EFFECT OF DOXORUBICIN-INDUCED APOPTOSIS ON GENDER

By

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This thesis is dedicated to:

Eun-ah Lee, for her love and support. I'm extremely lucky to have her in my life.

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Doxorubicin is a powerful anthracycline antibiotic used to treat a multitude of human neoplasms. However, doxorubicin causes severe cardiac toxicity, which compromises its clinical usefulness. Females are believed to be better protected against cardiovascular insults. This study examined the gender differences in doxorubicin-induced apoptosis. We administered doxorubicin at clinical levels (10mg/kg of body weight) to male and female rats. After one day and four days later, we measured the oxidant production and examined different apoptotic pathways. Females produced less oxidants in isolated mitochondria compared to males and were able to scavenge oxidant faster than male rats.

We assessed the apoptotic index by measuring DNA fragmentation. Male rats had a significantly increased level of apoptosis one day after doxorubicin treatment, but no changes were seen in females rats in both one and four days after the treatment. The effectors of apoptosis, caspase-3 activity were significantly increased at day four, in both

males and females. These data suggest that caspase-independent pathway might be involved in doxorubicin-induced apoptosis.

Mitochondrial-mediated pathway was not involved in activating capase-3 at day four. Cytochrome *c* release was prevented by anti-apoptotic protein Bcl-2 at day four in males. No changes were detected in receptor-mediated pathway. The initiator caspase-8 and its inhibitor cFLIP did not change in response to doxorubicin administration. These findings suggest that doxorubicin induces apoptosis through other novel apoptotic pathways such as sarcoplasmic reticulum mediated pathway.

A better understanding of gender difference in doxorubicin-induced pro- and anti-apoptotic signaling pathways in cancerous and non-cancerous cells may lead to new and improved therapeutic protocols for mitigating the toxic side effects of doxorubicin.

CHAPTER 1
INTRODUCTION

Introduction

The anthracycline antibiotic doxorubicin (adriamycin) is one of the most effective chemotherapeutic agents for treating human neoplasms such as leukemia, lymphomas, breast cancer, and many solid tumors. However, chronic use can become associated with acute and chronic cardiotoxicities (1). The cardiotoxicity is dose dependent and causes irreversible myocardial damage, resulting in dilated cardiomyopathy with fatal congestive heart failure (1, 2). The exact mechanism of doxorubicin-induced cardiomyopathy remains unclear, but most of the evidence indicates that reactive oxygen species (oxidants) are involved (3). It is believed that mitochondrial derived oxidants play a significant role in triggering this toxicity (4, 5). Isolated heart mitochondria have been shown to shuttle single electrons to doxorubicin, giving rise to oxygen radicals through the autoxidation of adriamycin semiquinones. Evidence suggests that NADH dehydrogenase associated with complex I of the electron transport chain is intrinsically involved in this one electron transfer to doxorubicin further generating free radicals (oxidants) (6). Reactive oxygen species have been reported to cause irreversible tissue damage by inactivating key proteins and enzymes present in cardiac sarcoplasmic reticulum and mitochondria, and they are also believed to induce apoptosis (7).

Apoptosis is an evolutionary conserved form of cell suicide through which multicellular organisms eliminate redundant, damaged, or infected cells (8-10). The central component of this form of cell death is a proteolytic system involving a family of

cysteine proteases called caspases (11). Interest in the control of apoptosis has grown significantly since the realization that disturbed apoptosis may contribute to cancer, degenerative diseases, and cardiomyopathies with the chronic use of doxorubicin (12, 13). Investigations in experimental animal models have demonstrated that apoptosis is one of the mechanisms of myocyte cell death with ischemic cardiac injury and heart failure (13). Further, apoptosis is now being implicated in anthracycline induced cardiotoxicity (Figure 1-1), which is one of the major limitations to the use of this otherwise highly efficacious antineoplastic drug (7).

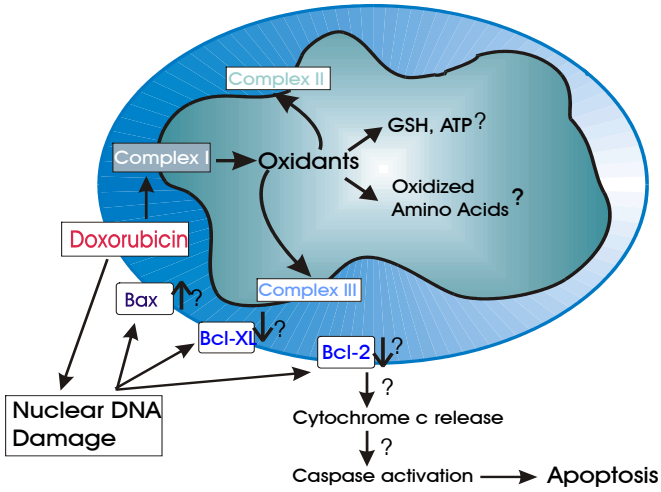

Figure 1-1. Doxorubicin-induced mitochondrial damage and apoptosis.

Oxidants, such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\bullet}$), and peroxynitrite ($ONOO^-$) may be generated by doxorubicin toxicity. Oxidants will affect mitochondrial redox status and may cause extensive oxidative damage to proteins. Furthermore, the mitochondrial transition pore can open releasing cytochrome c and possibly affecting mitochondria function. Cytochrome *c* can activate cytosolic caspases to induce apoptosis. Doxorubicin may induce anti-apoptotic (Bcl-2, Bcl-X_L) and pro-apoptotic (Bax, Bad) proteins.

Compelling evidence from several epidemiological and clinical studies indicates a substantially higher incidence of heart failure and cardiovascular diseases in men compared with age-matched women (14-16). The basis for these differences have been attributed in part, to the cardioprotective effects of estrogen (17). The assumption that

female sex hormones are largely responsible for the low incidence of cardiovascular disease in pre-menopausal women arises from the absence of similar cardiovascular protection in post-menopausal women, an occurrence paralleled by declining estrogen levels (16). However, many other mechanisms may exist and this requires further investigation. The benefits of estrogen gradually shift from the vascular system to the myocardium (15). This view is supported by the fact that functional estrogen receptors have been detected in the myocardium (18). In addition, females have higher levels of telomerase activity after adulthood and therefore an enhanced ability to preserve cardiac myocytes viability following an injury. This may increase the potential for growth and could certainly become critical factor to ensure an longer life span (19). In recent years, several investigations have documented that female hearts are inherently protected by estrogen against apoptotic cell death (20). It is speculated that estrogen reduces the activity of ICE-like protease caspase-3, which is an effector / downstream mediator of apoptosis (18, 21). In addition, gender differences in myocardial activation of Akt/PKB can also subsequently inhibit apoptosis by phosphorylating the Bcl-2 family member Bad and caspase-9, which are key components of the intrinsic cell death machinery (22).

Although much of the existing research has focused on trying to unravel the mechanisms responsible for doxorubicin induced cardiotoxicity and the numerous ways estrogen may afford protection to the cardiovascular system in females, no experiments have been carried out to determine if male and female hearts respond differently to an acute dose of doxorubicin. Therefore, we attempted to determine this possibility in these proposed experiments.

Specific Aims

This study will investigate whether there are sex differences following doxorubicin treatment in oxidant production, mitochondrial function, pro- and anti- apoptotic proteins and the overall incidence of apoptosis in male and female Fischer 344 rats.

Question 1

Are there sex differences in oxidant production, mitochondrial function and responses by antioxidant defenses one day and four days after doxorubicin administration?

Hypothesis 1

Female rats will have less oxidant production, less mitochondrial dysfunction, and an enhanced antioxidant defense adaptation following doxorubicin treatment.

Question 2

Are there differences in the overall incidence in apoptosis and caspase-3 activation between male and female rats following administration of doxorubicin?

Hypothesis 2

Female hearts will have exhibited less apoptotic cell death and caspase-3 activation.

Question 3

Are there differences in the adaptation of pro- and anti-apoptotic regulatory proteins in response to doxorubicin cardiotoxicity?

Hypothesis 3

Female hearts will have higher expression of anti- apoptotic proteins as compared to male hearts, which may partly explain differences in apoptosis.

CHAPTER 2
REVIEW OF LITERATURE

Doxorubicin and Cardiotoxicity

Doxorubicin (Adriamycin) is a powerful anthracycline antibiotic, originally isolated from the fungus *streptomyces peucetius*. It is used to treat many human neoplasms, including acute leukemias, lymphomas, stomach, breast and ovarian cancers, Kaposi's Sarcoma, and bone tumors (23). However, doxorubicin causes severe cardiac toxicity, which compromises its clinical usefulness (24). Chronic toxic effects of doxorubicin often develop after several weeks or months of treatment, and sometimes even 4 to 20 years after discontinuation of the treatment. Thus, the risk of developing heart failure in cancer patients treated with doxorubicin remains a life-long threat (2).

Since the first report of doxorubicin-induced cardiomyopathy (25), extensive clinical as well as basic research efforts have been focused on understanding the pathophysiology of congestive heart failure caused by this drug. A number of mechanisms have been proposed to explain the development of doxorubicin-induced cardiomyopathy including direct DNA damage and interference with DNA repair (26), change in adrenergic function (27), abnormalities in the mitochondria (28), lysosomal dysfunction (2), altered sarcolemmal Ca^{2+} transport, Na^{+}-K^{+} ATPase and Ca^{2+}ATPase, imbalance in myocardial electrolytes (2), free radical formation (2, 28, 29), reduction in myocardial antioxidant enzyme activities (29), lipid peroxidation (30), and apoptosis (28, 29, 31). Furthermore, the cytotoxic action by doxorubicin involves the cytoskeleton of both tumor cells and cardiomyocytes (32). Cytoskeletal changes following doxorubicin

administration include reduction in density of myofibrillar bundles (33), alterations on the Z-disc structure (33), and disarray and depolymerization of actin filaments (33, 34). This list demonstrates that the cause of doxorubicin-induced cardiomyopathy is probably multifactorial and the mechanism is complex. However, most of these changes have an underlying cause, which is highly likely to be damage from reactive oxygen species. Moreover, damage to either the mitochondria or the sarcoplasmic reticulum could create a favorable environment for the induction of apoptosis (28).

Doxorubicin and Oxidants

Heart mitochondria are thought to play an important role in mediating oxidative damage. The enzyme NADH dehydrogenase, a major player in transferring electrons to harmful oxidants interacts with doxorubicin. The quinone ring of doxorubicin, a part of its tetracyclic moiety, undergoes redox cycling between quinone and semiquinone. During this process, oxidizing agents, including oxygen, capture free generated electrons and then initiate a chain reaction that leads to the generation of superoxide anion production. The superoxide anion radical generated can undergo dismutation to hydrogen peroxide. Specifically, in the presence of redox active free metal ions, formation of the highly reactive hydroxyl radical could account for membrane damage (35). If subjected to oxidant insult, mitochondria can then be triggered to release cytochrome c and other apoptogenic factors that activate a cascade of initiator and effector caspases to induce apoptosis.

Apoptosis

Adult cardiomyocytes are post-mitotic cells that once destroyed, are slowly replaced. Consequently, their loss can contribute to the functional decline of the myocardium leading to heart disease (36). Until recently, the mode of cell death involved

in cardiomyocyte loss was not clear and was attributed to necrosis (13). It is now believed that apoptosis is also involved in cardiomyocyte cell death, and it plays an important role (37).

Necrosis is a rapid and irreversible process that occurs when cells are severely damaged. Necrosis involves swelling of the cell and its organelles, disruption of mitochondria, membrane rupture, and cell lysis (38). It is a destructive process, as release of cellular content into the surrounding environment can cause further damage or death to neighboring cells. Apoptosis, on the other hand, is a highly organized, energy dependent mechanism whereby a cell commits suicide without causing damage to surrounding tissue and it occurs normally during development, tissue turnover, and in the immune system (12). Apoptosis is characterized by cellular condensation while maintaining intra-organelle integrity, membrane blebbing, DNA fragmentation into oligo- and mono-nucleosomes, destruction of the cytoskeleton, and formation of apoptotic bodies which are endocytosed by macrophages and neighboring cells (12). The apoptotic process is mediated by the activation of cysteine proteases known as caspases, which cleave each other and other proteins after an aspartate residue within a specified amino acid sequence (11). There are approximately 14 caspases identified that participate in the apoptotic process depending on the signaling pathways (11).

Various stimuli such as oxidative stress (29, 39), mitochondrial dysfunction (28), elevated intracellular Ca^{2+}, excessive DNA damage and various cytokines can induce apoptosis, and different signaling pathways have been described in various cell types. The two most widely studied pathways in cardiac myocytes are the mitochondrial mediated and the death receptor mediated pathways.

Mitochondrial Mediated Pathway

Mitochondrial dysfunction is a well-known stimulus for mitochondrial-mediated apoptosis initiating the release of apoptogenic factors from the mitochondria. Release of cytochrome *c*, as well as other apoptogenic factors, from mitochondria to the cytosol initiates mitochondrial apoptosis (28). Cytochrome *c* then forms an apoptosis initiating complex with apoptosis protease-activating factor (Apaf-1), dATP and procaspase-9, resulting in the self-cleavage and activation of caspase-9 (28). The active caspase-9 then cleaves and activates procaspase-3. This, in turn, activates a cascade of caspases, internally breaking down the cell (11). Some of the targeted proteins of caspase-3 are procaspase-6 and procaspase-7, poly ADP ribose polymerase (PARP), inhibitors of DNA fragmentation factor (DFF) and caspase activated DNase (CAD) resulting in DNA fragmentation (11).

The Bcl-2 family of proteins regulates the release of cytochrome *c* (40). These include Bax, Bad, and Bid, which are pro-apoptotic proteins that favor cytochrome *c* release and Bcl-2 and Bcl-$_X$L which are anti-apoptotic proteins that inhibit cytochrome *c* release (40, 41). The mechanisms of how Bcl-2 family proteins regulate release of cytochrome *c* and apoptogenic factors are under investigation. Some of the hypotheses include: 1) physical rupture of the outer membrane, 2) a channel formed by pro-apoptotic Bcl-2 family proteins such as Bax, and 3) opening of a pore via a membrane permeability transition pore (MPT) characterized by loss of the mitochondrial membrane potential (11, 41).

The mitochondria also release other apoptogenic factors such as apoptosis inducing factor (AIF) which is a caspase-independent mitochondrial death effector (42). AIF can only be released upon MPT. These pro-apoptotic factors translocate to the nucleus where

they induce DNA chromatin condensation and large-scale DNA fragmentation into approximately 180-200 kbp. Previous studies have confirmed this observation in a variety of cell types (40, 42).

Death Receptor Mediated Pathway

Various receptors can mediate apoptosis such as tumor necrosis factor receptor 1 TNFR1) and Fas/CD95. Ligand binding to TNFR1 or Fas can induce apoptosis in an effector cell by the activation of procaspase-8, which cleaves and activates procaspase-3 to initiate the caspase cascade (43). Alternatively, binding of ligand to TNFR1 can induce a pro-inflammatory/anti-apoptotic response mediated through the cytosolic transcription factor NF-κB. The presence or recruitment of adaptor proteins to TNFR1 determines the outcome: caspase activation or NF-κB activation. Thus, whether the cell chooses a survival pathway or a death pathway in response to TNFR activation depends on the interaction of various signaling pathways and regulators of these pathways (43).

It has been shown that Fas antigens were overexpressed in myocytes of dilated-cardiac myopathies, chronic heart failure, and myocardial infarction (36). Nakamura *et al.* (44) observed apoptotic cell death via Fas-mediated pathway in adriamycin-induced cardiac myopathy rats.

Regulators of Apoptosis

Apoptosis is a sequential, multi step process made up of many different layers of regulation. This is of particular importance in post-mitotic cardiac myocytes in order to avoid unnecessary death of salvageable cells and to promote apoptosis in response to irreversible cellular damage, as opposed to necrosis, which could further harm the myocardium. The final steps of apoptotic death are highly conserved and likely to be

mediated by a similar set of caspases. Various inhibitory regulatory mechanisms exist within cells that target caspases. These include cFLIP and the inhibitors of apoptosis proteins (IAP) family (40). These inhibitors are present in various cell types and may also play an important role in the heart (13). The known IAPs include XIAP, cIAP, and cIAP2 (40). It is believed that these IAPs bind to cleaved/activated caspases and inhibit their activity. XIAP is consired one of the most active inhibitors of capse-3. Alternatively cFLIP inhibits the activity of caspase-8 and is highly expressed in the heart under normal physiological conditions but is degraded after ischemia/reperfusion (45).

Recently an inhibitor of apoptosis that is expressed almost exclusively in skeletal muscle and heart has been characterized. ARC (apoptosis repressor with caspase recruitment domain) was first shown to interact with caspase-8 and -2 and to attenuate apoptosis induced by stimulation of death receptors (46). More recently it was demonstrated that ARC inhibits cytochrome *c* release from mitochondria and protects against hypoxia-induced apoptosis (47). This study suggested that ARC can exert its effect at different levels in the apoptotic pathway and may be a key regulator of apoptosis in the heart.

Gender Related Differences in the Cardiovascular System

There are significant sex differences in the incidence of a variety of cardiovascular diseases and acute myocardial injuries (16). In recent years, several lines of evidence strongly suggest that loss of myocytes occur with heart failure, ischemia/reperfusion injury and aging. This loss of cardiac cells may occur because of both necrosis and apoptosis. This emerging concept of cardiac myocyte death by apoptosis may have important implications in terms of studying gender-based differences. It is well established that the female ovarian steroid hormone estrogen has strong cardio-protective

properties (48). Possible mechanisms behind this protectection might include systemic effects such as improvement in the lipid profile, reduction in lipid peroxidation, and stimulation of endothelium dependent NO production (18). Grohe *et al.* (18) demonstrated through immunofluorescent assays that cardiac myocytes and fibroblasts express functional estrogen receptor proteins in both male and female rats. Biological effects of estrogen generally require the presence of estrogen receptor, a ligand dependent transcriptional factor that regulates the expression of genes transduced by estrogen. However, additional mechanisms, may exist to explain the increased tolerance of female rats to a myocardial insult (48).

Gender Differences in Apoptosis

There is evidence that estrogen plays an important role in modulating certain cell-death related signals to inhibit apoptosis. Using estrogen (17β estradiol) at physiological concentration has shown to inhibit apoptosis in cardiac myocytes (17). Pelzer *et al*.(17) studied apoptosis in cultured cardiac myocytes induced by staurosporine, a tyrosine kinase inhibitor and a potent pro-apoptotic agent. A significant reduction in apoptosis was observed in cells that were simultaneously treated with estrogen and staurosporine. 17β-Estradiol has been found to reduce the activity of ICE-like protease caspase-3 an effector and downstream mediator of apoptosis. Moreover, Camper-Kirby and coworkers (22) recently reported a significant difference between sex in myocardial activation of Akt. Both localization of phospho Akt in the myocardial nuclei and cytosolic localization of phospho-forkhead, a downstream nuclear target of Akt, were found elevated in sexually mature female mice compared to that in male mice. Akt, also known as Protein Kinase B, is a serine/threonine kinase, which lies at the intersection of multiple cellular

signaling pathways involved in the regulation of glucose metabolism, gene transcription, and cell survival (49). It is a down stream effector molecule for signal transduction initiated by survival factors such as IGF-1.

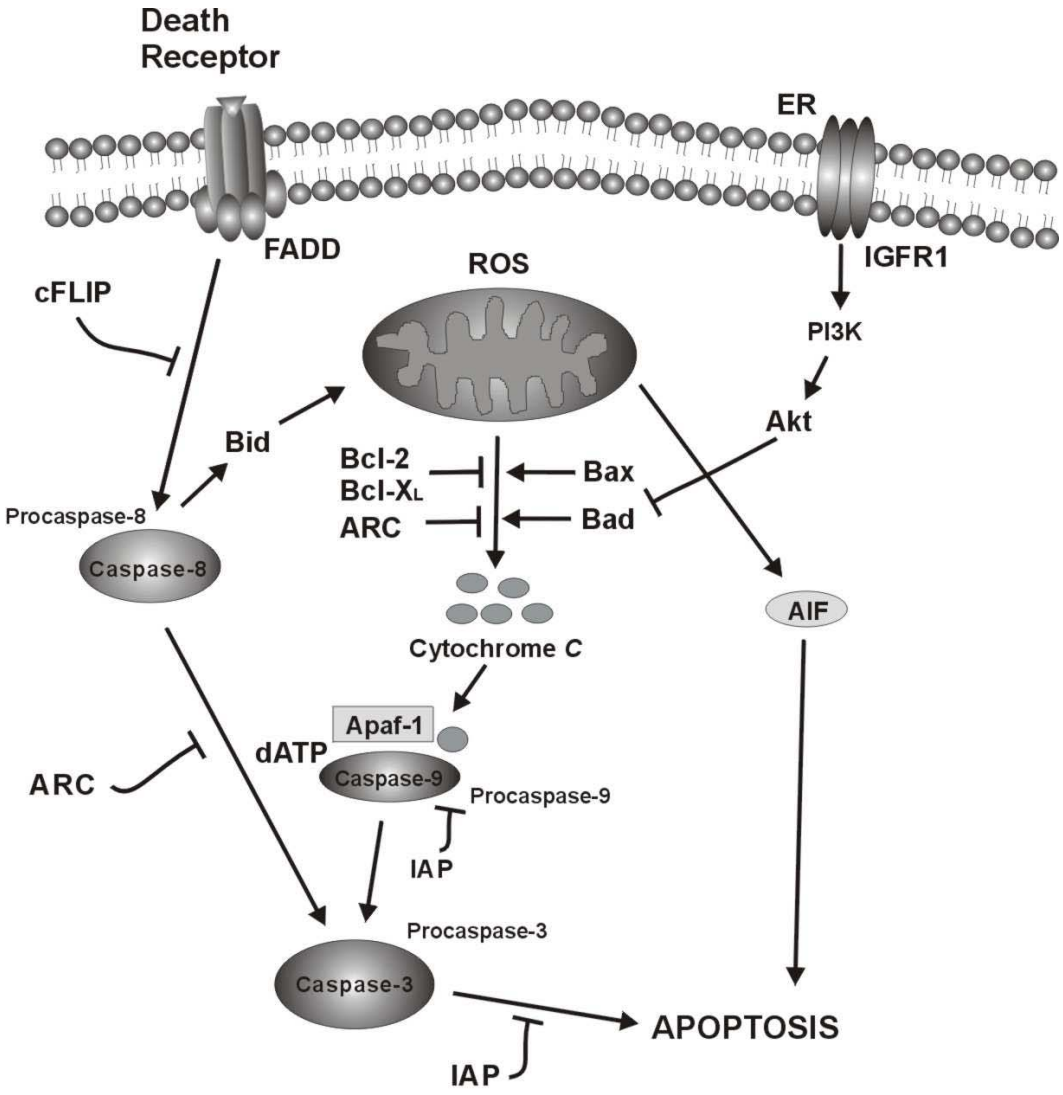

Figure 2-1. Overview of apoptotic pathways.

These factors bind to their respective cell surface receptors triggering the activation of several kinases including the PI3K. This pathway then activates Akt through phosphorylation which can subsequently inhibit apoptosis by phosphorylating Bcl-2 family member Bad and caspase-9, both of which are components of intrinsic cell death

machinery and the forkhead transcription factor (FKHRL1). FKHRL1 in a dephosphorylated state translocates to the nucleus where it induces target genes such as Fas ligand and triggers apoptosis. Hence, estrogen, by bringing about Akt-dependent phosphorylation and inactivation of FKHRL1 (Figure 2-1), suppresses the transcription of death genes and promotes survival (50).

Summary

A great deal of effort has been expended in trying to prevent or mitigate the cardiotoxic side effect of doxorubicin. However, it is imperative that any method designed to minimize the cardiotoxic effect of doxorubicin also maintains its antineoplastic efficacy. Surprisingly, no studies have been conducted to examine if sex differences exist in oxidative stress and apoptosis following the use of this drug.

CHAPTER 3
METHODS

Animals and Experimental Design

Male and female Fisher 344 rats (National Institute of Aging colony, Harlan Sprague Dawley, Indianapolis, IN) were used in this study. The animals were housed individually in a temperature (18-22°C) and light-controlled environment with a 12-hour light/dark cycle and were provided with food and water *ad libitum*. Animals from both male and female groups were randomly assigned either to a control or to a doxorubicin-treated group. Doxorubicin hydrochloride (Sigma Chemical Co., St. Louis, MO) was dissolved in saline and administered by intraperitoneal injection at a dose of 10 mg/kg to the group receiving treatment. Male and female animals were sacrificed one day after doxorubicin injection (n= 12). In order to see if there were adaptations additional groups of male and female rats were sacrificed four days after the injection (n=12). The control group was injected with an equal volume of saline and they were sacrificed on day one (n=12) and day four (n=12).

Tissue Harvesting

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight). The chest was opened and blood removed directly by cardiac puncture. This was followed by severing the inferior vena cava and perfusion of the heart with 10 ml of ice-cold antioxidant buffer containing 100 μM diethylenetriaminepentaacetic acid (DTPA), 1 mM butylated hydroxytoluene (BHT), 1%

ethanol, 10 mM 3-aminotriazole, and 50 mM $NaHPO_4$ (pH 7.4). After perfusion, the entire heart was excised, rinsed in antioxidant buffer to remove any remaining blood, blotted dry, and weighed.

Cellular Fractionation

Isolation of Mitochondrial and Cytosolic Fractions.

The atria of the heart was removed and stored at –80°C until analysis. A 500mg portion of the left ventricle was used for isolation of mitochondria. Tissue was weighed and minced in 5 volumes of isolation buffer (0.225M mannitol, 0.075 M sucrose, 0.2% fatty acid free bovine serum albumin, pH 7.4). The tissue was homogenized in a Potter-Elvehjem glass homogenizer and centrifuged for 10 minutes at 700g. The resulting supernatants were centrifuged again for 10 minutes at 8,000g. The supernatant (cytosolic fraction) was aliquotted and stored at –80°C. The mitochondrial pellet was resuspended in 5 mL of isolation buffer and centrifuged for 10 minutes at 8000g. The final mitochondrial pellet was resuspended in 1 mL of isolation buffer, aliquotted and stored for later analyses.

Protein Concentration

Cytosolic and mitochondrial protein concentrations was determined using the method developed by Bradford (51).

Mitochondrial Functional Parameters

Mitochondrial Respiratory Function

To assess mitochondrial damage due to isolation procedures, we calculated the respiratory control ratio (state 3 respiration / state 4 respiration), which is commonly used as an index for mitochondrial function. Mitochondrial respiration was monitored at 37°C in incubation buffer (145 mM KCL, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1

mM EGTA, 0.1 % fatty-acid free albumin, pH 7.4), 2.5 mM pyruvate, 2.5 mM malate, and 0.25 mg mitochondrial protein for a total volume of 500 µl. State 4 respiration (no ADP) was read for 2 minutes and monitored using a chart recorder. State 3 respiration (with ADP) was measured in the presence of 500 µM ADP for 10 minutes or until the oxygen pressure equals zero. Oxygen consumption was calculated as ng atom O_2 consumed/mg protein/minute. Respiratory measurements were completed within 3 hours after mitochondrial isolation and performed in duplicate.

ATP Content and Production

ATP production in isolated mitochondria was measured using a luminometer (model TD-20/20, Turner Designs, Sunnyvale, CA). The assay uses firefly luciferase, which fluoresces in proportion to the presence of ATP. Freshly isolated mitochondria were added to a cuvette containing 1 mM ADP, 1 mM pyruvate, 1 mM malate, and a Luciferin-Luciferase ATP monitoring reagent (ATP Determination Kit A-6608, Molecular Probes, Eugene, OR). A blank cuvette containing no metabolic substrate was assayed to account for nonspecific ATP production. A known ATP concentrations was used to establish a standard curve. Results were expressed as nmol ATP produced/mg protein/minute. The P/O ratio used as an index of mitochondrial efficiency was calculated by taking nmol ATP produced/mg protein/min divided by ng atoms of oxygen consumed/mg protein/min of state 3. The P/O ratio represents the number of ADP molecules phosphorylated per mole of oxygen atoms consumed.

Mitochondrial Hydrogen Peroxide (H_2O_2) Production

The rate of mitochondrial H_2O_2 production was measured according to Barja (52) using a fluorescent microplate reader (GeminiXS, Molecular Devices, Sunnyvale, CA).

Incubation buffer (145 mM KCl, 3 mM $MgCl_2$, 5 mM KH_2PO_4, 30 mM Hepes, 0.1 mM EGTA, 0.1% BSA, pH 7.4), mitochondria (0.25 mg/ml), horseradish peroxidase (5.7 U/ml), and homovanilic acid (0.1 mM) were added to test tubes. The reaction was initiated by the addition of pyruvate and malate (2.5 mM each) and the tubes are placed in a shaking water bath for 15 minutes at 37°C. The reaction was stopped by placing tubes on ice and adding 0.5 ml of stopping solution (0.1 M glycine, 25 mM EDTA, pH 12.0). Fluorescence was determined using a fluorescent microplate reader and a standard curve was generated for each analysis using glucose-glucose oxidase.

Biochemical Assays

Estrogen (Estradiol)

Plasma estradiol concentration was measured using radioimmunoassay (RIA) kit at Yerkes Endocrine Laboratory (Atlanta, GA). All samples and standards were measured in duplicates.

Cytosolic Mono- and Oligo-nucleosomes

DNA fragmentation was quantified by measuring the content of cytosolic mono- and oligonucleosomes (180 base pair nucleotides or multiples) using a Cell Death ELISA (Roche Molecular Biochemicals, Germany) according to instructions from the manufacturer. The assay is based on the quantitative sandwich-enzyme-immunoassay-principle. Wells are coated with a monoclonal anti-histone antibody. Nucleosome in the sample binds to the antibody followed by the addition of anti-DNA-peroxidase, which reacts with the DNA, associated with the histones. The amount of peroxidase retained in the immunocomplexes is determined photometrically with ABTS (2.2' –azino-di-[3-ethylbenzthiazoline sulfonate]) as a substrate. All samples were read in triplicates. Results were reported as arbitrary OD units/mg protein.

Western Blots Analysis

Proteins were separated on a 4-20% precast polyacrylamide gel (BMA, Rockland). Proteins were transferred onto a nitrocellulose membrane. Nitrocellulose membranes were blocked overnight using a blocking solution containing 0.05% Tween and 5.0% dry milk. Membranes were incubated for 90 minutes with the primary antibody at the desired dilution. Membranes were incubated for 90 minutes in anti-rabbit or mouse Ig horseradish peroxidase (Amersham Life Science) with an appropriate dilution. Blots were developed using ECL (Amersham Pharmacia Biotech, England). The protein bands were analyzed using Kodak Image Station 440CF (Eastman Kodak, Rochester, NY).

Cytosolic Cytochrome *C*

Cytosolic cytochrome c was quantified using an ELISA kit from R&D Systems (Minneapolis, MN) which employs the sandwich enzyme immunoassay technique. Data were reported as nmol/mg cytosolic protein.

Caspase-3 Activity

Caspase-3 activity was measured using the synthetic peptide n-acetyl-DEVD-AMC (BD PharMingen, San Diego, CA). This assay detects activated caspase-3 and, to a lesser extent, caspases -6, -7, and -8. Active caspases cleave the AMC from the peptide and the free AMC fluoresces. Standards of active caspase-3 were prepared. Briefly, 1 mL of assay buffer (20mM HEPES, 10% glycerol, 1 M DTT, and 14 μL of Ac-DEVD-AMC/mL of buffer) and 50 μL of sample will be added to a microcentrifuge tube and protected from the light. Samples were incubated at 37°C for 60 minutes after which fluorescence will be measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

Caspase-3 Content

A Western blot protocol was used to detect the full length and the activated (cleaved) caspase-3. A polyclonal anti-caspase-3 antibody (Stressgen, Canada) was used.

Caspase-8

A Western blot protocol was used to detect the full length and the activated (cleaved) caspase-8. A polyclonal anti-caspase-8 antibody (Steersmen, Canada) was used.

Inhibitors of Apoptosis (XIAP, FLIP, ARC)

Endogenous inhibitors of apoptosis were measured using Western blot. The cytosolic fraction was used to assess the content of these inhibitors. The following antibodies will be used: monoclonal XIAP (MBL, Watertown), polyclonal ARC (Ab-1) (Oncogene, Boston) and antiserum FLIP (Alexis, San Diego).

Bcl-2 and Bax

The content of Bax and Bcl-2 were measured using Western blots. The following antibodies were used: polyclonal Bax (Ab-1) and polyclonal Bcl-2 (Ab-4) (Santa Cruz Biotechnology).

Antioxidant Enzyme Assay

Glutathione Peroxidase Activity

Selenium-dependent glutathione peroxidase activity was assayed according to Nakamura *et al.* (53) with modification, using H_2O_2 as the substrate.

Statistical Analysis

Two-way ANOVA was used for comparisons between groups. Bonferroni *post hoc* test was performed if significance was found. A p-value of <0.05 were considered significant.

CHAPTER 4
RESULTS

Morphological Characteristics

We determined if there were sex differences in body weight, heart weight, heart weight-to-body weight ratio (Table 4-1), and estradiol levels (Table 4-2). The body weights of the male rats were significantly greater than in the female rats ($p < 0.05$). Moreover, a significant gender difference was observed in heart weight. The hearts of males were (36%) greater then female hearts ($p<0001$). However, the female rats had a significantly higher heart weight-to-body weight ratio ($p<0.001$). Furthermore, body weight was not different one day after doxorubicin treatment in male or female rats. However, there was a significant decrease (10%) in body weight four days after doxorubicin treatment in the male rats ($p=0.004$), but not in female rats. Furthermore, the heart weights of the male and female rats remained unaltered 1 day following doxorubicin treatment. However, in both sexes, there was a significant decrease in heart weight four days after doxorubicin treatment. The males showed a 15% decline ($p<0.001$) and the females showed a slightly smaller (13%) decrease ($p<0.05$). The heart weight-to-body weight ratio in male and female rats was not affected after 1 day or 4 days after doxorubicin treatment (Table 4-1).

Table 4-1. Body weight, heart weight, and heart weight to body weight ratio of male and female rats treated with doxorubicin or saline.

	Male			Female		
	Control	**Dox Day 1**	**Dox Day 4**	**Control**	**Dox Day 1**	**Dox Day 4**
Body weight	358.8±31.0	357.2±24.0	323.9±25.0†	197.6±10.0 *	190.6±14.0 *	176.0±7.0 *
Heart weight	0.81±0.07	0.78±0.05	0.69±0.04†	0.52±0.03 *	0.51±0.05 *	0.45±0.01† *
HW:BW	0.0022± 0.0001	0.0021± 0.0008	0.0023± 0.0007	0.0026± 0.0001 *	0.0027± 0.0002 *	0.0026± 0.0009 *

Body weight, heart weight, and the heart to body weight ratio (HW:BW) of male (n=12) and female rats (n=12) sacrificed one day and four days after administration of 10mg/kg doxorubicin or equal volume of saline (Mean ± SEM). Male (n=12) and female (n=12) animals were injected with equal volume saline on day1 (n=6) and day 4 (n=6). *Significant sex difference (p<0.05). †Significantly different from saline injected rats (p<0.05). Units: gram.

Plasma Estrogen Levels in Male and Female Rats

17-Beta estradiol was measured in the plasma using a radioimmunoassay (RIA) method to determine the differences of this sex hormone between the male and female rats. Moreover, since estradiol has cardio-protective effects we wanted to know the variability with each group. Surprisingly, there was only a 20-30% difference between young male and female rats (p<0.05). Furthermore, the variability between the individual female rats was only approximately (20-30%), with a maximum level of estradiol of 13.8 pg/mL and the minimum level of 5.4 pg/mL.

Table 4-2. Plasma 17-β estradiol level.

	Control	**Dox Day 1**	**Dox Day4**
Male	5.7±0.6	6.0±1.5	6.1±0.7
Female	8.4±2.7*	8.7±2.4*	7.0±1.4*

Plasma estrogen (estradiol) in male and female rats sacrificed one day and four days after injection with 10mg/kg doxorubicin vs. controls (Mean±SEM). * Significant sex difference (p<0.05). Units: pg/ml

Mitochondrial Function

We determined the effect of doxorubicin treatment on mitochondrial function in male and female rats (Table 4-3). Mitochondrial oxygen consumption was measured in state 4 (no ADP) and during state 3 respiration (0.5 mM ADP). There were no sex differences between State 4 and State 3 measurements. Moreover, doxorubicin treatment following either 1 day or 4 days did not effect State 4 or State 3 respiration. The amount of ATP produced by the mitochondria was used as a parameter to determine sex differences in energy production and as a parameter to assess mitochondrial function following doxorubicin administration. There was a significant sex difference in ATP production; the female rats produced significantly less ATP per milligram of protein of freshly isolated mitochondria determined in State 3 (p=0.0032). However, rates of ATP production in isolated mitochondria one day or four days after doxorubicin treatment were not different from those of controls. The P/O ratio, which compares the amount of ATP that is phosphorylated with the amount of oxygen consumed, also did not differ between groups.

Table 4-3. The effects of doxorubicin administration on mitochondrial function.

	Male			Female		
	Control	**Dox Day 1**	**Dox Day 4**	**Control**	**Dox Day 1**	**Dox Day 4**
State 4 VO_2	8.40±1.36	3.01±5.10	8.49±2.51	9.35±3.04	7.20±4.75	8.73±3.14
State 3 VO_2	42.21±28.61	47.51±26.1	34.62±24.81	31.78±22.46	25.94±17.18	21.08±6.74
ATP	31.75±8.81	38.08±6.84	33.32±8.47	27.81±8.74 *	26.75±12.37 *	23.42±6.33 *
P/O Ratio	1.15±0.37	1.06±0.52	1.48±0.80	1.34±0.50	0.89±0.32	1.18±0.33

State 4 and State 3; nmol oxygen consumption/mg protein/minute; ATP production; nmol ATP/mg protein/minute. Values presented are mean ± SEM. *Significant gender difference. ($p<0.05$)

Hydrogen Peroxide Production in Isolated Mitochondria

In order to measure the rate of reactive oxygen species production by heart mitochondria, we measured hydrogen peroxide (H_2O_2) formation in freshly isolated mitochondria (Figure 4-1). Interestingly, the amount of H_2O_2 production was significantly different between the two sexes. Male rats produced significantly higher levels of hydrogen peroxide compared to female rats (p=0.003). Doxorubicin did not change hydrogen peroxide production in the male rats after day 1. In striking contrast, there was a significant decrease in hydrogen peroxide production after day 1 in the female rats (p<0.05). Males did show a significant drop (p<0.001) in hydrogen peroxide production after day 4, but females remained at approximately the same level of hydrogen peroxide production as compared to day 1.

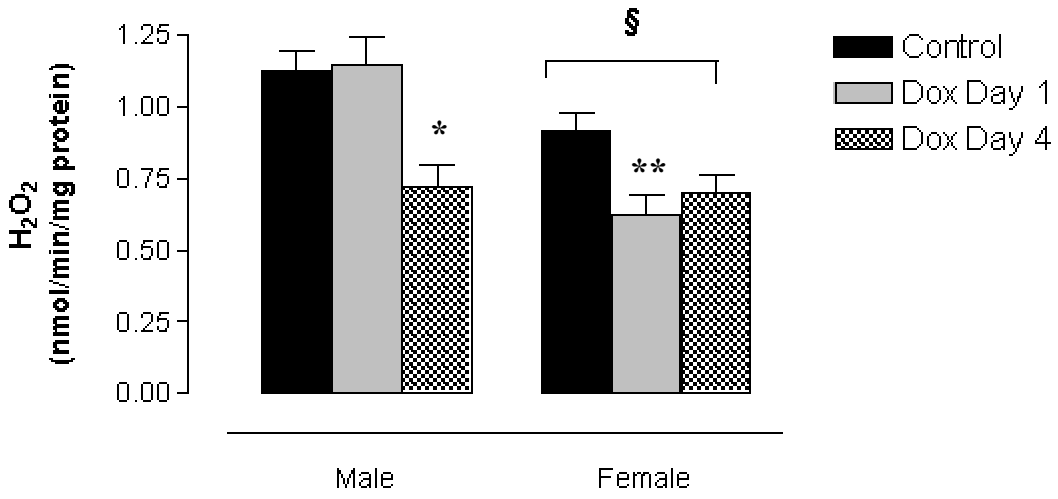

Figure 4-1. The effect of doxorubicin administration on heart mitochondrial oxidant production.

Mitochondria were isolated on day 1 and day 4 after the injection of doxorubicin 10mg/mL) or equal volume of saline in control animals. Values presented are mean ± SEM. * Significantly different from the control group (*p<0.001) and (**p<0.05). § Significant gender difference (p<0.05).

Glutathione Peroxidase Activity

To assess adaptations to mitochondrial hydrogen peroxide production, we determined mitochondrial glutathione peroxidase (GPX) activity (Figure 4-2). Male rats had significantly higher level of GPX activity compared to female rats (p=0.001). There was no significant difference in GPX activity detected after doxorubicin treatment on any of the days following doxorubicin treatment.

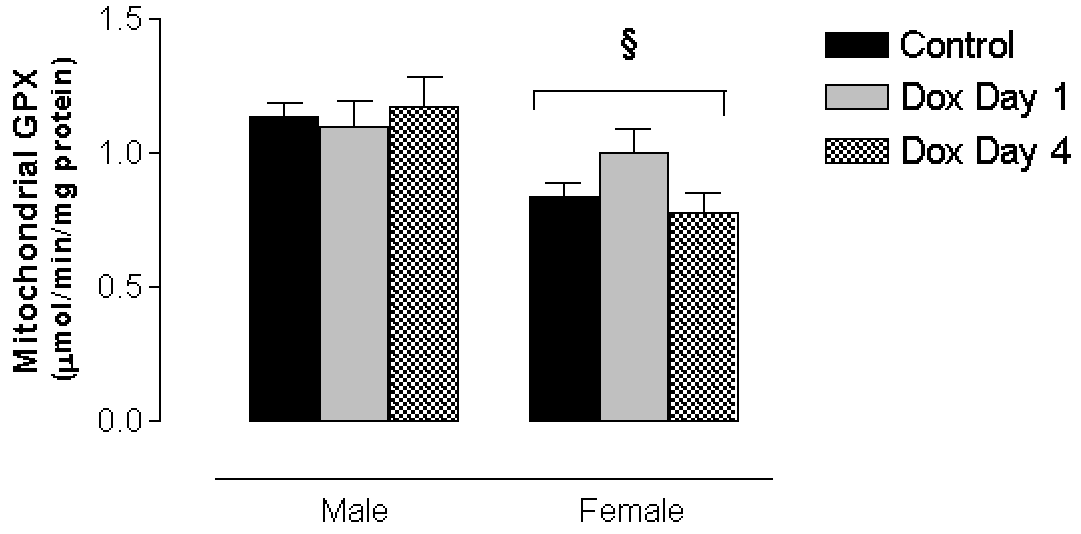

Figure 4-2. The effect of doxorubicin administration on heart mitochondrial glutathione peroxidase activity (GPX).

Values presented are ±SEM. § Significant gender difference (p<0.05).

Apoptosis Determined by Mono and Oligo-nucleosomes

To assess the overall incidence of apoptotic cell death we determined the levels of mono- and oligo-nucleosome contents in the isolated cytosolic fraction of the heart. Apoptosis is characterized by the formation of mono- and oligo-nucleosome in the nucleus. These DNA fragments are transported to the cytosol for degradation. Levels of mono- and oligo-nucleosome were similar in the male and female control animals.

However, male rat hearts contained greater levels of mono- and oligo-nucleosome content one day after doxorubicin treatment ($p<0.01$) and levels returned back to those seen in control animals after day 4. In contrast, female rats showed no increases in the levels of mono- and oligo-nucleosome after one day or four days after doxorubicin administration, suggesting that female hearts were more resistant to apoptosis (Figure 4-3).

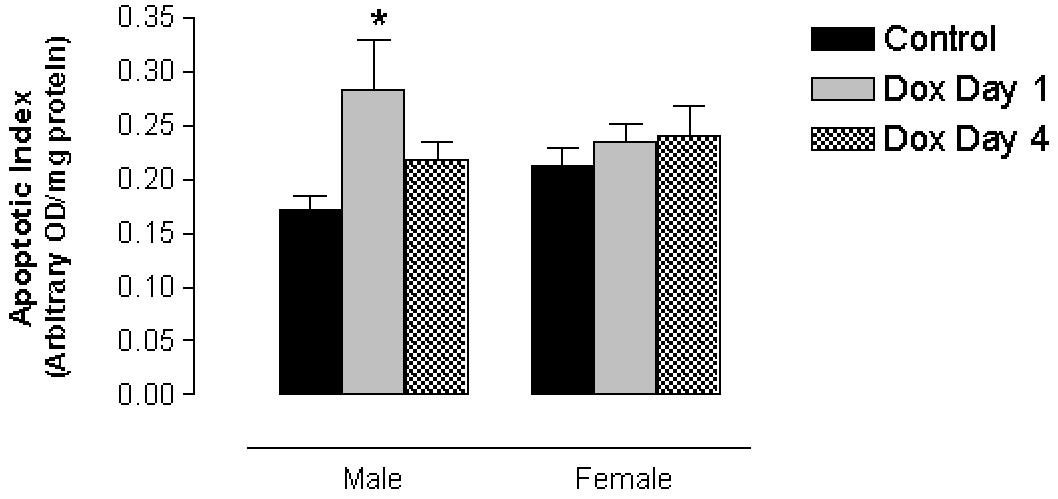

Figure 4-3. The effect of doxorubicin administration (10mg/kg) on the content of mono- and oligo-nucleosomes in the heart cytosol.

Values presented are ±SEM. * Significantly different from the control group ($p<0.05$).

Caspase-3 Activity and Caspase-3 Content

Caspase-3 is considered a central caspase of apoptosis, because most caspases are able to activate this caspase to initiate apoptosis. There were no differences in caspase-3 proteolytic enzyme activity between male and female control rats and 1 day after doxorubicin administration. However, four days after the administration of doxorubicin there were significant increases in both the male and female rats ($p<0.01$) (Figure 4-4).

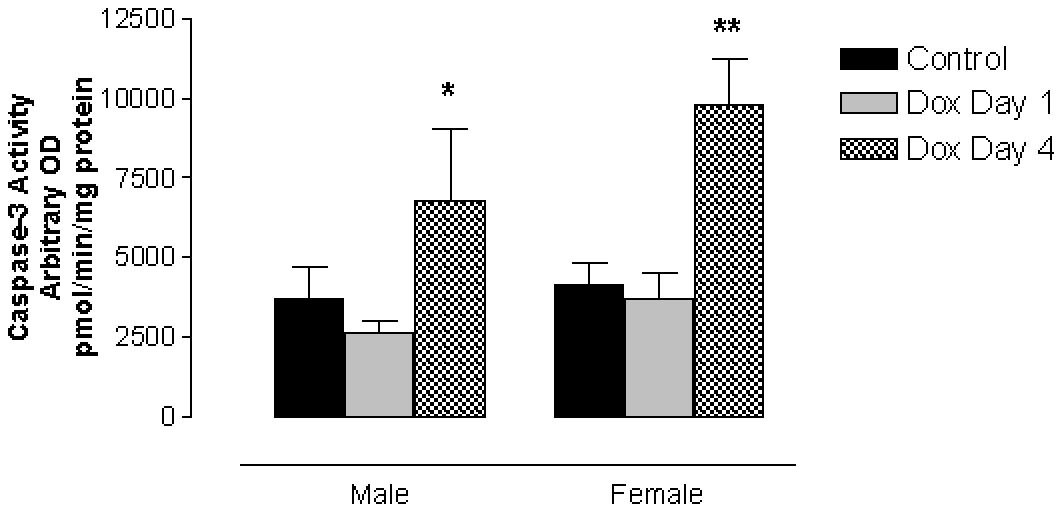

Figure 4-4. The effect of doxorubicin administration (10mg/kg) on caspase-3 activity. Caspase-3 activity measured using fluorescence ELISA method. Values presented are mean ± SEM. * Significantly different from the control group (*p<0.05) and (**p<0.05)

To determine if the rate of synthesis of caspase-3 was altered we measured the protein levels of the zymogen or pro-caspase-3 and the cleaved caspase-3 content (Figure 5). The total levels of the zymogen form of caspase-3 remained unaltered (Figure 4-5A), suggesting that the absolute protein levels of this caspase were not altered during the experimental phase in any of the groups. However, the content of the cleaved active caspase-3 was significantly increased after day four in both the male and female rats (Figure 4-5B).

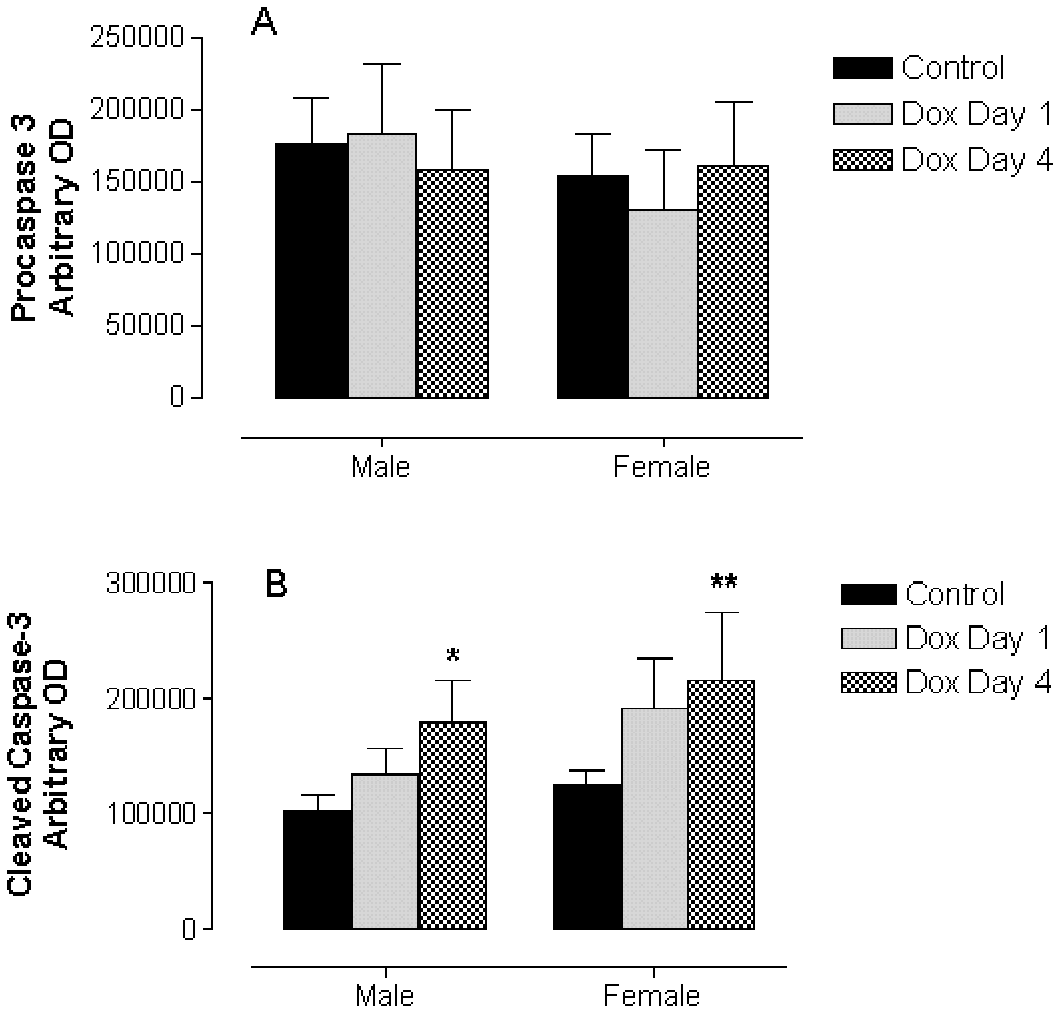

Figure 4-5. The effect of doxorubicin administration on the caspase-3 content (A) and cleaved caspase-3 concentration (B) determined by Western blot analysis.

Values presented are mean ± SEM. * Significantly different from the control group (*$p<0.05$) and (**$p<0.05$).

We determined if gender differences and changes to doxorubicin treatment existed in the levels of caspase inhibitor, X-linked inhibitor apoptotic protein (XIAP) a repressor of caspase-3 activity (Figure 4-6). We found no differences due to sex or doxorubicin treatment in any of the groups.

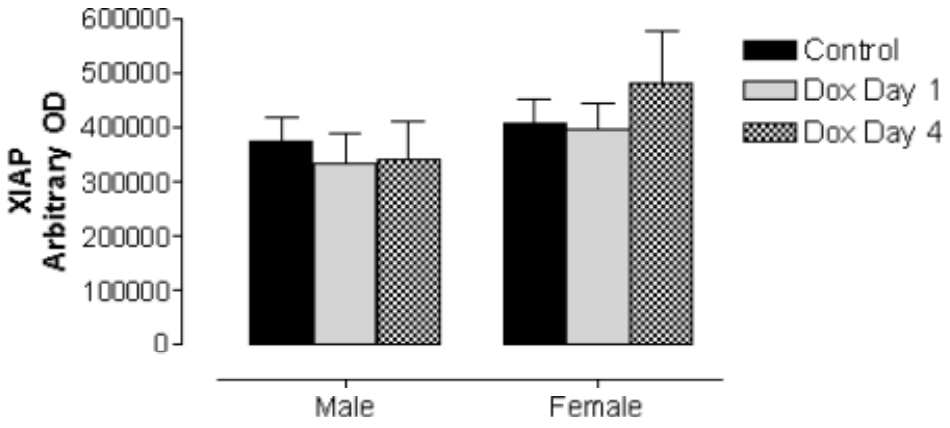

Figure 4-6. The effect of doxorubicin administration (10mg/kg) on the amount of anti-apototic proteins XIAP measured in cytosolic fraction using Western method.

Values presented are ±SEM.

Mitochondrial Mediated Pathway of Apoptosis

Apoptosis in cardiac myocytes is often associated with the release of cytochrome *c* from the mitochondria (Figure 4-7). Female control rats had significantly lower cytosolic cytochrome *c* levels compared to male rats ($p<0.05$). The dose of 10mg/mL of doxorubicin did not result in increased levels of cytosolic cytochrome *c* determined after 1 day or 4 days of doxorubicin treatment.

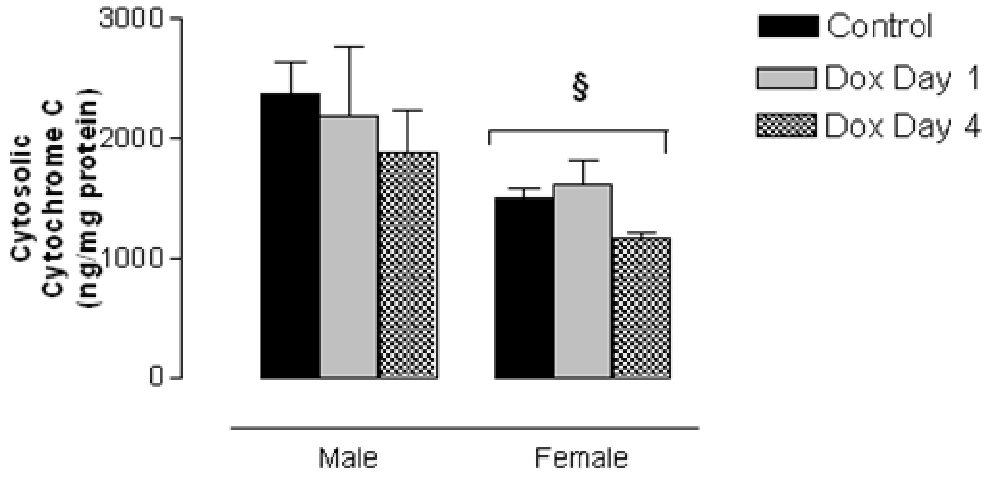

Figure 4-7. The effect of doxorubicin administration (10mg/kg) on cytochrome *c* concentration in the cytosol.

Values presented are ± SEM. § Significant gender difference ($p<0.05$).

Mitochondrial Regulators of Cytochrome *C* Release

The levels of Bcl-2, an anti-apoptotic protein, and Bax, a pro-apoptotic protein were determined in isolated cardiac mitochondria (Figure 8).

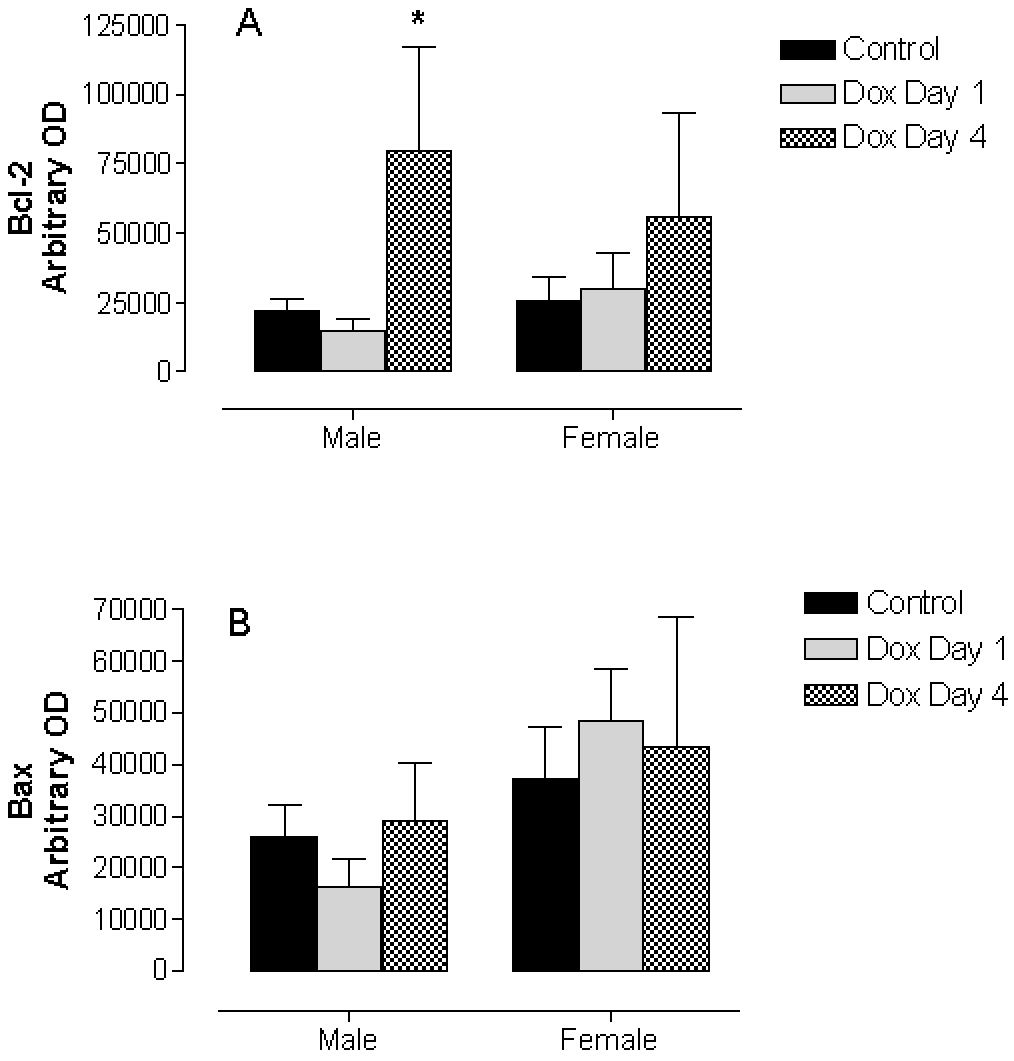

Figure 4-8. The effect of doxorubicin administration (10mg/kg) on the content of Bcl-2 (A) and Bax (B) measured in the cardiac mitochondria using Western Blot analysis.

Values presented are ±SEM. * Significantly different from control animals (*p<0.05).

There were no differences in the levels of these proteins due to gender. However, there was an increase in the levels of Bcl-2 four days after doxorubicin treatment in the male and female, but these changes were only statistically significant in the male animals (Figure 8A). The levels of Bax were not significantly different between gender and doxorubicin had no significant effect on this pro-apoptotic protein (Figure 8B).

Receptor Mediated Pathway of Apoptosis

To investigate the possibility if receptor mediated cell death was involved following doxorubicin administration we determined the content of capase-8 (Figure 4-9 A&B). Pro-caspase-8 content was not different between male and female rats (Figure 4-9 A). Moreover, doxorubicin treatment did not result in a significant change in the inactive procaspase-8 concentration, suggesting no increase in the synthesis rate of this pro-caspase during the 4-day experiment phase. Moreover, the cleaved caspase-8 did not differ significantly between sexes (Figure 4-9B). Furthermore, doxorubicin treated rats did not show a statistically significant change in the levels of the cleaved form. We also measured cFLIP, which is a potent inhibitor of caspase-8 activation and found no differences in cFLIP due to gender or doxorubicin treatment (Figure 4-10).

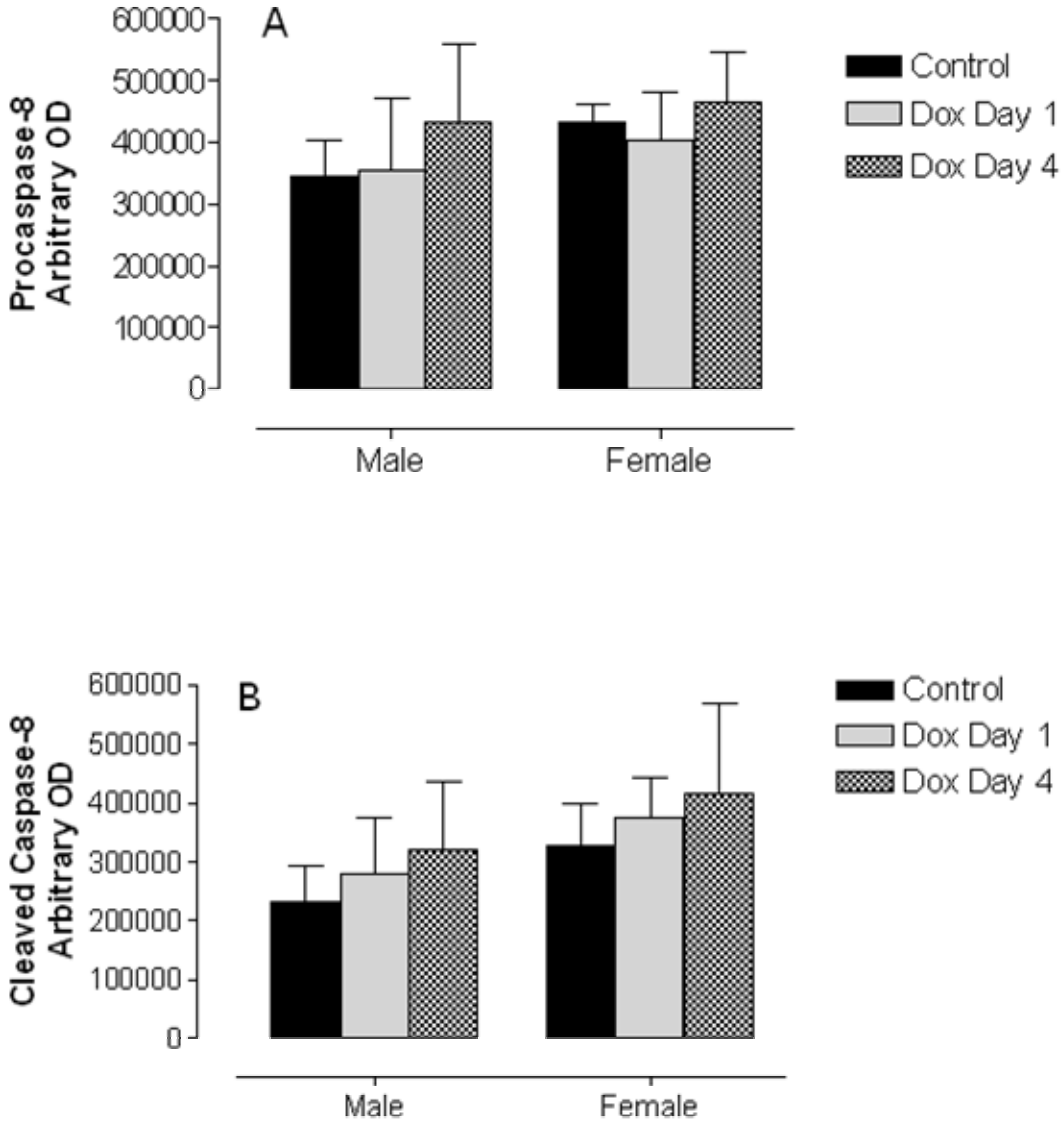

Figure 4-9 The effect of doxorubicin administration (10mg/kg) on the content (A) Procaspase-8 and (B) cleaved caspase-8.

Values presented are mean ± SEM.

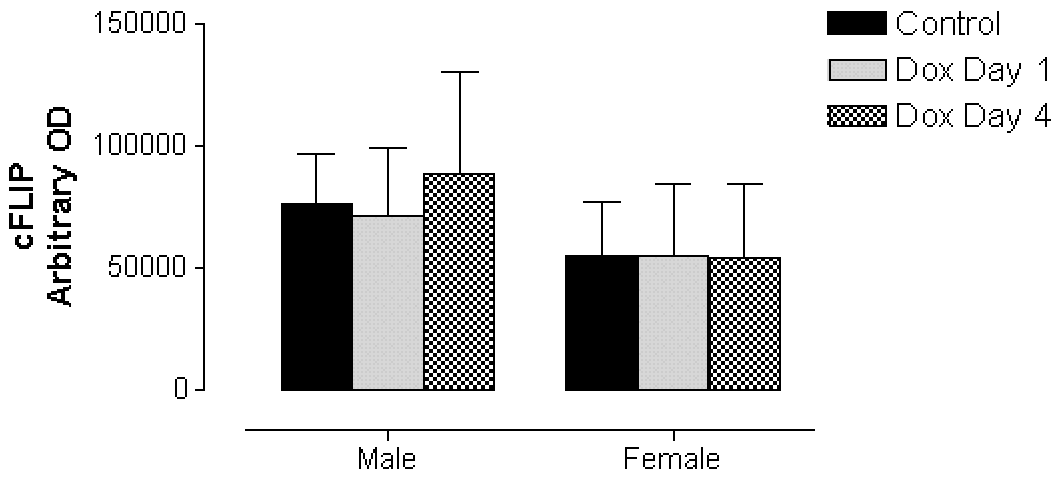

Figure 4-10. The effect of doxorubicin administration (10mg/kg) on the content of cFLIP measured in cytosolic fraction using Western blot method.

Values presented are mean ± SEM.

CHAPTER 5
DISCUSSION

Overview of Principle Findings

These are the first experiments to examine if gender differences exist in cardiotoxicity induced by doxorubicin as determined by mitochondrial function, oxidative stress, and apoptosis. No significant differences were observed in mitochondrial function following doxorubicin treatment in any of the groups. However, our data shows that female rats produce lower levels of hydrogen peroxide one day after doxorubicin treatment compared to male rats. Moreover, the apoptotic index determined by the levels of mono- and oligo-nucleosomes was significantly increased in male rats, but not in female rats 1 day after doxorubicin treatment. In addition, caspase-3 activity and cleaved caspase-3 content were increased after 4 days in male rats as well as in female rats. Sex differences in mitochondrial oxidant production immediately after doxorubicin could have influenced the release of pro-apoptotic factors, such as cytochrome c, AIF, or Endonuclease G (54) and may explain the sex differences observed in DNA fragmentation pattern. However, levels of cytosolic cytochrome c remained unaffected in both the male and female rats, which could be explained by the increase of Bcl-2 content in the isolated mitochondria. Furthermore, these data show that the receptor mediated pathway is unlikely the major cause for the increased activation of caspase-3 *in vivo*.

Body Weight and Heart Weight

Doxorubicin administration caused a significant reduction in body mass in male rats, but not in female rats. Furthermore, four days after doxorubicin treatment the animals heart weights of both the male and female rats were significantly lower compared to those injected with saline. The loss in heart weight was probably due to 1) increased levels of apoptosis and 2) an inhibition of protein synthesis by doxorubicin (55). Other factors, such as dehydration and food consumption could have played a role in the loss of heart weight. Heart weight-to-body weight ratio was significantly higher in female rats, which could have reduced the absolute toxic effect of the drug (10 mg of doxorubicin/kg of body weight) on the female rats. However, drug metabolism and fat-to-lean-body mass ratio may have also played a role in the cardiotoxic effect of doxorubicin.

Oxidant Production and Antioxidant Enzymes

Female rats had significantly lower hydrogen peroxide (H_2O_2) production compared to the male group, which supported our initial hypothesis. Moreover, in response to doxorubicin, male and female rats showed a different rate of oxidant production. Hydrogen peroxide (H_2O_2) production was decreased one day after doxorubicin administration in females. A possible explanation for this observation might be that mitochondria could adapt to cytotoxic stress by enhanced electron coupling and a reduction in the free radical leak. In addition, it remains possible that mitochondrial biogenesis was significantly increased after doxorubicin treatment and that newly synthesized mitochondria adapted and produced less hydrogen peroxide.

In response to the reduction in mitochondrial reactive oxygen species in the control female rats, we found a similar decrease in mitochondrial glutathione peroxidase (GPX)

activity. Acute doxorubicin administration did not alter GPX activity in either male or female rats suggesting that GPX activity was not up regulated within the 1 or 4 day period after the oxidative stress insult. This result agrees with previous study by Li *et al.* (55) and recent data from our laboratory (29), which show that a single dose (20mg/Kg) of doxorubicin did not have any influence on the activity of GPX four days after administration.

Apoptosis Induced by Doxorubicin

The overall incidence of apoptosis in control animals measured by DNA fragmentation did not differ between sexes. However, in response to doxorubicin administration, male rats showed an increased concentration of cytosolic mono- and oligo- nucleosomes on day 1 only, whereas in female rats, no change was seen in DNA fragmentation after day 1 and day 4. This data suggests that female rats might have been able to prevent the loss in heart cells due to adriamycin-induced cardiotoxicity. Surprisingly, the key effector caspase-3, showed significant increase in activity and content after four days of doxorubicin treatment in both male and female rats. Hence, the caspase-3 data does not correlate with the levels of apoptosis detected by the concentrations of mono- and oligo-nucleosomes. Furthermore, we did not detect any changes in XIAP content due to gender or doxorubicin treatment to add an explanation to this finding. Therefore, we further investigated if the mitochondrial-mediated pathway of apoptosis was responsible for the differences seen in apoptosis and caspase-3 activation.

Mitochondrial Mediated Pathway of Apoptosis

To confirm which pathway may have been responsible for the activation of caspase-3 we examined the mitochondrial-mediated pathway. Cytosolic levels of cytochrome *c* in male and female rats showed a very similar response and therefore this

pro-apoptotic protein does not appear to be responsible for the increased DNA fragmentation seen in the male rats. However, it remains possible that changes in cytochrome *c* levels occurred immediately after doxorubicin treatment and that the effects of apoptosis, such as caspase-9 activation or AIF release occurred before day 1. Moreover, it appeared that the mitochondrial anti-apoptotic protein, Bcl-2 was significantly increased in response to doxorubicin administration in both male and female animals four days after treatment. Hence, it is possible that other adaptive response might have occurred during the four-day experimental period. For example, other inhibitory proteins such as cytosolic ARC could have been sequestered by the mitochondria and affected the release of cytochrome *c*, AIF, and endonuclease G (54) differently. ARC is known to inhibit cytochome *c* release from mitochondria and protects against hypoxia–induced apoptosis in heart (56). Therefore, up-regulation of this protein could have altered the release of cytochrome *c* from mitochondria.

Receptor-Mediated Pathway of Apoptosis

We investigated if the receptor-mediated pathway was affected by doxorubicin treatment and if gender differences were present. Others have shown that Fas-mediated activation of caspse-8 *in vitro* with doxorubicin treatment causes apoptosis (44). Although there were gradual increases in the levels of cleaved caspase-8 after day one and day four, these changes were not statistically significant. Therefore, from the changes observed in this study, using an *in vivo* model, it appears that this pathway was not a major player in the activation of caspase-3. Furthermore, no significant changes were detected in the caspase-8 inhibitor cFLIP due to gender or doxorubicin treatment. Hence, it remains possible that the dose injected in these rats (10mg/kg) did not induce a

sufficient stress response to significantly activate this pathway or this pathway does not play an essential role in inducing apoptosis.

Mitochondrial Function and Doxorubicin Treatment

Mitochondrial function and ATP production remained unaltered after acute doxorubicin treatment. This is in strong agreement with the current literature which suggest that mitochondrial DNA damage and damage to respiratory complexes needs to be excessive (exceed ~50% of it's function) before significant decreases in ATP production are observed (57). Rossignal *et al.* (57) and several others (58, 59) have shown that it was possible to inhibit the activity of a respiratory chain complex (~30-50%), up to a critical level, without affecting the rate of mitochondrial respiration and ATP synthesis (57). The mitochondria functional data in our present study supports this hypothesis.

Limitations in Present Study

One of the potential limitations to this study is that we did not control for the estrous cycle. Although, there were only moderate fluctuations (20-30% change in estrogen between males and females and within each group), the variability in estrous cycle could have been a confounding variable. Levels of estrogen found in the plasma were in pg/mL level, which might be too low to have any significant effect on attenuating oxidative stress compared to lipid and water soluble antioxidant found in plasma (60). However, most studies suggest that estrogen's protective effects may stem from binding to cell surface receptors and up-regulating a variety of cellular proteins, such as NOS (18, 61) and heat shock proteins (18, 62). Moreover, most studies show an anti-apoptotic effect of estrogen in cell culture models which might have very little relevance in a complex biological *in vivo* model, such as that used in this study (17, 18). In addition,

estradiol levels of Fischer 344 rats are at their highest levels around the age of 9 to 12-months (63). Our rats were 6 months of age and might not have been mature enough to receive the full protection of estrogen. Therefore, the levels of estrogen in this study may not have been sufficient to be cardioprotective and it would be worthwhile to investigate rats with ovariectomy and to see if supplementing estradiol may have an effect on apoptotic signaling pathways *in vivo*.

Conclusion and Future Direction

To our knowledge, these were the first experiments that look at the effects of gender and doxorubicin in relation to oxidative stress and cell death. The principle findings from this study include: 1) there are gender differences in doxorubicin-induced oxidant production and apoptosis; 2) mitochondrial pathways may have been involved, but the rapid up-regulation of Bcl-2 may have prevented significant cell death by this pathway and 3) receptor mediated cell death appears to play a minor role, since no adaptations in cFLIP and significant increases in caspase-8 were observed following doxorubicin treatment.

Other pathways, such as caspase-12, an endoplasmic reticulum-mediated pathway might be also partly responsible for the apoptosis observed in the male rats. The activation of the endoplasmic reticulum-mediated pathway causes the release of calcium and the activation of caspase-12. Indeed, numerous studies have reported that doxorubicin can alter Ca^{2+} homeostasis (2, 7, 64). Therefore, more research is needed to further elucidate other possible mechanisms and to determine the rate of mitochondrial biogenesis, mitochondrial proteolytic degradation; and activation of autophagy through lysosomal pathways following doxorubicin-induced cardiotoxicity. A better understanding of gender difference in doxorubicin-induced pro- and anti-apoptotic

signaling pathways in cancerous and non-cancerous cells may lead to new and improved therapeutic protocols for mitigating the toxic side effects of doxorubicin.

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BIOGRAPHICAL SKETCH

Youngmok C. Jang was born in Seoul, South Korea, where he lived until moving to New York, in 1984. Youngmok returned to Korea in 1988 where he graduated from Korea University, Seoul, Korea, with a Bachelor of Science degree in physical education. In August 2001, Youngmok began his graduate studies in exercise physiology at the University of Florida where he taught for Human Physiology Laboratory and also worked as a research assistant under Dr. Christiaan Leeuwenburgh. After earning a Master of Science in Exercise and Sport Sciences degree, Youngmok plans to pursue his doctoral degree in the same field at the University of Florida.