 ± 3.75	4.28 ± 0.52*	107.28 ± 3.72	3279.88 ± 108.9	-2566.07 ± 114.6
	3	9	96.34 ± 1.37*	2.05 ± 0.72	94.29 ± 1.54*	2935.78 ± 95.73*	-2270.70 ± 71.68*
	5	12	99.47 ± 4.74*	1.25 ± 0.31	98.23 ± 4.64*	3029.98 ± 160.2*	-2357.55 ± 148.0
	7	10	104.24 ± 4.74*	-0.11 ± 0.28	104.34 ± 4.66	3343.82 ± 164.1	-2616.42 ± 149.5
	9	10	90.60 ± 2.34*	0.99 ± 0.90	89.61 ± 2.47*	2993.21 ± 149.9*	-2068.54 ± 80.99*

Values are M ± SEM
SED+SAL, n = 9; SED+DOX, n = 61; TM+DOX, n = 58; WR+DOX, n = 51
ESP = End systolic pressure
EDP = End diastolic pressure
LVDP = Left ventricular developed pressure
dP/dt_{max} = Maximal rate of pressure developed
dP/dt_{min} = Maximal rate of pressure decline
* significant difference from SED+SAL

Left ventricular DOX accumulation.

Day	SED+DOX (ng DOX/g LV)	TM+DOX (ng DOX/g LV)	WR+DOX (ng DOX/g LV)
1	702.16 ± 24.90[*]	416.88 ± 42.89 [a]	525.68 ± 30.61[a]
3	484.49 ± 36.14[*]	219.64 ± 21.61[a]	173.25 ± 11.57 [a]
5	178.15 ± 21.09[*]	83.94 ± 17.19 [a]	54.67 ± 7.82 [a]
7	97.37 ± 24.59[*]	N/D [a]	N/D [a]
9	N/D	N/D	N/D

Values are M ± SEM

SED+DOX, n = 65; TM+DOX, n = 61; WR+DOX, n = 61

N/D = Not detectable

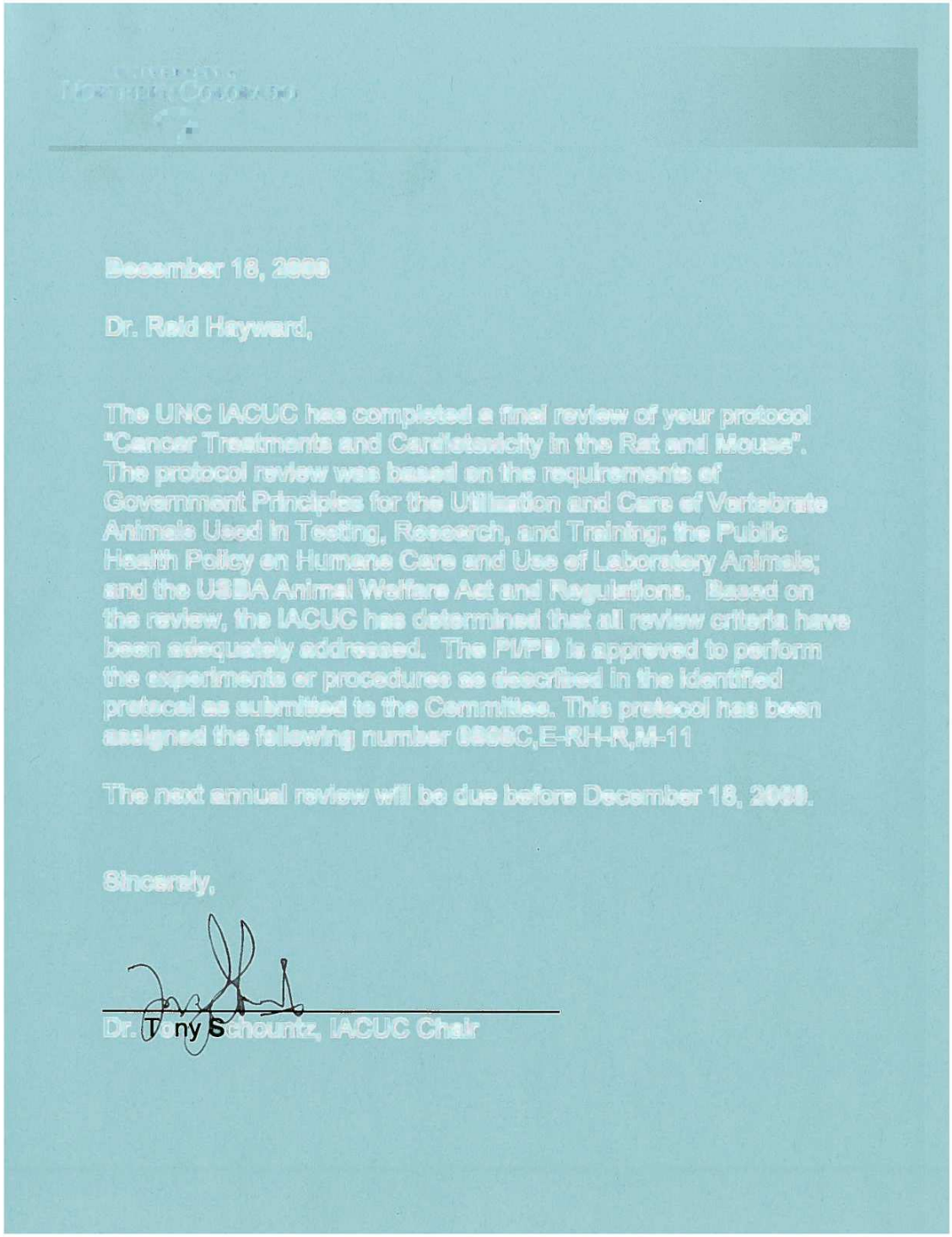
[*] Significant difference between groups ($p < 0.001$)

[a] Significantly less than SED+DOX ($p < 0.05$)

No significant differences were observed between TM+DOX and WR+DOX ($p > 0.05$)

APPENDIX B

Institutional Animal Care and Use Committee Approval Form



December 18, 2008

Dr. Reid Hayward,

The UNC IACUC has completed a final review of your protocol "Cancer Treatments and Cardiotoxicity in the Rat and Mouse". The protocol review was based on the requirements of Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the Public Health Policy on Humane Care and Use of Laboratory Animals; and the USDA Animal Welfare Act and Regulations. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. This protocol has been assigned the following number 08-C,E-RH-R,M-11

The next annual review will be due before December 18, 2009.

Sincerely,

Tony S

Dr. Tony Schountz, IACUC Chair