GENDER DIFFERENCES IN THE RESPONSE TO SHORT TERM β-ADRENERGIC INDUCED CARDIOMYOCYTE APOPTOSIS AND NECROSIS IN RATS

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Abstract

Background: Males have a higher prevalence of cardiovascular diseases compared to premenopausal women. However, postmenopausal women are at equal risk to men. It has therefore been suggested that estrogen is cardioprotective. Although the exact mechanisms of the purported cardioprotective effects of estrogen are unknown, estrogen administration has been reported to suppress beta-adrenergic receptor up-regulation in ovariectomized female rats. As beta-adrenergic activation induces cardiomyocyte apoptosis and necrosis, and hence adverse cardiac remodelling and heart failure, I aimed to determine whether the extent of beta-adrenergic induced apoptosis and necrosis differs between males and females.

Methods: 27 male Wistar rats were assigned to one of two groups: ISO M (n=14) receiving a beta-adrenergic receptor agonist, isoproterenol (0.02mg/kg) and CON M (n=13) receiving vehicle (saline, 0.2ml). 29 female Wistar rats were assigned to one of two groups: ISO F (n=15) receiving a beta-adrenergic receptor agonist, isoproterenol (0.02mg/kg) and CON F (n=14) receiving vehicle. Isoproterenol and saline were administered by means of daily subcutaneous injections for 5 days. On the 5^{th} day, cardiac geometry and function were assessed before and after ISO or saline administration using echocardiography. Rats were then terminated under anaesthesia within 30 minutes of ISO (or vehicle) administration and blood samples collected for the determination of serum estrogen concentration (ELISA). Female rats were terminated in proestrus which corresponds to peak estrogen concentrations. Cardiac myocyte apoptosis was assessed histologically using the DeadEndTM Colorimetric TUNEL system (Promega, Madison, WI, USA). The number of apoptotic cardiomyocyte nuclei was expressed as a percentage of the total number of cardiomyocyte nuclei per slide (heamotoxylin and eosin stain). Necrosis and fibrosis (pathological score) were assessed by assigning a pathological score to sections stained for fibrosis (van Gieson). Groups were

compared using two-way (gender and regimen; and including repeated measures for echocardiography data) ANOVA followed by the Tukey-Kramer *post hoc* test.

Results: As expected estrogen concentrations were higher in female compared to male rats (mean±SEM, $pg.ml^{-1}$; ISO M: 7.04±1.41; CON M: 7.14±0.53; ISO F: 23.00±3.47; CON F: 19.31±3.66; $p<0.01$). Five days of ISO or saline administration had no effect on cardiac function or geometry in either the male or the female rats. Inotropic effects (increased heart rate and cardiac function) were observed in response to acute ISO administration in both male and female rats. The female rats had slower heart rates ($p<0.05$) and showed a greater heart rate response to acute ISO administration than the male rats ($p<0.05$). But the acute ISO induced increments in cardiac function were similar between genders. Five days of ISO administration induced cardiomyocyte apoptosis in male rats but not in female rats (mean±SEM, % ; ISO M: 0.086±0.013; CON M: 0.030±0.004; ISO F: 0.053±0.004; CON F: 0.041±0.007; $p<0.05$). Furthermore, 5 days of ISO administration induced cardiomyocyte necrosis in male rats but not in female rats (mean±SEM, pathological score; ISO M: 1.21±0.21, CON M: 0.46±0.14, ISO F: 0.50±0.11, CON F: 0.68±0.12, $p<0.01$).

Conclusion: Male rats are more susceptible than female rats to beta-adrenergic induced cardiomyocyte apoptosis and necrosis. The protective effects of estrogen against the adverse effects of beta-adrenergic activation on the heart, may explain the lower risk of cardiovascular disease in premenopausal women compare to men; however, the possible role of progesterone cannot be ignored.

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science of Medicine, in the Faculty of Medicine, University of Witwatersrand, Johannesburg. The work contained in this thesis, has not been submitted for any degree or examination in this university, or any other university.

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I certify that the study contained in this thesis has the approval of the Animal Ethics Committee of the University of Witwatersrand, Johannesburg. The ethics approval number is 2007/28/'04

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Table of Content

List of Tables

List of Figures

Dedication

This dissertation is dedicated to my loving and one of a kind family who make every day worthwhile, and to the special man in my life for his continuous help, love, support and encouragement, without you this thesis would never have come together.

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List of Abbreviations

ANP	atrial naturetic peptide
CAD	coronary artery disease
CHD	coronary heart disease
CVS	cardiovascular system
CON	control
DNA	deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
F	female
FS end	endocardial fractional shortening
FS mid	midwall fractional shortening
HDL chol	high density lipoprotein cholesterol
HERS	Heart and Estrogen/progestin Replacement Study
HR	heart rate
HRT	hormone replacement therapy
ISO	isoproterenol
KEEPS	Kronos Early Estrogen Prevention Study
LAD	left anterior descending
LDL chol	low density lipoprotein cholesterol
LVEDD	left ventricular end diastolic diameter
LVESD	left ventricular end systolic diameter
M	male
MI	myocardial infarction

ER-α	estrogen receptor-α
ER-β	estrogen receptor-β
PWTd	posterior wall thickness in diastole
PWTs	posterior wall thickness in systole
ROS	reactive oxygen species
RWT	relative wall thickness
SHR	spontaneous hypertensive rat
SNS	sympathetic nervous system
TAC	transverse aortic constriction
TDT	terminal deoxynucleotidyl transferase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VSMC	vascular smooth muscle cell
WHI	Women's Health Initiative
WKY	Wistar-Kyoto
WT	wild-type

Preface

It is well known that premenopausal women are at a lower risk for cardiovascular incidents, such as heart attacks, as compared to men. However, the latter is not true for post-menopausal women. Indeed, studies have revealed that in post-menopausal women the cardiovascular risk profile is equal to that of men. These data allude to the possibility that the female hormone estrogen may be cardioprotective (protects the heart). Currently, various human and animal studies have been conducted to determine the exact cardioprotective mechanisms of estrogen and the overall benefits of estrogen. Results have revealed that estrogen protects damaged blood vessels, decreases lipid plaque formation (a negative predictor of coronary artery disease), controls blood pressure and prevents bone degeneration; all of which are positive outcomes. However, the question still remains how exactly does estrogen elicit such cardioprotective effects?

In order to evaluate how estrogen is cardioprotective various clinical trials have assessed the effects of hormone replacement therapy. Currently, these trials are controversial with certain studies revealing beneficial outcomes associated with hormone replacement. However, other studies have highlighted the detrimental effects of estrogen use such as increased risk of breast cancer, heart attacks and lung complications. Plausible explanations for the rather conflicting data obtained from clinical trials include differences in the choice of hormone administered, study design, characteristics of women, and the age of women at initiation of hormone replacement therapy. In this regard some studies have assessed estrogen combined with progesterone; whereas others have assessed estrogen only. The problem with administering estrogen and progesterone together, is one cannot conclude which hormone (estrogen or progesterone or both) is responsible for the beneficial or detrimental effects of hormone replacement therapy. In addition, various clinical trials have used different hormone concentrations, the duration of the trials vary widely and the route of administration of

hormone replacement therapy is not consistent (either tablets, transdermal patches or injections). With regards to the women, some trials have assessed the effects of hormone replacement therapy in post-menopausal women; whereas others have assessed peri-menopausal (just before menopause starts) women. Moreover, in some studies the women enrolled had intact uteruses and were healthy; while in other studies the women had a prior history of cardiovascular disease. With such variations between studies, it is not surprising that the outcomes are inconsistent and hence no decisive conclusions can be drawn.

In order to overcome the controversies of clinical human trials, animal or laboratory based studies have been conducted in an attempt to determine the cardioprotective effects of estrogen and the mechanisms thereof. Indeed, great strides have been made with studies demonstrating beneficial effects of estrogen especially on the heart, blood pressure and lipid concentrations. However, animal studies also have some limitations. Studies using cell cultures (*in vitro* model) are valuable for assessing specific physiological systems in isolation; nevertheless the data obtained cannot be extrapolated to a complete organism (for example an intact animal). Moreover, *in vivo* animal studies have chosen aggressive animal models of cardiovascular disease which therefore fail to mimic the natural slow progression of heart failure. In addition, previous animal studies which have attempted to investigate the effects of estrogen, have either removed the ovaries (which produce estrogen), administered excessively high levels of exogenous estrogen, or have used estrogen receptor over-expression models. Thus, currently no study has assessed the cardiovascular effects of the naturally cycling endogenous estrogen.

Hence, my dissertation is aimed at investigating the impact of gender on an adrenergic-induced model of cardiovascular disease. The sympathetic nervous system (SNS), more specifically beta-adrenergic receptors, plays an important role in the development and progression of cardiovascular diseases and is therefore a crucial physiological system to

investigate. The daily administration of a beta-adrenergic agonist (isoproterenol), mimics the adrenergic activation which occurs in slowly progressive heart failure in humans. My aim was to determine whether gender impacts upon isoproterenol-induced changes in the heart. As one of the early changes which occur in the heart is the death of cardiomyocytes, I compared the impact of a short period of isoproterenol administration on the geometry and function of the heart, as well as cardiomyocyte death by apoptosis and necrosis, in male and female rats.

Chapter 1

Introduction

1 Introduction

A pronounced gender difference exists in the risk of cardiovascular diseases. Males have a higher risk than females prior to menopause, after which the gender discrepancy in risk of cardiovascular disease is diminished (Hulley et al 1998; Rossouw et al 2002). Many studies have been conducted in an attempt to determine the exact cause of this gender difference. These studies have primarily focused on the physiological advantages and disadvantages of the exogenous administration of female sex hormones, either estrogen plus progesterone in combination or estrogen alone.

A number of clinical trials have assessed the effects of hormone replacement therapy (HRT) in peri- and post-menopausal women. The results are inconsistent in that some trials have demonstrated cardioprotective effects (Grady et al 1992); whereas others have shown a detrimental effect of HRT on cardiovascular diseases (Hulley et al 1998; Rossouw et al 2002). In an attempt to clarify the controversies which have arisen from the results of clinical trials, various animal- or laboratory-based studies have assessed the effects of estrogen administration on the cardiovascular system and the physiological mechanisms thereof.

In this regard, research has indicated a relationship between estrogen and β-adrenergic receptors in the heart; whereby estrogen is thought to down-regulate the β-adrenergic receptors (Thawornkaiwong et al 2002). Importantly, the sympathetic nervous system (SNS) is known to play a crucial role in cardiovascular diseases. Indeed, increased β-adrenergic receptor activation induces myocardial infarction, heart failure and progressive myocardial dilatation. Nevertheless, the cardiovascular effects of estrogen in the presence of increased β-adrenergic receptor activation are currently unknown. As chronic β-adrenergic receptor activation is the most important factor that promotes progressive heart failure (Badenhorst et al 2003), further research to assess the potential benefits of estrogen is crucial.

Although, the most effective way to counteract the effects of SNS activation on the cardiovascular system is by the use of β-adrenergic receptor blockers (Metra et al 2000), only 15% of all patients with heart failure are able to tolerate these agents because they reduce myocardial contractility (Metra et al 2000). Hence, it is important to understand the mechanisms (pathways downstream from β-adrenergic and estrogen receptors) which may be responsible for progressive heart failure or the prevention thereof, as well as which patients are most likely to benefit from these agents.

1.1 The controversies regarding the cardiovascular effects of oestrogen administration

1.1.1 Human clinical trials

Numerous clinical trials have been performed to determine the potential cardiovascular benefits of HRT. A summary of the most pertinent human studies is provided in Table 1. The results of these clinical trials are inconsistent. Some studies demonstrate cardioprotective effects of estrogen, such as lowered atherosclerotic plaque progression, improved lipid profile (Mansen et al 2007), and reduced risk for myocardial infarction (Varas-Lorenzo et al 2000) and coronary artery disease (Grady et al 1992). Conversely, other studies have shown HRT to have detrimental consequences, such as increased risks of breast cancer, coronary heart disease and pulmonary emboli (Hulley et al 1998; Roussow et al 2002). It is therefore imperative to critically analyse the clinical trials that have been performed thus far, to try and understand the reasons underlying the controversies related to the effects of estrogen administration.

The Heart and Estrogen/Progestin Replacement Study (HERS) (Hulley et al 1998), a randomised blinded control study, in post-menopausal women younger than 80 years of age, who had previously undergone a hysterectomy and who had established coronary heart

Table 1: Summary of human trials

Author	Patient type	HRT combination	Duration	Outcome	Overall conclusion
Arnlov et al 2006	Middle aged white men with no CVD at baseline (mean age 56 years)	None →assessed endogenous estradiol levels	Ten year follow-up	• Higher estradiol levels were associated with lower risk of CVD events in older men. • Testosterone and DHEA-S levels did not alter risk of CVD events.	↑ estradiol = ↓ CVD risk
Grady et al 1992	Peri- menopausal and post menopausal women	Meta- analysis of studies. Regimens included: 1. estrogen only 2. estrogen and progestin	Studies ranged from 3-10 years	• Estrogen therapy reduced the risk of CHD and hip fracture • Long term estrogen use was associated with increased risk of endometrial and breast cancer	Estrogen ↓ CHD but estrogen ↑ cancer risk
Hulley et al 1998 (HERS)	Post- menopausal with established coronary disease and intact uterus mean age 66.7 years (55-80 years)	Experimental group: Conjugated equine estrogen and progesterone (medroxyprogesterone) Control group: Placebo	Span of four years with data recorded in year one and four	• No difference between groups in primary and secondary outcomes • Lower low-density lipoprotein levels in experimental group • Increased CHD events within the first year followed by lower CHD events in the fourth and fifth years in the experimental group	HRT ↓ CHD after 4 years
Manson et al 2007 (sub study of WHI)	Early post menopausal women with hysterectomy aged 50-59 years	Experimental group: Conjugated equine estrogen Control group: Placebo	Follow-up period seven years	• Experimental group had 30-40% less coronary artery calcium build up compared to placebo group • Experimental group required less procedures to reopen clogged arteries	HRT ↓ atherosclerosis

Author	Patient type	HRT combination	Duration	Outcome	Overall conclusion
Miller et al 2008 (KEEPS)	Healthy menopausal women aged 42-58 years	Experimental group: estrogen only HRT Control: placebo	Four years	• Still awaiting results	-
Psaty et al 1994	Post-menopausal women with a history of fatal or non-fatal MI aged 68 years	Group 1: HRT nonusers Group 2: estrogen only HRT Group 3: estrogen + progestin HRT	Period of two years	• Risk ratio of MI in estrogen only users was 0.69 • Risk ratio of MI in estrogen and progestin users was 0.68	Estrogen= estrogen + progestin
Roussouw et al 2002 (WHI)	Post-menopausal with no history of cardiovascular disease with intact uterus aged 50-79 years	Experimental group: Conjugated equine estrogen and progestin (medroxyprogesterone) Control group: Placebo	Follow-up period of five years	• Lower low-density lipoprotein levels in experimental group • Global index of benefit versus risk revealed HRT to be harmful (CHD events and breast cancer)	HRT ↑ CHD but ↓ LDL cholesterol
Varas-Lorenzo et al 2000	Post- menopausal women with no history of cardiovascular disease (aged 50-74 years)	1. Non- users 2. HRT users →oral Estrogen alone Estrogen + Progestin (conjugated) → transdermal Estradiol Estradiol + Progestin (conjugated) →estradiol implants	Follow-up from six months to three years	• Overall HRT users had a reduced risk for MI compared to non-users	HRT ↓ risk of MI

CHD, coronary heart disease; CVD, cardiovascular disease; DHEA-S,dehydroepiandrosterone sulfate; HERS, Heart and Estrogen/Progestin Replacement Study; HRT, hormone replacement therapy; KEEPS, Kronos Early Estrogen Prevention Study; LDL, low density lipoprotein; MI, myocardial infarction: WHI, Women's Health Initiative.

disease (defined as one or more of the following: myocardial infarction, coronary artery bypass graft surgery, percutaneous coronary revascularisation or angiographic evidence of at least 50% occlusion of one or more major coronary arteries), assessed the effects of HRT (estrogen and progesterone) on coronary heart disease (CHD). The primary outcomes were the occurrence of non-fatal myocardial infarction (MI) or CHD deaths (described as fatal MI, sudden death within one hour of symptoms, death due to coronary revascularization procedures or congestive heart failure) over a period of 5 years. Secondary outcomes were coronary revascularization, unstable angina, congestive heart failure, resuscitated cardiac arrest, stroke and peripheral arterial disease. The participants were monitored every 4 months however results were expressed per year. Overall the results revealed no significant differences between the two groups (HRT versus placebo) with respect to primary outcomes. The HRT group however, were noted to have decreased low-density lipoprotein (LDL) and increased high-density lipoprotein (HDL) cholesterol concentrations compared to the placebo group after 1 year and after 5 years. Moreover, the trial revealed that the HRT group had increased incidence of CHD within the first year of the study; however in the fourth and fifth years of follow-up the HRT group had decreased CHD events compared to the placebo group. Although, HRT had no effect on the prevalence of the primary outcomes after 1 year, long-term HRT use could offer possible myocardial protection, as noted from the decrease in CHD events in the 4th and 5th years of the study. The HERS study failed to assess heart failure progression. Indeed, in this trial the results were assessed after a maximum of 5 years, which may have been too short a time period to fully determine the potential cardiovascular benefits of HRT in preventing heart failure progression (Hulley et al 1998). Moreover, as the experimental group where receiving combination HRT (estrogen plus progesterone), the potential benefits of estrogen alone could not be assessed.

A question which arose from the HERS study is whether HRT would potentially be beneficial in healthy post-menopausal women. Thus, the Women's Health Initiative (WHI) trial evaluated the effects of HRT (estrogen plus progestin) on CHD events in healthy post-menopausal women aged from 50-79 years, who had an intact uterus but had no history of CVD (Rossouw et al 2002). To determine the health benefits versus risks of HRT a global index was determined and compared between the experimental and placebo groups. The global index was described as the earliest occurrence of CHD (more specifically acute MI requiring overnight hospitalisation, silent MI or CHD death), invasive breast cancer, stroke, pulmonary embolism, endometrial cancer, colorectal cancer, hip fracture, or death due to other causes. The trial was stopped prematurely as a significant portion of the participants in the experimental group (estrogen plus progestin) presented with breast cancer, CHD, stroke and pulmonary emboli. However, some of the results were similar to those of the HERS trial, in that the experimental group had lower LDL and increased HDL cholesterol levels. With respect to the global index, the experimental group had lower hip fracture rates compared to the placebo group; however, the experimental group had higher incidences of non-fatal MI, strokes, venous thrombo-embolism and non-fatal events compared to the placebo group. Thus the global index demonstrated an overall harmful effect of HRT. The unfavourable outcomes of this trial may be related to certain limitations namely: (i) the doses of estrogen and progestin may have been too high [indeed the authors suggested that transdermal administration could have been more physiologically favourable (Rossouw et al 2002)]; (ii) the effects of estrogen alone or progesterone alone could not be determined as HRT was administered as a combination. It is plausible that progestin could have masked any beneficial effects of estrogen. Hence, similar to the HERS trial; whether estrogen alone was cardioprotective could not be assessed in WHI trial.

Some of the issues that may have contributed to the conflicting results obtained in the HERS and WHI trials (decreased versus increased CHD events respectively), were addressed in a population-based case-control study conducted by Varas-Lorenzo et al (2000). The participants enrolled in this study were similar to those enrolled in the WHI trial. Participants were between 50-74 years of age, had no history of cardiovascular events, and either had an intact uterus or their uterus had been surgically removed. The participants were stratified according to the type of HRT used, namely: oral estrogen alone; transdermal estrogen alone; implants of estradiol alone; estrogen plus progestogen; and non-users. The aim of this study was to assess the incidence of MI and to correlate the incidence of MI with the route of administration, dose and duration of HRT. The results revealed that the age-adjusted odds ratio (OR) for MI in participants taking HRT, irrespective of route of administration, over a period of 6 months was 0.72 compared to non-users. Compared to non-users, those participants that had used HRT for more than 1 year had an OR of 0.68, and those that had used HRT for more than 3 years had an OR of 0.59. In addition the protective effects of long-term (>1 year) HRT use were better for fatal events than non-fatal events (OR=0.38 and 0.77 respectively). In addressing some of the limitations of the WHI trial this study showed that long-term HRT (>1 year) had lower risk for MI compared to short-term use (6 months). These results are in agreement with the HERS trial where long-term (4 and 5 years) administration had more favourable cardiovascular results compared to short-term use (1 year). A possible explanation for the latter is described by the meta analysis of Grady et al (1992) that stated that long-term (more than 3 years) estrogen therapy was imperative for improving CHD as it is a chronic progressive disease (further explanation is found on page 11). Regarding the route of administration, transdermal users had an OR of 0.75 and oral users an OR of 0.66. It is important to note however, that with respect to the latter results transdermal users were only 21% of the users and hence a sample size of 22, compared to the

oral estrogen group which had a larger sample size of 63 participants. The dosage of the hormones was also assessed and revealed that high doses of HRT had an OR of 0.75, medium doses an OR of 0.59, and low doses an OR of 0.96. Hence, medium doses were optimal, confirming the concerns raised by the authors of the WHI trial (Rossouw et al 2002), that the unfavourable outcomes observed in WHI may be the result of doses of estrogen and progestin that were too high.

In this population-based case-control study, Varas-Lorenzo et al (2000), also evaluated the effects of opposed estrogen (estrogen plus progestogen) and unopposed estrogen (estrogen alone) HRT regarding the risk of MI. The results revealed that unopposed HRT had an OR of 0.52 compared to opposed HRT which had an OR of 0.79 and as stated by the authors unopposed therapy had a greater risk reduction (Varas- Lorenzo et al 2000). These data showing that unopposed estrogen had a lower risk of MI, is crucial as it addresses one of the many limitations of the WHI trial where only opposed estrogen HRT (estrogen plus progestin) was used. Thus to summarise, when estrogen HRT was assessed alone, the route of administration (transdermal versus oral), the dosage and the duration of use influenced the results obtained with respect to risk of MI. Importantly, Varas-Lorenzo et al (2000) demonstrated a cardioprotective effect (decreased risk of MI) of long-term, medium-dose, estrogen alone HRT in healthy post-menopausal women. However, whether the same results would be observed in post-menopausal women with CHD remained to be assessed.

Bearing in mind the favourable effects of HRT on cholesterol levels (HERS and WHI trials), and the fact that the presence of atheromatous plaques increases the risk of cardiovascular events, a sub-study of the WHI trial determined the effect of conjugated equine estrogen HRT on calcified plaque formation in the coronary arteries of healthy, younger (aged 50-59 years) post-menopausal women after a follow-up period of 7.4 years (Manson et al 2007). The experimental group had significantly lower plaque formation

compared to the placebo group. The beneficial effects of HRT observed in this trial were in accordance with the lower risk of MI observed with medium-dose estrogen alone (Varas-Lorenzo et al 2000) and hence add plausibility to the cardioprotective effects of estrogen. However, as only younger post-menopausal women were assessed, whether the same outcomes would occur in older post-menopausal women is not known. Moreover whether conjugated estrogen HRT has an effect on established atheromatous plaques was not evaluated in this study as only healthy (no history of CVD) post-menopausal women were assessed (Manson et al 2007).

A question which therefore arises is whether the effects of HRT are dependent on the presence or absence of CVD. In this respect Psaty et al (1994), conducted a population-based, case-control study in post-menopausal women who had previously experienced either a fatal or a non-fatal MI. In this study cases were post-menopausal women who presented with a fatal or non-fatal MI, and controls were age-matched to cases. The participants were stratified according to HRT use namely: nonusers; estrogen alone; or estrogen plus progestin (combined therapy). The aim of this study was to determine whether HRT has cardioprotective effects after adjusting for major coronary risk factors and co-morbid conditions. The results demonstrated that the risk ratio for an MI was not different between the estrogen alone users and combination HRT users (risk ratios of 0.69 and 0.68 respectively). However it is important to note that the study had a small sample size and was a case-control design as opposed to a randomised control study (HERS and WHI trials), two factors which limit the statistical power of the study. Moreover, the authors did not calculate the relative risk of the non-user group, thus failing to assess whether non-users did in fact have a higher risk ratio for an MI as compared to the HRT users (Psaty et al 1994).

A further study which provides some clarity on the possible factors contributing to the contradictory outcomes of HRT clinical trials is a meta-analysis of 32 trials. Grady et al

(1992) designed a meta-analysis to assess the benefits and risks of HRT in peri-menopausal and post-menopausal women and assessed studies investigating the effects of estrogen therapy alone and combination therapy on endometrial cancer, breast cancer, coronary artery disease (CAD) and stroke. This meta-analysis revealed that estrogen therapy alone lowered the risk for CAD and was beneficial for women who had undergone hysterectomies as well as patients with CHD or at high risk of CHD (Grady et al 1992). Grady et al (1992) stated that long-term (more than 3 years) estrogen therapy was imperative for improving CHD as it is a chronic progressive disease. Indeed, in the HERS trial, the decrease in CHD events was noted in the fourth and fifth years of follow-up but not in the first year of follow-up. In accordance with these data, Varas-Lorenzo et al (2000) demonstrated that the odds ratios for an MI were significantly decreased in participants who had been taking HRT for 3 years as compared to only 6 months or 1 year.

In addition to the duration of HRT, Mendelsohn and Karas (2005) revealed that the age at which HRT is started is extremely important. The "timing hypothesis" as it is known states that if HRT is started early, before an atherosclerotic plaque has advanced, HRT can be beneficial by reversing endothelial dysfunction. However, if the plaque is advanced, HRT may induce inflammatory and haemostatic complications (Mendelsohn and Karas 2005). This "timing hypothesis" could be a plausible explanation for the discrepancies between the trials discussed above. Indeed, in the trials that have shown beneficial outcomes, the participants were younger when HRT was initiated (Manson et al 2007; Varas-Lorenzo et al, 2000) as compared to the participants in the trials in which HRT was shown to be detrimental (Rossouw et al 2002). Thus the timing of initiation and duration of HRT plays a significant role in the effects of HRT on cardiovascular outcomes in patients.

Another issue with respect to HRT is whether estrogen is gender specific or only elicits a cardioprotective effect in females. To investigate the possible impact of gender on

cardiovascular effects of estrogen, Arnlov et al (2006) conducted a prospective cohort study in men. The effect of endogenous estrogen levels on risk of CVD in men was determined. The results revealed that older men (>56 years) with higher estrogen levels had decreased risk for CVD events, even after adjustments for cardiovascular risk factors. Unlike testosterone levels which decrease with age in men, estrogen concentrations in men remain relatively constant. Hence, with age the ratio of estrogen to testosterone concentration would increase and estrogen could therefore elicit a beneficial response (Arnlov et al 2006). Plausible mechanisms of how estrogen is cardioprotective in men include decreases in blood pressure, lipid and glucose levels (Arnlov et al 2006). An important issue which needs to be addressed is whether testosterone is harmful to the myocardium. Indeed, Cavasin et al (2003) revealed that castrated male mice and intact male mice receiving exogenous estrogen post MI had increased ejection fraction and reduced left ventricular dimensions as compared to sham castrated mice; thus suggesting that estrogen administration post MI prevents deterioration of cardiac functioning and remodelling and conversely testosterone exacerbates myocardial dysfunction and remodelling (Cavasin et al 2003). Plausible mechanisms of how testosterone is harmful to the myocardium include an impaired coronary artery relaxation (Ceballos et al 1999), androgen receptor induced increases in myocyte hypertrophy (Marsh et al 1998) and enhanced myocyte apoptosis (Zaugg et al 2001). Although it has been suggested that testosterone may be detrimental to the myocardium (Cavasin et al 2003), this data is controversial. Webb et al (2008) demonstrated in a randomised, placebo controlled, crossover design that men aged 40-75 years with known CHD and low levels of testosterone had increased myocardial perfusion through unobstructed coronary arteries after administration of testosterone for a period of 8 weeks. However, testosterone treatment had no effect on overall myocardial perfusion, global endothelial function, quality of life and angina symptoms (Webb et al 2008). The authors stated that an increased myocardial perfusion could impact

on arterial stiffness which is a predictor of cardiovascular outcomes. However, oral testosterone had no effect on endothelial function or angina symptoms thus revealing that the benefits of testosterone are limiting. Indeed, further research is crucial in understanding the mechanisms and effects of testosterone on the cardiovascular system.

Lastly, it is possible that differences in study design and characteristics of the participants may contribute to the controversies about the potential benefits of HRT (see Table 1). In order to resolve the controversies between observational / case-control (Psaty et al 1994; Varas-Lorenzo et al 2000) and randomised control studies (Hulley et al 1998; Rossouw et al 2002; Manson et al 2007), the Kronos Early Estrogen Prevention Study (KEEPS) is a randomised, double-blind placebo control trial which is currently enrolling healthy (no CHD risk factors) women participants aged 42-58 years old, between 6-36 months from their final menses, and with intact uteruses (Miller et al 2008). The aim of KEEPS is to address the controversies between the WHI trial and observational studies by starting HRT at a young age and at the start of menopause and evaluating whether early intervention of HRT will lower the risk of CHD events and the formation of atherosclerotic plaques (Miller et al 2008). The trial will be completed in 2010 and will hopefully demonstrate whether early intervention is the key to estrogen being cardioprotective. However, even if the KEEPS trial does reveal that estrogen HRT is beneficial, the need for further experimental research is critical in order to determine the mechanisms of the cardioprotective effects of estrogen.

Thus in conclusion it can be seen that although numerous clinical trials have been performed the evidence from the different trials is controversial (Table 1). To summarise, many of the trials are heterogeneous and difficult to repeat as the participants' characteristics differ (i.e. presence or absence of existing CVD). In addition many of the trials use estrogen and progestin together and thus the effect of either hormone alone cannot be determined. The

regime and dosage of HRT is also diverse amongst trials and therefore results are not comparable between studies. Furthermore, in those studies including participants with CHD, the CVD is heterogeneous in aetiology. The latter is important to consider as the consequences of an MI are different from those of a stroke, and hence the potential impact of estrogen on these CVD may differ. Thus to overcome these confounding factors, it is crucial to conduct well controlled studies. In this regard, laboratory bred animals and animal models of disease are more homogenous than their human counterparts. Moreover in animal studies, as invasive and terminal experiments are possible, one can assess specific physiological mechanisms that could possibly explain the potential cardioprotective effects of estrogen.

1.2 Animal research studies

As previously discussed, the outcomes of studies conducted in humans are controversial possibly because of associated confounding factors that are difficult to avoid. However, animal models allow for the control of many of these confounding factors. Moreover, in animal studies, but not in human studies, specific physiological systems can be isolated and manipulated, hence allowing for the assessment of the possible mechanisms of the potential cardioprotective effects of estrogen. Therefore it is crucial that animal studies be conducted to try and elucidate whether estrogen is cardioprotective and the exact mechanisms thereof. In this regard, numerous animal studies have investigated the role of estrogen on various physiological systems. These studies, which are summarised in Table 2, will be discussed in the subsequent sections. Although many of these animal studies have demonstrated a beneficial effect of estrogen on CVS risk, these studies present with numerous limitations pertaining to the model of CVS disease assessed or the methods used to manipulate estrogen concentrations. Hence, the animal studies will be discussed with regards to the impact of the different models used and the methods of estrogen manipulation.

Table 2: Summary of animal studies

Author	Animal Model	Manipulation of estrogen	*In vivo/ Ex vivo*	Outcome	Overall conclusion
Booth et al (2003)	Left anterior descending coronary artery ligation(LAD)	Ovariectomised female rabbits receiving endogenous 17β estradiol	In vivo	• 17β estradiol treated rabbits had smaller infarct sizes compared to controls	17β estradiol→ ↓ infarct size
Booth et al (2005)	Left anterior descending coronary artery ligation(LAD)	Ovariectomised female rabbits receiving endogenous 17β estradiol, ER selective agonists (α and β).	In vivo	• Rabbits treated with ER-α agonists had smaller infarct sizes compared to ER-β and 17β estradiol treated rabbits	ER-α possibly mediates the cardioprotective effects of estrogen
Dubey et al (1998)	Isolated cardiac fibroblasts	17 β-estradiol, progesterone and other estrogen metabolites administered to cardiac fibroblasts	Ex vivo	• 17 β-estradiol and progesterone inhibited cardiac fibroblast growth an effect not noted with the other metabolites	17β estradiol+ progesterone → ↓ cardiac fibroblast growth
Gao et al (2003)	Over-expression of β-adrenergic receptors	None	In vivo	• Lower survival rates in transgenic mice compared to controls • Male transgenic mice had worse cardiomyopathy phenotypes compared to female transgenic mice	↑ survival in female transgenic mice vs. male mice
Kim et al (2006)	Hypoxic re-oxygenation (H/R)	17β estradiol administration to cardiomyocyte cultures	Ex vivo	• Isolated cardiomyocytes receiving 17β estradiol had decreased apoptosis compared to control cardiomyocytes	17β estradiol ↓ cardiomyocyte apoptosis
Osadchii et al (2007)	Short term β-adrenergic agonist administration (isoproterenol)	None	In vivo	• Myocardial fibrosis and apoptosis	ISO → ↑ apoptosis/ fibrosis

Author	Animal Model	Manipulation of estrogen	*In vivo/ Ex vivo*	Outcome	Overall conclusion
Pelzer et al (2005)	-	Ovariectomised female mice receiving exogenous 17β estradiol	In vivo	• Knockout mice receiving 17 β-estradiol had higher mortality rates, body weight, fluid retention and biomarkers of heart failure compared to controls.	Estrogen receptor β is important in heart failure progression
Ueyama et al 2007)	-	Ovariectomised female mice receiving exogenous 17β estradiol	In vivo	• 17β estradiol treated rats had lower heart rates, blood pressure compared to controls • Increased cardioprotective markers in rats receiving 17β estradiol	17β estradiol→ ↓ heart rate/blood pressure 17β estradiol→ ↑ cardioprotective markers
Van Eickels et al (2001)	Transverse aortic constriction (TAC)	Ovariectomised female mice receiving exogenous 17β estradiol	In vivo	• Mice receiving 17β estradiol had less myocardial hypertrophy compared to controls	17β estradiol→ ↓ myocardial hypertrophy
Van Eickels et al (2003)	LAD (coronary artery ligation)	Ovariectomised female mice receiving exogenous 17β estradiol	In vivo	• 17β estradiol treated mice had smaller infarct sizes and cardiomyocyte apoptosis and with lower mortality rates and myocardial remodelling compared to controls	17β estradiol→ ↓ infarct size/ apoptosis
Veliotes et al (2005)	Long term β-adrenergic agonist administration (isoproterenol)	None	In vivo	• Chronic isoproterenol administration induced cardiac dilation and heart failure. • Cardiac dilation was prevented with an aldosterone receptor antagonist (spironolactone)	Chronic ISO→heart failure→prevented by spironolactone

H/R, hypoxic re-oxygenation; IMO, immobilisation stress model; ISO, isoproterenol; LAD, left anterior descending; SHR, spontaneous hypertensive rat; TAC, transverse aortic constriction.

1.2.1 Ex vivo models of CVS diseases and manipulation of estrogen in *ex vivo* models

Cell cultures are often used to elucidate the specific physiological mechanisms of the effects of various substances. However, with regards to investigating the mechanisms of the effects of estrogen, only a few studies have been performed. Kim et al (2006), in a study of isolated cultured rat cardiomyocytes, revealed that prolonged hypoxic reoxygenation induced cardiomyocyte apoptosis (cell death), which was attenuated by 17β estradiol. Hypoxic reoxygenation injury generates mitochondrial reactive oxygen species (ROS) which are potent stimulators of apoptosis. The latter is important as apoptosis has been shown to occur in patients with MI or end-stage heart failure (Guerra et al 1999). Although this study demonstrated the possible cardioprotective effects of estrogen, the hypoxic reoxygentation model is a very aggressive model for producing cell death. Indeed the percentage apoptosis was 3-4% as compared to percentages of 0.25% in males and 0.085% in females as reported in patients with MI or end-stage heart failure (Guerra et al 1999). Moreover, the amount of apoptosis may have been falsely elevated as a result of the absence of other physiological systems such as the influence of nitric oxide on the vasculature (Dubey et al 1998) or the role of the renin angiotensin aldosterone system (RAAS) on the myocardium and vasculature (Cohn and Colucci 2006) which would normally be present in an *in vivo* model. In this regard, one of the limitations of *ex vivo* studies is that cells are assessed in an isolated system which fails to address all of the natural physiological processes that maintain homeostasis.

Nevertheless, *ex vivo* studies have provided some interesting mechanistic information. In addition to estrogen's protective effects against hypoxic reoxygenation induced cardiomyocyte apoptosis, the growth of cultured cardiac fibroblasts is inhibited by 17β-estradiol, its metabolites and progesterone in both genders (Dubey et al 1998). As increased cardiac fibroblast growth occurs in patients with hypertension and in patients with MI (Dubey

et al 1997), these effects of estrogen and progesterone, as demonstrated in cultured cardiac fibroblasts, are of clinical relevance. However, whether these effects were due to estrogen alone, progesterone alone or the combination of estrogen plus progesterone could not be identified. In addition, estrogen has been shown to inhibit the growth of vascular smooth muscle (VSM) cells (Nishigaki et al 1995). Hence, estrogen could possibly attenuate myocardial and vascular remodelling by decreasing cardiac fibroblast (Dubey et al 1998) and VSM growth (Nishigaki et al 1995) respectively.

Although the maintenance of cell cultures in an isolated system is certainly beneficial in terms of identifying mechanisms independent of possible confounding factors; such systems fail to address the impact of a substance (e.g. estrogen) in the presence of other factors involved in normal physiological processes. Hence, whether the results observed in cell culture systems can be extrapolated to *in vivo* models is questionable. The benefits of estrogen observed *ex vivo* may not occur *in vivo* due to for example the presence of other possible opposing hormones. Hence to determine whether estrogen has protective effects on the CVS, *in vivo* studies need to be performed.

1.2.2 *In vivo* animal models of cardiovascular diseases and manipulation of estrogen in *in vivo* models

1.2.2.1 In vivo animal models of cardiovascular disease

In order to induce a cardiovascular insult in animals various methods have been employed. Most of these methods may be too aggressive hence resulting in rapid progression to heart failure, which does not truly represent the fairly slow progression of CVS disease and heart failure in humans. For example, animal models of MI have been induced by acute ligation of the left anterior descending (LAD) coronary artery (Booth et al 2003); whereas in humans MI is most frequently the consequence of progressive atherosclerosis. Alternatively transverse aortic constriction (TAC) has been used to induce pressure overload (Van Eickels

et al 2001); which fails to resemble the slow development of essential hypertension in humans. Nevertheless, in both of these models estrogen has been shown to be protective. In their study to assess the effects of 17β-estradiol on MI size in ovariectomised female rabbits, Booth et al (2003) observed smaller MI and normal sarcomere structures in the 17β-estradiol treated ovariectomised rabbits as compared to vehicle treated ovariectomised rabbits (all female rabbits in this study were ovarectomised and received either 1)17β estradiol; 2)vehicle; 3)17α estradiol; 4)17β estradiol receptor antagonist + 17β-estradiol; or 5) 17β estradiol receptor antagonist + vehicle). In addition, ovariectomised rabbits treated with a 17β-estradiol receptor antagonist plus17β-estradiol had similar MI size as vehicle treated ovariectomised rabbits. Similarly, Van Eickels et al (2001) identified that pressure overload (TAC) – induced myocardial hypertrophy in ovariectomised female mice, was attenuated by both four and eight weeks of 17β-estradiol administration (female mice in all groups were ovarectomised and received either: 1)TAC + placebo; 2) TAC + 17β estradiol; 3) sham-no TAC + placebo; or 4) sham-no TAC +17β estradiol). As myocardial hypertrophy is a positive predictor of cardiac mortality (van Eickels et al 2001), these beneficial effects of estrogen are important. Although, the results of these studies highlight the cardioprotective effects of estrogen *in vivo*; they fail to identify the mechanisms of these effects.

As discussed above, both Van Eickels et al (2001) and Booth et al (2003) used aggressive methods to induce myocardial damage (i.e. TAC and LAD coronary artery ligation). However, heart failure is normally a slow progressive disease; hence experimental models that acutely occlude the aorta or coronary blood vessels and subsequently induce myocardial hypertrophy or ischemia such as TAC and LAD coronary artery ligation models do not mimic the natural progression of heart failure. Hence alternative models which have a slower progression are preferable.

In this regard, an alternative to the surgical induction of CVS disease (i.e. TAC or LAD coronary artery ligation), is the use of overexpression of the adrenergic receptors. It is well established that heart failure is accompanied by an increase in circulating noradrenaline which consequently increases the SNS and induces β-adrenergic receptor up-regulation (Communal et al 1998). Hence, Gao et al (2003) investigated the effects of β_2-adrenergic receptor overexpression in male and female mice using a transgenic mouse model. Survival rates were lower in the transgenic mice compared to the controls, and moreover male transgenic mice had lower survival rates compared to female transgenic mice. The study also revealed that transgenic male mice developed LV hypertrophy and LV dilation as well as increased myocyte fibrosis, collagen and apoptosis, an effect not demonstrated in the female transgenic mice. Although this model of β_2-adrenergic receptor overexpression highlights the deleterious effects of β_2-adrenergic receptor overstimulation, it is unlikely to represent natural physiology as the animals are genetically manipulated. Moreover, β_2-adrenergic receptor overexpression does not allow for the natural progression of heart failure as only one of the physiological mechanisms that play a role in influencing heart failure progression is targeted.

A more physiological animal model which mimics the increased concentrations of noradrenaline observed in heart failure is the daily administration of isoproterenol (ISO), a β-adrenergic agonist. Isoproterenol is an informative drug to use in experimental animal models of CVS disease, as detrimental cardiovascular effects are produced with both short-term (5-7 days) and long-term (5 to 7 months) administration. Short-term administration induces myocardial fibrosis and apoptosis (cell death) (Osadchii et al 2007). As apoptosis and fibrosis are early indications of heart failure, they are important features to investigate. In this regard, future research which leads to early detection and prevention of mechanisms which induce heart failure is imperative.

Chronic or long-term isoproterenol administration induces cardiac dilatation and heart failure (Veliotes et al 2005). As mentioned previously the benefit of using isoproterenol is it physiologically mimics the slow progression of heart failure and is not an aggressive model unlike other methods namely TAC and LAD coronary artery ligation. However, the isoproterenol dosage is also important as extremely high doses induce myocardial arrhythmias, premature death and do not mimic natural cardiac compensatory mechanisms (Cao et al 2000). Therefore, when using isoproterenol to produce an animal model of CVS disease, the dosage should be carefully chosen. Indeed, it is preferable to use a low dose as established by Woodiwiss et al (2001) and Veliotes et al (2005). In the latter studies ISO was administered at a low dose of 0.02-0.04mg/kg for a period of 4.5 or 7 months. The results revealed an increase in LV cavity volumes and reduced pump function. Moreover, long-term low-dose ISO administration decreased LV relative wall thickness and increased myocardial collagen concentrations (more specifically total, non-crosslinked, type I and type III collagen) (Veliotes et al 2005; Woodiwiss et al 2001); characteristics which are observed in patients with cardiac dilation and heart failure (Schwartzkopff et al 2002). Thus, when using ISO administration to produce an animal model of slowly progressive CVS disease (as opposed to acute MI), long-term low-dose ISO administration is preferable. Nevertheless, to date no studies have assessed the role of estrogen in the CVS effects associated with low-dose ISO administration.

In summary, although progress has been made in determining the potential cardioprotective effects of estrogen; the results of the current animal studies (either *ex vivo* or *in vivo*) are not transferable to human CVS diseases. It is therefore imperative to assess the effects of estrogen on the cardiovascular system in an animal model of CVS disease that closely resembles human CVS disease. In addition, the potential role of estrogen in

modifying the specific mechanisms that influence heart failure progression such as cardiomyocyte apoptosis and necrosis needs to be elucidated.

1.2.2.2 Manipulation of estrogen in *in vivo* models

In order to determine the potential cardioprotective effects of estrogen, animal studies have focussed on different methods of manipulating estrogen concentrations. Models include estrogen receptor knockouts; ovariectomised females receiving exogenous estrogen; and intact animals in which the effects of endogenous estrogen is assessed. Although most of the studies have used the approach of ovariectomy with exogenous estrogen administration, as will be discussed, the disadvantages of this approach is the inhibition of the normal cyclical fluctuations in estrogen concentrations. Indeed, some studies have suggested that the contradictory detrimental effects of estrogen are related to constant high doses as opposed to concentrations which range from high to low at different periods of a normal menstrual cycle (Marcondes et al 2002). Nevertheless, cardioprotective effects of estrogen have been demonstrated in studies assessing the effects of exogenous estrogen administration in the presence of ovariectomy.

The effects of exogenous estrogen administration to ovariectomised animals include decreases in blood pressure and heart rate in rats with increases in circulating adrenaline and noradrenaline (Ueyama et al 2007); decreases in cardiomyocyte apoptosis, infarct size, ventricular remodelling and mortality rates in mice with LAD coronary artery ligation (van Eickels et al 2003) and decreased myocardial hypertrophy in mice with TAC (van Eickels et al 2001). Furthermore, in a study using a combination of estrogen receptor knockout, ovariectomy and exogenous estrogen administration; increases in mortality, body weight, fluid retention and biochemical markers of heart failure were observed in comparison to wild-type mice (Pelzer et al 2005). However, this study did not demonstrate whether the effects

observed were as a result of estrogen receptor knockout or secondary to other physiological effects such as renal fluid retention or increases in blood pressure.

Although beneficial CVS effects of estrogen were demonstrated in these studies, it cannot automatically be assumed that similar results would be observed in the presence of naturally cycling endogenous estrogen. In this regard, the concentrations of exogenous estrogen administered far exceeded the normal physiological range. Indeed, Jang et al (2004) revealed that the mean value for normal physiological concentrations of endogenous estrogen was 8.4pg.ml^{-1} in female rats and could have possibly been higher as the levels of endogenous estrogen fluctuate throughout the rat estrus cycle. To explain further, the female rat estrus cycle lasts approximately four to five days and consists of various stages. The four stages are proestrus, estrus, metestrus and diestrus. Estrogen levels start increasing in metestrus, peak during proestrus and return to baseline in estrus. Conversely, van Eickels et al (2003) demonstrated that ovariectomized female mice receiving exogenous 17β-estradiol had excessively high concentrations of 81pg.ml^{-1} which the authors described as being within the physiological range a contrast from levels described by Jang et al (2004) . Administering exogenous estrogen is problematic as it is always at a constant concentration and does not fluctuate like the normal female menstrual (rodent estrous) cycle. Hence, although the animal models discussed in this section have determined the plausible CVS benefits of estrogen; they however fail to provide an understanding of the role of naturally cycling endogenous estrogen in cardioprotection. Thus it is important to specifically address the influence of endogenous estrogen on the myocardium in an animal model.

1.3 The relationship between estrogen receptors and the myocardium

Having established from human and animal studies that estrogen appears to be cardioprotective (at least at constant high doses), it is important to discuss the evidence which

supports a direct mechanistic effect of estrogen on the heart. Estrogen is a complex hormone which activates different receptors and functions to protect the body in varying organs and even in both genders. The identified beneficial effects of estrogen, as well as the different estrogen receptors, will therefore be discussed in the context of function, location and cardioprotective mechanisms.

1.3.1 Estrogen receptors

There are two estrogen receptors, namely α and β. Both receptors bind estrogen with the same affinity, are expressed in the heart and vasculature, and have various functions (Mendelsohn and Karas 1999). Studies have been conducted to assess the function and possible cardioprotective mechanisms of the two estrogen receptors (Figure 1). Certain studies favour estrogen α-receptors while other studies emphasize estrogen β-receptors as the cardioprotective receptors. The two receptors are discussed below in the context of research that has been conducted to date.

1.3.1.1 Estrogen α-receptors

The main effects of estrogen α-receptors (ER-α) are on the vasculature. The functions mediated by ER-α are the protection of damaged blood vessels by enhancing re-endothelialization, decreasing atherosclerotic plaque formation and inhibiting smooth muscle cell proliferation and matrix deposition (Mendelsohn and Karas 2005). In addition, ER-α interact with cytoplasmic proteins, are important in cardiomyocyte structure and stability, and play a role in cell-cell interactions (Mahoodzadeh et al 2006). ER-α are reported to be up-regulated during aortic stenosis and dilated cardiomyopathy (Mahoodzadeh et al 2006). Interestingly, Mahoodzadeh S et al (2006) revealed that dilated cardiomyopathy in both genders invokes mRNA up-regulation of ER-α. In addition, alterations in the localisation of these receptors occur, such that there is a loss of ER-α from the intercalated discs and a co-localization with β-catenin. It has therefore been postulated that the up-regulation of ER-α

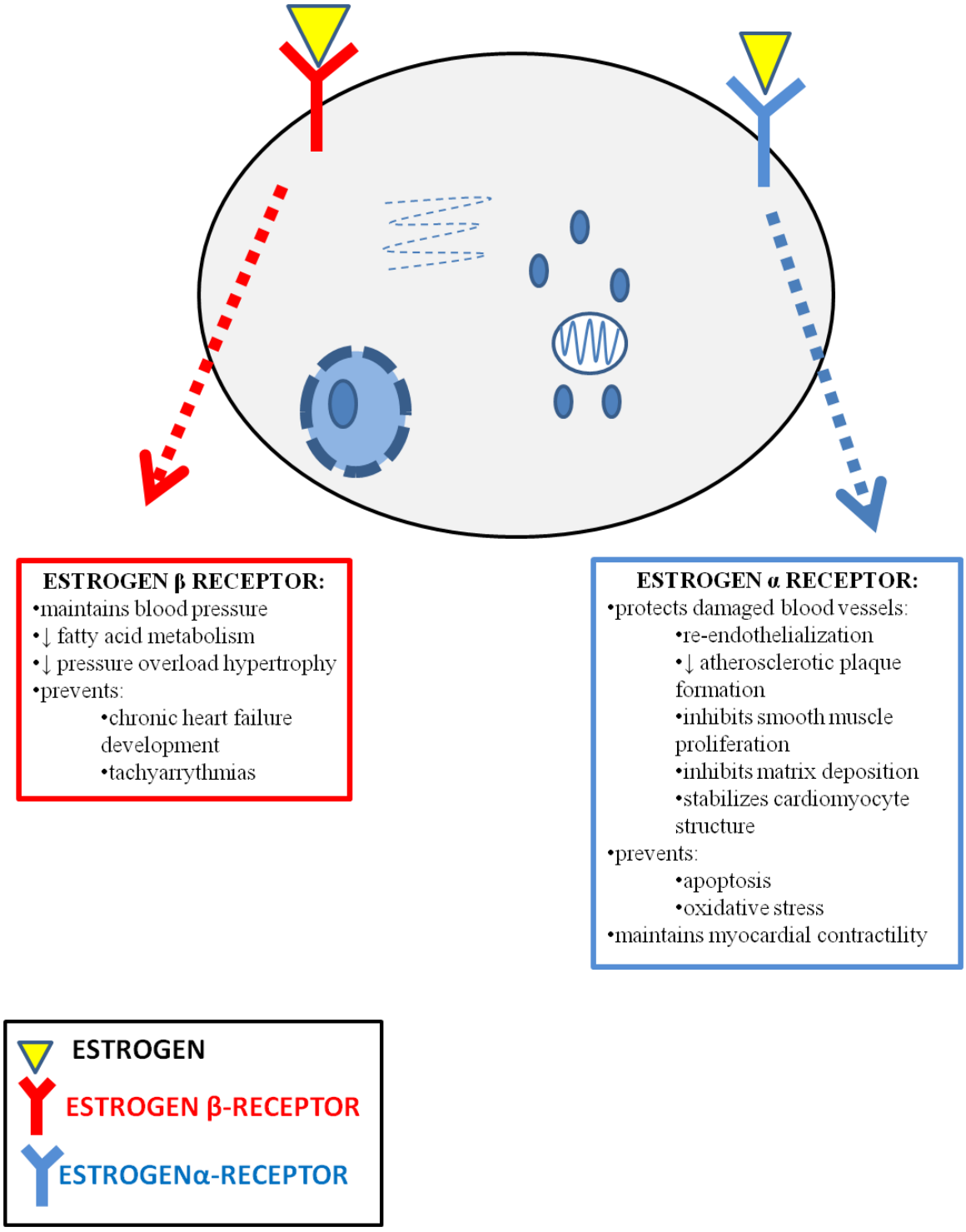

Figure 1: Beneficial effects mediated by estrogen receptors (α and β) on cardiomyocytes

may be a possible cardioprotective mechanism in response to dilated cardiomyopathy and aortic stenosis. Furthermore, during heart failure, the stability of the intercalated discs is weakened, and therefore ER-α are up-regulated and their localisation is altered to cope with the extra load exerted on the heart (Mahoodzadeh et al 2006).

The effects of ER-α on post-ischaemic myocardial function, inflammatory signals and apoptotic signalling have also been evaluated (Wang et al 2006). In a study comparing ER-α knockout mice of either gender to wild type mice, the male wild type mice as well as the ER-α knockout mice of either gender had myocardial contractile dysfunction; whereas the female wild type mice had no myocardial functional impairment. It is important to note that the female wild type (who had normal cycling endogenous estrogen), had lower levels of the pro-apoptotic protein, JNK and elevated levels of extracellular signal-regulated protein kinase (ERK), which assists in myocardial functional recovery after ischaemic reperfusion injury.

In this regard, Booth et al (2005) demonstrated the cardioprotective effects of ER-α in an ischaemic-reperfusion model (LAD coronary artery ligation for 30 minutes followed by reperfusion). Intact rabbits as well as ovariectomised female rabbits were used in the study. Three groups of rabbits were used: group 1 consisted of intact rabbits receiving ER-α agonist, estrogen β-receptor (ER-β) agonist, 17 β-estradiol, and vehicle; group 2 consisted of intact rabbits receiving ER antagonist+ ER-α agonist, ER antagonist + 17β-estradiol, and ER antagonist + vehicle; group 3 consisted of ovariectomized female rabbits and received the same regimen as group 1. The results demonstrated that infarct sizes were smaller in intact rabbits treated with ER- α agonist and 17β-estradiol but not ER-β agonist. In addition, ER- α agonist and 17β-estradiol treated rabbits had similar infarct sizes when treated with the ER antagonist, thus highlighting the cardioprotective effects of estrogen and more specifically ER-α. The results also revealed that ER-α agonist and 17β-estradiol treated rabbits had decreased levels of C-reactive Protein (CRP) and cardiac-specific Troponin I (cTn1);

markers of myocardial damage, an effect not observed in the ER- β agonist treated rabbits. These results suggest that the cardioprotective effects of estrogen are mediated by estrogen α-receptors but not by estrogen β-receptors. Interestingly, ovariectomized female rabbits treated with vehicle had larger infarct sizes as compared to intact female rabbits also receiving vehicle after ischemic-reperfusion. The latter emphasises the cardioprotective effects of endogenous estrogen.

A further study confirmed that the cardioprotective effects of estrogen are mediated by ER- α (Jeanes et al 2008). In ovariectomised rats an ER-α agonist significantly reduced infarct size (in an ischaemic-reperfusion model induced by LAD coronary artery ligation followed by reperfusion) and oxidative stress. In comparison an ER-β antagonist produced no effect on the ischaemia-induced myocardial injury. The lack of effect of the ER-β antagonist (Jeanes et al 2008) in combination with data showing no effect of ER-β agonist on infarct size (Booth et al 2005), suggests that ER-β play no role in myocardial ischaemia reperfusion injury. However, there is still a possibility that ER-β may be cardioprotective in other models of CVS diseases.

1.3.1.2 Estrogen β-receptors

Estrogen β-receptors (ER-β) have been shown to play a role in vasodilatation and the control of blood pressure in both genders (Mendelsohn and Karas 2005). In addition, the role of ER-β in the development of chronic heart failure after MI (LAD coronary artery ligation) has been demonstrated in ovariectomised ER-β knockout mice. Eight weeks after the MI, mortality rates were higher in the ovariectomised ER-β knockout mice compared to wild-type mice; however, no differences in infarct size, cardiac geometry or function (hemodynamic and echocardiographic results) were noted between the groups. The higher mortality rates in the ovariectomised ER-β knockout mice were associated with increases in body weight, pleural effusions, ascites, atrial naturetic peptide levels and phospholamban expression; all of

which are markers of heart failure. Hence, although ER-β have no effect on infarct size either after MI (Pelzer et al 2005) or in an ischaemic-reperfusion model (Booth et al 2005), ER-β demonstrates cardioprotective effects with regards to the development of heart failure. In addition, ER-β, but not ER-α, prevent increases in pressure-overload (via TAC) induced hypertrophy (Skavdahl et al 2004). Interestingly, the increase in pressure-overload induced hypertrophy as a consequence of ER-β knockout was noted in female but not in male mice (Skavdahl et al 2004). In addition, male mice had significantly increased pressure-overload induced hypertrophy compared to female mice, hence highlighting the underlying cardioprotective effects of endogenous estrogen mediated by the ER-β.

Additional cardiovascular effects noted to be mediated by ER-β include decreases in lipoprotein lipase expression (and hence fatty acid metabolism) (Skavdahl et al 2004). Moreover, ER-β knockout mice were shown to have prolonged ventricular repolarisation, decreased ventricular spontaneity and decreased expression of the voltage-gated potassium channels (Korte et al 2005). These effects mediated by ER-β may explain the decreased incidence of fatal ventricular tachyarrhythmias in women compared to men (Coa et al 2000).

In summary, both estrogen receptors play a role in cardioprotection. However these roles differ in that the ER-α maintain myocardial contractility, inhibit apoptotic signalling, decrease oxidative stress and reduce myocardial infarct size (Pelzer et al 2005; Booth et al 2005; Jeanes et al 2008; Mahoodzadeh et al 2006); whereas ER-β decrease mortality in chronic heart failure, pressure overload hypertrophy, lipoprotein lipase and tachyarrythmias (Skavdahl et al 2004; Korte et al 2005). Neither receptor has been established as being more important with regards to cardioprotection; however the interaction of these receptors with other physiological systems such as the SNS may differ.

1.3.2 β-adrenergic receptors and estrogen

Having established that the most appropriate animal model of slowly progressive CVS disease is low-dose ISO administration, it is important to discuss the evidence which supports a possible interaction between estrogen and β-adrenergic receptors. Several studies have examined the relationship between the sympathetic nervous system (SNS), its receptors and estrogen. The two receptors of the SNS which mediate cardiovascular effects are the α_1 and β_1 receptors. In brief the primary cardiovascular effects mediated by α_1-receptors are vasoconstriction of peripheral blood vessels and hence increases in blood pressure and by β_1-receptors are enhanced myocardial contractility, lusitrophy (relaxation), dromotrophy (conduction) and heart rate (Dorn 2002).

With respect to β_1-adrenergic receptors, a relationship with estrogen has been demonstrated. Indeed, in ovariectomised mice, a significant up-regulation of β_1-adrenergic receptors was noted after 10 weeks (Thawornkaiwong et al 2003). In addition, ovariectomy induced an increase in calcium responsiveness of cardiac myofilaments. In this regard, bearing in mind that β_1-adrenergic receptors increase calcium responsiveness of the myocardium (Saito et al 2000) and that long-term β_1-adrenergic receptor stimulation is detrimental to the myocardium (Communal et al 1998), it is plausible that estrogen protects the myocardium from β_1-adrenergic receptor over-stimulation.

This interaction between β_1-adrenergic receptors and estrogen was confirmed in a study in which isoproterenol (a β_1-adrenergic receptor agonist) was administered to ovariectomised rats (Kam et al 2003). β_1-adrenergic receptor expression, density and affinity, cyclic AMP expression as well as infarct size (induced by LAD coronary artery ligation) were increased in ovariectomised rats. However, exogenous 17β-estradiol administration to ovariectomised rats suppressed these effects. In support of a role of estrogen in moderating

β_1-adrenergic receptor responses, Vizgirda et al (2002) demonstrated increased β_1-adrenergic receptor responses to isoproterenol in male rats compared to female rats.

An important study confirming the interaction between adrenergic receptors and estrogen *in vivo* was that of Ciric and Susic (1980), in which the heart rate and blood pressures of female rats were determined at different stages of the estrus cycle. Female rats were assigned to one of 5 groups: group 1, female rats in proestrus; group 2, female rats in metestrus; group 3, ovarectomised rats receiving placebo; group 4, ovarectomised receiving estradiol; group 5, ovarectomised receiving progesterone. Measurements of heart rate and blood pressure were obtained before and after the injection of ISO. The results demonstrated no difference in baseline measurements (heart rate and blood pressure) between groups 1 and 2, both groups had a positive inotropic response to ISO with an increase in heart rate however; rats in proestrus had higher heart rates in response to ISO and the effect of ISO on heart rates lasted longer as compared to rats in metestrus. In groups 3, 4 and 5, ISO produced a fall in blood pressure. Interestingly, ovarectomised rats receiving exogenous estradiol had lower resting heart rates compared to placebo and progesterone treated rats. After ISO administration all three groups had a positive inotropic response however, rats treated with estradiol had a greater increase in heart rate as compared to progesterone and placebo treated groups. In summary, rats in proestrus which correlates to peak estrogen concentrations and ovarectomised rats receiving exogenous estradiol had a greater inotropic response to ISO as compared to the other groups. The authors suggested that such a response could be due to an interaction between estrogen and β-adrenergic receptors in that estrogen enhances myocardial functioning. Moreover, the authors cautioned that this was a short term study and that long term estradiol administration could have produced a different response. However, this was one of the first studies to show the importance of the estrus cycle as well as a relationship between estrogen and β-adrenergic receptors.

Although research has focused on β_1-adrenergic receptors there is evidence to suggest that β_2-adrenergic receptors also play a role in the myocardium. Gao et al (2003) explored the effect of β_2-adrenergic receptor (β_2-AR) overexpression in both genders and the subsequent influence on survival rate and myocardial function. The results demonstrated that survival was greatest in the WT mice compared to both gender overexpression groups. In addition, male transgenic mice had significantly lower survival rate compared to female β_2-AR overexpression mice, thus alluding to the fact that a gender difference does exist and that estrogen is cardioprotective. Moreover male transgenic mice had enlarged left ventricular dimensions, increased hypertrophy and dilatation compared to the female transgenic mice. The study also demonstrated increased amounts of fibrosis and apoptosis in the male transgenic mice compared to the other groups (WT and female transgenic mice). The researchers performed orchiectomy (removal of the testes) in the male transgenic mice and ovariectomised the female transgenic mice. Interestingly, male transgenic mice had increased survival rates compared to their female counterparts, and hence raises the questions whether testosterone is detrimental to the myocardium. Furthermore, whether estrogen is only cardioprotective in the early stages of a myocardial insult and not during chronic development also needs to be assessed. In another study Xydas et al (2006) confirmed that a β_2-AR agonist (clenbuterol) attenuated cardiomyocyte apoptosis and ventricular remodelling in LAD ligation induced heart failure. The latter effect was also noted in rats receiving a β_1-receptor antagonist (metoprolol), thus demonstrating the importance of the β_1-receptor.

In summary, the above studies (Gao et al 2003; Xydas et al 2006; Kam et al 2003; Wong et al 2007; Thawornkaiwong et al 2003; Vizgirda et al 2002) have clearly demonstrated that a relationship does exist between β_1-adrenergic receptors and estrogen; however the effects of estrogen on the mechanisms of the detrimental effects of increased β_1-adrenoceptor stimulation warrant investigation. In this regard, the effects of estrogen on β_1-

adrenoceptor induced cardiomyocyte death (apoptosis or necrosis) and myocardial collagen production are not known.

1.4 Cardiomyocyte apoptosis and necrosis

Apoptosis, or programmed cell death, is a physiological process designed to remove dead or damaged cells and maintain a homeostatic environment. Apoptosis is controlled genetically by the activation of specific genes and enzymatic proteins (Dlamini et al 2004). Distinct morphological features of apoptosis are; cell shrinkage, peripheral chromatin condensation, DNA fragmentation, cytoplasmic "blebbing" and the formation of apoptotic bodies. Apoptotic bodies are recognised by specific immune cells and are removed without the induction of an inflammatory response (Dlamini et al 2004). In contrast, necrosis is accidental cell death and results in dysregulation of the cell structure; that is cell swelling, rupturing of the plasma membrane, spillage of cellular contents, surrounding cell damage and the induction of an inflammatory response (Dlamini et al 2004; Jugdutt and Idikio 2000). Moreover, necrosis is closely associated with fibrosis. Weber and Brilla (1992) define fibrosis as an abnormal increase in collagen concentration which is either reactive, that is increased *de novo* collagen synthesis; or replacement, that is formation of scar tissue by replacement of necrotic myocytes. Both types of fibrosis negatively influence myocardial compliance (Bos et al 2004, Suzuki et al 2002).

Current research demonstrates that increases in both cardiomyocyte apoptosis and necrosis occur in myocardial infarction and heart failure (Communal et al 1998). The consequences of increased apoptosis and necrosis are that viable, functioning cardiomyocytes are destroyed and consequently cardiac remodelling is induced (Francis 2000). Prolonged cardiac remodelling results in myocardial dilatation, pump dysfunction and ultimately end stage heart failure (Cohn and Colucci 2006). An important question is therefore, what is

responsible for the increases in cardiomyocyte apoptosis and necrosis prior to end stage heart failure?

It is well established that patients in heart failure present with increased levels of noradrenaline. The initial increase in noradrenaline is a compensatory effect to improve myocardial contractility and hence cardiac function; however prolonged stimulation of β-adrenergic receptors induces severe myocardial remodelling and cardiac dilatation (Tan et al 2004). Moreover, high levels of noradrenaline have been shown to induce cardiomyocyte apoptosis both *in vitro* (Communal et al 1998) and *in vivo* (Goldspink et al 2004) as well as cardiomyocyte necrosis (Goldspink et al 2004). The noradrenaline or ISO-induced increases in apoptotic cardiomyocytes were shown to be prevented by the pre-treatment of cardiomyocytes with the β-blocker, propanolol (Communal et al 1998). Three to six hours after the administration of high doses of ISO to rats, cardiomyocyte apoptosis was identified; whereas cardiomyocyte necrosis only occurred 18 hours after the administration of ISO (Goldspink et al 2004). Hence, excess stimulation of β-adrenergic receptors induces cardiomyocyte apoptosis and necrosis.

1.4.1 Estrogen effects on cardiomyocyte apoptosis

Bearing in mind the lower incidence of heart failure in premenopausal women compared to men, the effects of estrogen on cardiomyocyte apoptosis and necrosis have been investigated (Figure 2). Kim et al (2006) reported that 10nM estrogen prevented hypoxia-induced apoptosis in cultured rat cardiomyocytes, an effect which was inhibited by an estrogen receptor antagonist. Estrogen was shown to inhibit the activity of pro-apoptotic kinase p38α (Kim et al 2006), which is a crucial protein involved in the pro-apoptotic pathway. In addition, estrogen was shown to decrease apoptosis by enhancing the activity of kinase p38β which promotes cell survival (Kim et al 2006). The production of ROS (reactive oxygen species), which are potent stimulators of apoptosis, was also decreased by estrogen

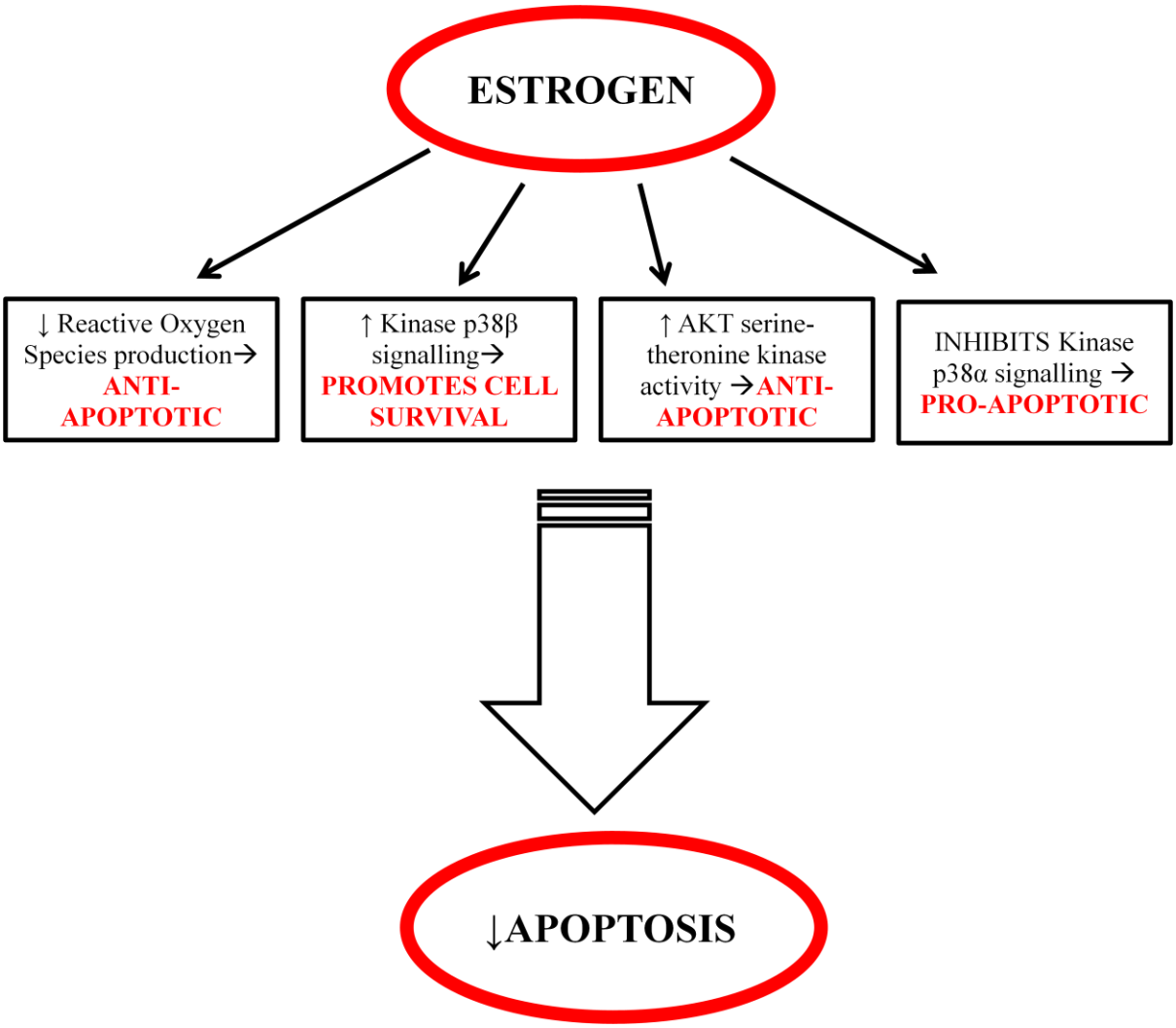

Figure 2 Effects of estrogen on apoptotic signalling pathways

(Kim et al 2006). Although, this study clearly highlights the positive role estrogen plays in the inhibition of cardiomyocyte apoptosis; it fails to assess the effect of estrogen in an *in vivo* model which could present with different results as other physiological systems would be involved.

Hence, in order to assess the effects of estrogen in an *in vivo* model, van Eickels et al (2003) performed a study using ovariectomised female mice receiving 17β-estradiol. Smaller myocardial infarctions (LAD coronary artery ligation) were observed after six weeks of 17β-estradiol administration. However, the mice receiving 17β-estradiol had increased LV remodelling and mortality despite the smaller infarct sizes. Nevertheless, cardiomyocyte apoptosis was decreased in the ovariectomised mice receiving 17β-estradiol. In this study apoptosis was assessed by using the TUNEL technique as well as by determining the concentrations of caspase-3 (an enzymatic marker of apoptosis). These results are controversial as exogenous 17β-estradiol administration decreased myocardial infarct size as well as cardiomyocyte apoptosis but failed to prevent cardiac remodelling or to decrease mortality rates. Indeed, previous studies have demonstrated that 17β-estradiol is anti-hypertrophic and decreases cardiac remodelling (Booth et al 2005; van Eickels et al 2001; Pelzer et al 2005). Moreover, reductions in cardiomyocyte apoptosis (TUNEL and caspase-3) have been reported following 17β-estradiol administration to ovarietomised mice with MI induced by coronary artery ligation (Patten et al 2004). The Akt (serine-threonine kinase) pathway, which plays a crucial role in anti-apoptotic effects, was enhanced at 24 and 72 hours post-MI in the mice receiving 17β-estradiol versus the controls, an effect which was inhibited by an estrogen receptor antagonist (Patten et al 2004). This study further demonstrated that 17β-estradiol activated Akt through estrogen α-receptors and not through estrogen β-receptors. In contrast to the study performed by van Eickels et al (2003), Patten et

al (2004) demonstrated the protective effect of estrogen against cardiomyocyte apoptosis and caste some light into one of the specific apoptotic pathways mediating this effect.

Although both van Eickels et al (2003) and Xydas et al (2006) reported a reduction in cardiomyocyte apoptosis due to estrogen, in both of these studies cardiomyocyte apoptosis was induced by LAD coronary artery ligation. However, LAD coronary artery ligation is an extremely aggressive model, in that it induces very high percentages of cardiomyocyte apoptosis. Indeed, van Eickels et al (2003) reported cardiomyocyte apoptosis ranging from 3.85% in control to 17.7% in LAD, and Xydas et al (2006) from 0.2% in control to 21% in LAD. Importantly, a 0.2% increase in cardiomyocyte apoptosis per day, if sustained for a year, would result in a loss of half of the functioning cardiomyocytes and consequently severe myocardial damage would occur (Colucci 1996). Hence, high percentages of apoptosis are not physiologically compatible with normal or even compromised myocardial function. Therefore, LAD coronary artery ligation models are not likely to be representative of human heart failure. In comparison to the high percentages of apoptosis reported only 6 weeks after LAD coronary artery ligation (van Eickels et al 2003; Xydas et al 2006); Veliotes et al (2005), in a model of heart failure induced by 4.5 months of ISO administration, demonstrated lower levels of apoptosis with percentages ranging from 1.99% in control to 5.37% with ISO. Thus in comparison to the model of LAD coronary artery ligation, chronic ISO administration (Veliotes et al 2005) is more likely to mimic the natural progression of human heart failure. Nevertheless, in order to elucidate whether cardiomyocyte apoptosis is a cause or a consequence of chronic ISO-induced cardiac failure, the effects of short-term (~7 days) ISO administration on cardiomyocyte apoptosis need to be assessed. Indeed Osadchii et al (2006) assessed the effects of short term ISO administration on cardiomyocyte apoptosis. The results demonstrated that 5 days of ISO induced 5.02% cardiomyocyte apoptosis

(Osadchii et al 2006). However, to date the effects of estrogen administration on short-term (~7 days) ISO administration on cardiomyocyte apoptosis have not been assessed.

1.4.2 Estrogen effects on cardiomyocyte necrosis

In addition to the effects of estrogen on cardiomyocyte apoptosis, the impact of estrogen on cardiomyocyte necrosis and replacement fibrosis has also been investigated. In ovariectomized rabbits receiving 17β-estradiol (which decreases cardiovascular mortality, see end of section 1.3.1.2, page 27 and Figure 1), infarct size (induced by LAD coronary artery ligation) was decreased in comparison to that of ovariectomized rabbits receiving 17α-estradiol (which decreases apoptosis and myocardial infarction, see end of section 1.3.1.2, page 27 and Figure 1) or sham operated female rabbits (Booth et al 2001). The reduction in infarct size with 17β-estradiol was associated with the prevention of necrosis as determined by sarcomere and mitochondrial structure as well as decreased neutrophil accumulation in the affected infarct areas (Booth et al 2001). Hence, estrogen, via stimulation of the estrogen β-receptor, decreases infarct size and the degree of necrosis.

Similarly, in a model of ischaemic-reperfusion injury, exogenous 17β-estradiol administration to ovariectomized rats reduced necrosis, neutrophil infiltration and oxygen free radicals (Jeanes et al 2008). However, in this study by Jeanes et al (2008) the beneficial effects attributed to estrogen were shown to be mediated by estrogen α-receptors and not estrogen β-receptors. Indeed, Hale et al (1996) demonstrated that ovariectomised female rabbits receiving exogenous 17β-estradiol after coronary occlusion, had smaller infarct sizes and decreased myocardial necrosis as compared to rabbits receiving placebo. Although the results are favourable, the concentrations of 17β-estradiol that were used were extremely high with levels of 392 $pg.ml^{-1}$. Such concentrations fail to correlate with normal physiological

levels as described by other studies (Jang et al 2004). Hence, although these studies demonstrated that exogenous 17β-estradiol decreased necrosis *in vivo* in ovariectomized mice, the possible cardio-protective effects of estrogen released during the natural estrus cycle remain to be elucidated.

The studies discussed above on the role of estrogen in cardiomyocyte apoptosis and necrosis have all been animal based. Hence, what is the evidence of estrogen mediated cardiomyocyte apoptosis or necrosis in human hearts. In myocardial samples, collected from male and female patients undergoing heart transplantations for heart failure, males had a twofold higher rate of cell death (both apoptosis and necrosis) than females (Guerra et al 1999). In addition, the women were noted to have had a later onset of myocardial decompensation and an increased time frame between heart failure and the need for heart transplantation compared to the men (Guerra et al 1999). Although, this study revealed a gender difference in cardiomyocyte apoptosis and necrosis in humans; the women participants were aged 41-61 years old and whether they were pre- or post-menopausal or on HRT was not assessed. It is plausible that estrogen played a role in the cardioprotection of the female hearts; however, in the context of the limitations of this study, it is crucial to investigate the possible physiological mechanisms that facilitate female cardioprotection. At present no studies have assessed the effects of estrogen on short term ISO-induced apoptosis and necrosis and further research is required to elucidate whether estrogen can decrease β-adrenergic induced cardiomyocyte apoptosis and necrosis.

1.5 The relationship between estrogen, β-adrenergic receptors and apoptosis/necrosis

A relationship between increased β-adrenergic stimulation and cardiomyocyte apoptosis/necrosis, and the subsequent damaging effects on the myocardium (cardiac

dilatation and heart failure progression) has been demonstrated (Communal et al 1999). In addition, estrogen receptors are located in the myocardium and research has revealed the beneficial effects of estrogen therapy on myocardial induced insults (Booth et al 2005; Van Eickels et al 2001; Pelzer et al 2005). Moreover, studies have confirmed that estrogen down regulates the action of β-adrenergic receptors within the myocardium (Thawornkaiwong et al 2002). Studies have also revealed that estrogen decreases cardiomyocyte apoptosis and necrosis induced by hypoxic reperfusion (Kim et al 2006). However, at present no study has examined whether estrogen can attenuate β-adrenergic induced cardiomyocyte apoptosis or necrosis *in vivo*. Moreover, the effects of the natural estrus cycle on increased β-adrenergic stimulation, a potent catalyst of myocardial damage and heart failure, have not been assessed. Thus it is crucial to evaluate whether estrogen is cardioprotective due to possible inhibitory effects on β-adrenergic activation and a subsequent reduction in apoptosis and necrosis.

It is also important to note that currently the studies that have been performed have used ovariectomized female animals receiving exogenous estrogen, knockout or over expression of estrogen receptors (either β or α receptors) or cell cultures. Although the studies have yielded constructive results these animal models fail to assess the natural estrus cycle. In addition in models of females receiving exogenous estrogen, physiological levels are exceeded and the fluctuations during the normal estrus cycle are eliminated. Thus, although a relationship has been established between estrogen and β-adrenergic receptors as well as cardiomyocyte apoptosis/necrosis and β-adrenergic receptors, whether estrogen is cardioprotective through decreasing β-adrenergic induced apoptosis and necrosis is unknown.

1.6 Summary and study objectives

In summary, the results from human studies are inconsistent. Some studies demonstrate beneficial effects of HRT such as decreasing atherosclerotic plaque progression, improving the lipid profile (Manson et al 2007), reducing the risk of myocardial infarctions (Varas-

Lorenzo et al 2000) and coronary artery disease (Grady et al 1992). On the contrary, other studies reveal harmful effects of HRT namely; increased risk of breast cancer, coronary heart disease and pulmonary emboli (Hulley et al 1998; Roussow et al 2002). Moreover, there are variations between the human studies with respect to their study designs and the characteristics of the participants. In addition, treatment regimens for HRT vary between studies such as the duration of administration, route of administration (tablets or transdermal), dosages, and the combination of hormones (estrogen only or estrogen plus progesterone). Though, the general consensus from human studies is that estrogen rather than progesterone is the cardioprotective hormone (Grady et al 1992, Varas-Lorenzo et al 2000, Manson et al 2007). Nevertheless, data shows that males have a higher risk of cardiovascular disease than females prior to menopause, after which the gender discrepancy in risk of cardiovascular disease is diminished (Hulley et al 1998; Rossouw et al 2002). Therefore, it is essential to explore the potential cardioprotective effects of estrogen and the physiological mechanisms thereof. For the latter to be achieved animal studies are required.

Although, to date numerous animal studies have demonstrated beneficial effects of estrogen, such as decreases in infarct size (Booth et al 2003), cardiac fibroblast growth (Dubey et al 1998), apoptosis/necrosis (Kim et al 2006), heart rate (Ueyama et al 2007) and myocardial hypertrophy (Van Eickels et al 2001), these studies have certain limitations. Firstly, the animal models (e.g. transverse aortic ligation, immobilization stress models, left coronary artery ligation and hypoxic re-oxygenation) used to induce myocardial damage are extremely aggressive and hence fail to mimic the natural progression of cardiac failure. Secondly, cell cultures rather than intact animals are often used and hence the data fail to assess the interaction of all physiological systems, therefore rendering the results unlikely to represent data obtained in an *in vivo* model. Thirdly, the majority of *in vivo* studies have investigated the effects of exogenous estrogen administration to ovariectomized female

animals. The limitations of exogenous estrogen administration are the high non-physiological concentrations of estrogen that are used, as well as the loss of the normal cyclical fluctuations of estrogen during the estrus cycle. Indeed, the benefits versus risks of estrogen are dependent on the concentration administered (Varas-Lorenzo et al 2000), with higher concentrations conferring less benefit (Varas-Lorenzo et al 2000).

To address the three limitations discussed above, I chose to study an *in vivo* model which more closely mimics the natural slow progression of heart failure, namely daily ISO administration, in naturally cycling female compared to male rats. In order to identify mechanisms it is imperative to assess early markers of cardiac dysfunction, hence I chose to assess apoptosis and necrosis/fibrosis as my primary outcomes.

Hence, the aim of my study was to assess the impact of gender on short term β-adrenergic induced cardiomyocyte apoptosis and necrosis/fibrosis in rats *in vivo*.

The specific objectives of my study were to assess:

- the impact of short term (5 days) β-adrenergic receptor activation on **cardiomyocyte apoptosis** in naturally cycling female WKY rats during peak estrogen concentrations compared to male WKY rats
- the impact of short term (5 days) β-adrenergic receptor activation on **cardiomyocyte necrosis/fibrosis** in naturally cycling female WKY rats during peak estrogen concentrations compared to male WKY rats
- the impact of short term (5 days) β-adrenergic receptor activation on **cardiac function** in naturally cycling female WKY rats during peak estrogen concentrations compared to male WKY rats

- the impact of short term (5 days) β-adrenergic receptor activation on **cardiac geometry** in naturally cycling female WKY rats during peak estrogen concentrations compared to male WKY rats
- the impact of short term (5 days) β-adrenergic receptor activation on **acute responses to β-adrenergic receptor activation** in naturally cycling female WKY rats during peak estrogen concentrations compared to male WKY rats

Chapter 2

Materials and Methods

2 Materials and methods

A summary of the materials and methods used in this study is illustrated in Figure 3.

2.1 Groups and treatment regimen

All experimental procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC 2007/28/04). A total of 27 male and 29 female age-matched (2 month old) Wistar-Kyoto (WKY) rats were assigned to one of four groups. The four groups were named according to the regimen administered namely; daily subcutaneous injection of a β-adrenergic agonist isoproterenol [ISO (SIGMA-ALDRICH, South Africa); $0.02mg.^{-1}kg.^{-}1.day^{-1}$ administered in 0.2ml saline]; isoproterenol male (ISO M, n=14); isoproterenol female (ISO F; n=15) and daily subcutaneous injection of vehicle (saline, 0.2ml), control female (CON F, n=14) and control male (CON M, n=13). The isoproterenol or saline was administered for a period of 5 days. On the fifth day, rats were weighed and then anaesthetised with ketamine (Anaket, $75mg.kg^{-1}$, Centaur Labs, South Africa) and xylazine (Chanazine, $15mg.kg^{-1}$, Centaur Labs, South Africa) and echocardiography was performed to assess the effects of ISO administration on cardiac geometry and function (see 2.3 below).

As the primary aim of my study was to assess gender differences and more specifically, the effects of natural estrogen (estrogen concentrations in a natural estrus cycle) on cardiomyocyte apoptosis and necrosis, female rats were terminated at the peak of estrogen concentrations, in other words during proestrus (Marcondes et al 2002; Kramer and Bellinger et al 2009). Hence, the timing of the commencement of isoproterenol or saline administration had to be considered. The normal rat estrus cycle lasts approximately four to five days and consists of various stages. The four stages are proestrus, estrus, metestrus and diestrus. Estrogen concentrations start increasing in metestrus, peak during proestrus and return to

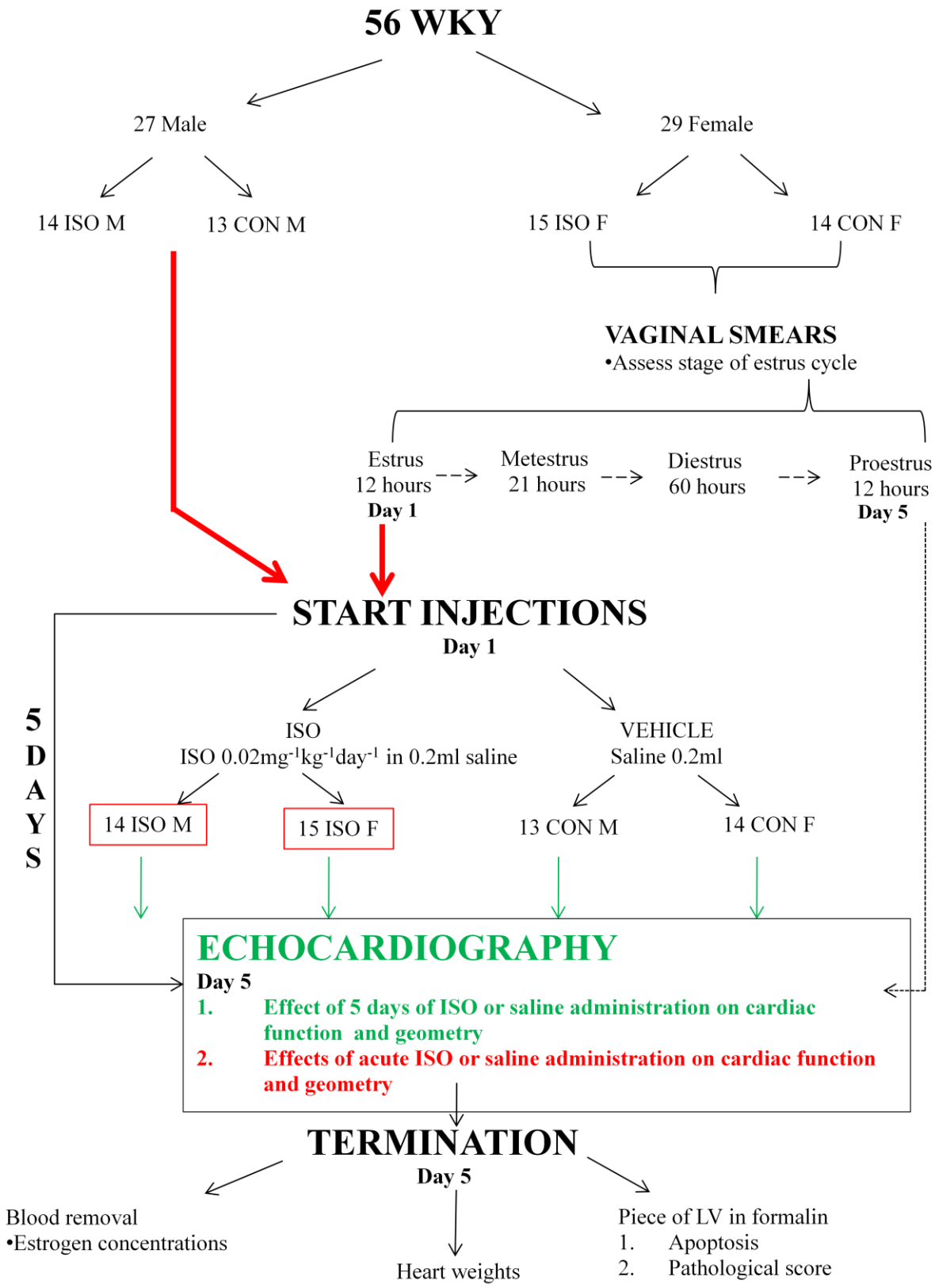

Figure 3: Summary of the materials and methods.

ISO, isoproterenol; F, female; LV, left ventricle; M, male; WKY, Wistar-Kyoto

baseline in estrus. In order to ensure termination of the female rats at peak estrogen concentrations (proestrus), isoproterenol or saline administration had to commence in estrus. Vaginal smears were therefore performed to assess the stage of estrus (see 2.2 below) prior to the commencement of isoproterenol or saline administration. The reason for using two month old rats are as follows: female rats start cycling at about six weeks of age, thus two month old female rats were used with aged matched male rats. The youngest age of termination was approximately three months of age therefore ensuring that rats were in a "pre-menopausal" state. Female rats stop cycling at about twelve months of age (Marcondes et al 2002) and are then considered to be in a "post-menopausal" state.

2.2 Vaginal smears

The four stages of the rat estrus cycle, namely proestrus, estrus, metestrus and diestrus last twelve, twelve, twenty one and sixty hours respectively, hence the estrus cycle lasts a total of 4 days and 19 hours (Marcondes et al 2002). The stage of diestrus was further subdivided into early and late diestrus. Each phase is characterised by specific vaginal epithelial cells that can be distinguished using light microscope (Marcondes et al 2002). Proestrus has round nucleated epithelial cells; estrus has large cornified, anucleated epithelial cells; metestrus has large cornified and elongated epithelial cells as well as small round leucocytes; early diestrus has only small leucocytes and late diestrus has small leucocytes as well as some round nucleated epithelial cells (Figure 4). To assess each stage of estrus, the rats were restrained manually (hand placed over back of rat) and the tail and hind legs elevated to expose the vaginal orifice.

A plastic dropper containing saline was inserted into the vagina and the cervix was washed several times to remove vaginal surface cells. The dropper was then used to aspirate

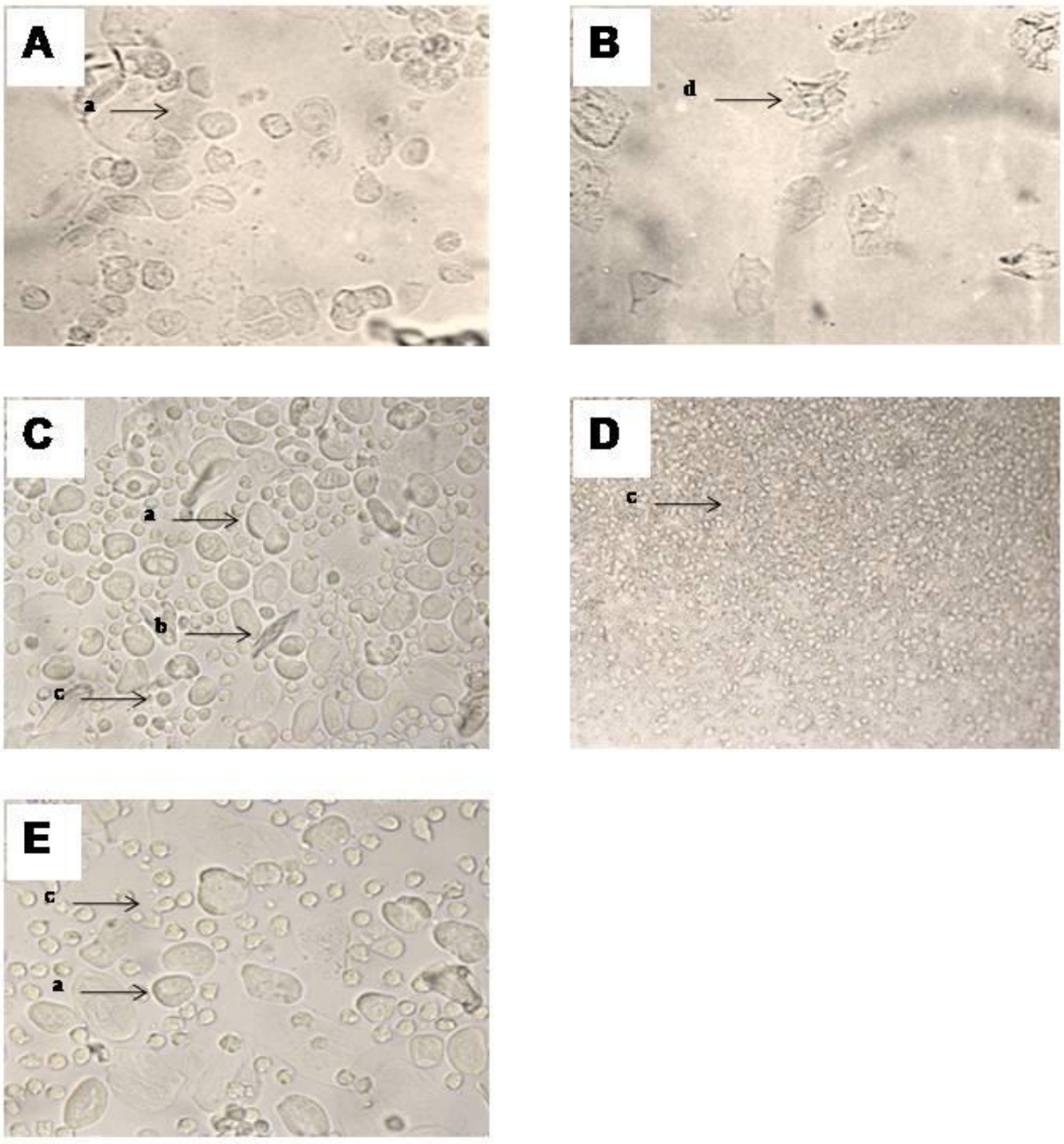

Figure 4: Representative examples of vaginal smears representing the four stages of the rat oestrus cycle.

A, Proestrus - round nucleated epithelial cells (a); B, Estrus - large cornified, anucleated epithelial cells (d); C, Metestrus - large epithelial cells (a), elongated epithelial cells (b) and a few leucocytes (c) ; D, Early Diestrus - many leucocytes (c); E, Late Diestrus - leucocytes (c) and a few nucleated epithelial cells (a).

saline containing vaginal surface cells. Once aspirated a drop of saline containing vaginal cells was placed on a slide and viewed instantly under a light microscope at 40X magnification. As peak estrogen concentrations occur during proestrus, to ensure that female rats were terminated when estrogen levels were at their peak, ISO or saline injections were started at estrus, which is approximately five days before proestrus. Vaginal smears were performed daily and once rats were cycling normally and together, ISO or saline injections were commenced at estrus.

2.3 Echocardiography

Echocardiography was performed to assess the effects of five days of ISO or saline administration on cardiac geometry and function *in vivo* in male versus female rats, as well as the effects of acute ISO or saline administration on cardiac function and geometry. The assessments of the acute effects served to confirm that the dose of ISO administered was sufficient to induce a positive inotropic response (increased contractility).

On the fifth day, rats were anesthetised with ketamine (Anaket, 75mg.kg^{-1}, Centaur Labs, South Africa) and xylazine (Chanazine, 15mg.kg^{-1}, Centaur Labs, South Africa) to ensure immobilisation. Two sets of echocardiography measurements were made, the first to assess the effects of five days of ISO or saline administration on cardiac geometry and function in male and female rats. The second sets of measurements were made after an injection of ISO or saline according to the group specification. In other words, ISO M and ISO F received a subcutaneous injection of ISO (0.02mg.kg^{-1} administered in 0.2ml saline) and CON M and CON F received a subcutaneous injection of saline (0.2ml). To allow for adequate responses to ISO or saline to occur, the second set of measurements were made at least 30 minutes after ISO or saline administration.

Echocardiography is a non-invasive technique which entails placing a high resolution probe on the chest wall of the rats and obtaining a two-dimensional guided M-mode image of the heart (7.5 MHz transducer, Siemens Medical Solutions, Mountain View, California, USA). By imaging the left ventricle in the short axis, images of the left ventricular dimensions and posterior wall thickness were acquired and used to assess left ventricular geometry and both chamber and myocardial systolic function (Norton et al 1997; Tsotetsi et al 2001; Woodiwiss et al 2001). From the images acquired measurements of left ventricular end diastolic diameter (EDD), left ventricular end systolic diameter (ESD), posterior wall thickness at end-diastole (PWTd), and posterior wall thickness at end-systole (PWTs) were made (Figure 5). These measurements were used to assess changes in left ventricular geometry in response to ISO or saline in male and female rats. In addition, in order to assess the degree of ventricular concentricity, chamber diastolic area (Area) and relative wall thickness (RWT) were calculated using standard formulae (see below).

To determine the effects of ISO or saline on chamber and myocardial systolic function in male and female rats, left ventricular endocardial fractional shortening (FSend) and left ventricular myocardial fractional shortening (FSmid) were calculated from the measurements of EDD, ESD, PWTd and PWTs, using standard echocardiographic formulae (Sahn et al 1978) (see below). In addition, to determine the effects of ISO and saline on heart rate in male and female rats, heart rate was calculated using a standard formula (see below).

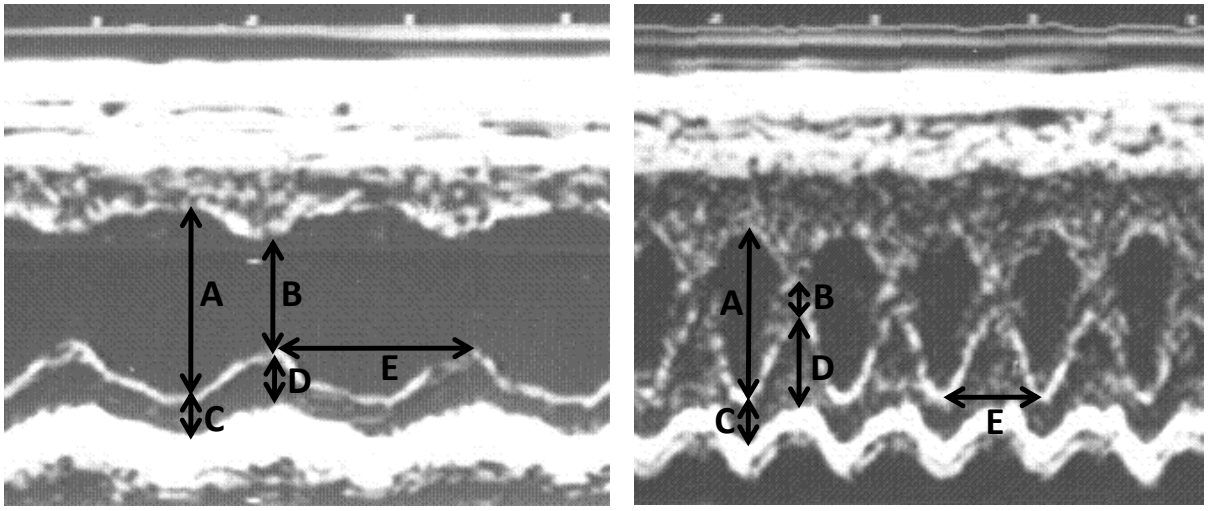

Figure 5: Representative example of M-mode echocardiographic images of the left ventricle before (1) and after (2) isoproterenol administration.

A, left ventricular end diastolic diameter (LVEDD); B, left ventricular end systolic diameter (LVESD); C, posterior wall thickness in diastole (PWTd); D, posterior wall thickness in systole (PWTs); E, cardiac cycle duration (R-R interval) used to calculate heart rate.

$$Area = \pi(\frac{EDD}{2})^2$$

$$RWT = \frac{PWTd}{(EDD/2)}$$

$$FS\ end\ \% = \frac{EDD-ESD}{EDD} \times 100$$

$$FS\ mid\ \% = \frac{(EDD+PWTd)-(ESD+PWTs)}{(EDD+PWTd)} \times 100$$

$$Heart\ rate\ (bts/min) = \frac{60}{cardiac\ cycle\ duration\ (min)}$$

2.4 Heart weights and blood removal

After all of the echocardiographic data had been obtained, a midline thoracotomy was performed after a second dose of ketamine and xylazine. The chest cavity was opened, the heart was rapidly excised and then placed in cold 0.9% saline.

The heart tissue was washed in the cold saline, excess tissue and fat were removed and the heart tissue was blotted dry. The whole heart was then weighed. The right ventricular free wall was then removed and weighed, and then the left ventricular free wall and septum were weighed. In order to correct for differences in body weights between the male and female rats, whole heart, left and right ventricular weights were also calculated per 100g body weight.

A longitudinal slice of the left ventricular free wall from the apex to the base was cut and placed in 4% formaldehyde (methanol free, 37% formaldehyde) in phosphate buffered saline (mmol.L^{-1}) NaCl 137; KCL 2.7; Na_2HPO_4 8; KH_2PO_4 1.5 at pH 7.4, for histological analyses (see 2.7 below).

2.5 Blood sampling

Immediately after extirpation of the heart, blood within the chest cavity was syringed and placed in evacuated tubes with no additives. The tubes were centrifuged and serum was removed and stored for later analysis of estrogen concentrations (see 2.6 below). The blood was removed from the chest cavity after removal of the heart, rather than directly from the heart via cardiac puncture, as the latter would have been likely to cause trauma to the heart which itself may induce cardiomyocyte apoptosis or necrosis.

2.6 ELISA estrogen concentrations

To confirm that the female rats were terminated at peak estrogen concentrations during proestrus, and to confirm that the male rats had lower estrogen concentrations compared to the female rats, serum estrogen concentrations were determined using an enzyme-linked immunosorbent assay (ELISA; DRG estradiol ELISA EIA-2693, Germany). This assay works on the principle of competitive binding. The serum samples were mixed with an estradiol horseradish peroxidise conjugate and then pipetted in duplicate into a microtiter 96 well plate which was coated with estrogen specific antibodies. These estrogen specific antibodies bind to the antigenic site of the estrogen molecule. Hence the serum sample and the estradiol horseradish peroxidase conjugate both compete for binding to the estrogen specific antibodies. After incubation the unbound conjugates were washed off. The amount of peroxidase conjugate that is bound to the antibodies is reverse proportional to the amount of estradiol in the sample. A substrate solution was then added to each well, which produces an enzymatic reaction and hence a colour reaction to occur. A stop solution was subsequently added, which stopped the enzymatic reaction. The optical density (absorbance) was then read at a wavelength of 450±10nm using a microtiter plate reader, within 10 minutes of adding the stop solution. The intensity of the colour is inversely proportional to the concentration of estradiol in the sample. A standard curve was constructed with absorbance values on the Y-

axis and concentration on the X-axis. The standard curve (see Figure 6) was used to determine the concentration of estrogen in each of the serum samples.

2.7 Histological techniques

The longitudinal slice of left ventricle that had been placed in 4% formaldehyde was embedded in paraffin wax. Histological sections (5µm thick) were then cut using a microtome and mounted onto slides prior to the various histological staining techniques (see 2.7.1 and 2.7.2 below).

2.7.1 TUNEL staining - cardiomyocyte apoptosis

2.7.1.1 Principle

Apoptosis was detected using a DeadEnd colormetric TUNEL system (Promega, South Africa) which works on the principle of DNA fragmentation. Briefly slides were dehydrated through a series of alcohol solutions and the tissues were primed for the staining technique. The tissues were permeabilized with proteinase K and labelled with biotinylated nucleotide mix which binds to the fragmented DNA in the presence of terminal deoxynucleotidyl transferase (TDT) enzyme. Subsequently streptavidin/horseradish peroxidase was added to bind to the biotinylated nucleotides. Finally the tissues were stained with the peroxidise substrate, hydrogen peroxide and chromogen diaminobenzidine which enables the apoptotic nuclei to be viewed under a light microscope (apoptotic nuclei appear brown) (Figure 7D) (Agarwala and Kalil 1998). The cardiomyocyte nuclei were easily identified due to their characteristic large size, cigar shape and speckled brown appearance (Figure 7B, cells labelled a). In comparison the fibroblasts nuclei and nuclei of other tissue cells were small and stained a solid dark brown (Figure 7B, cells labelled b).

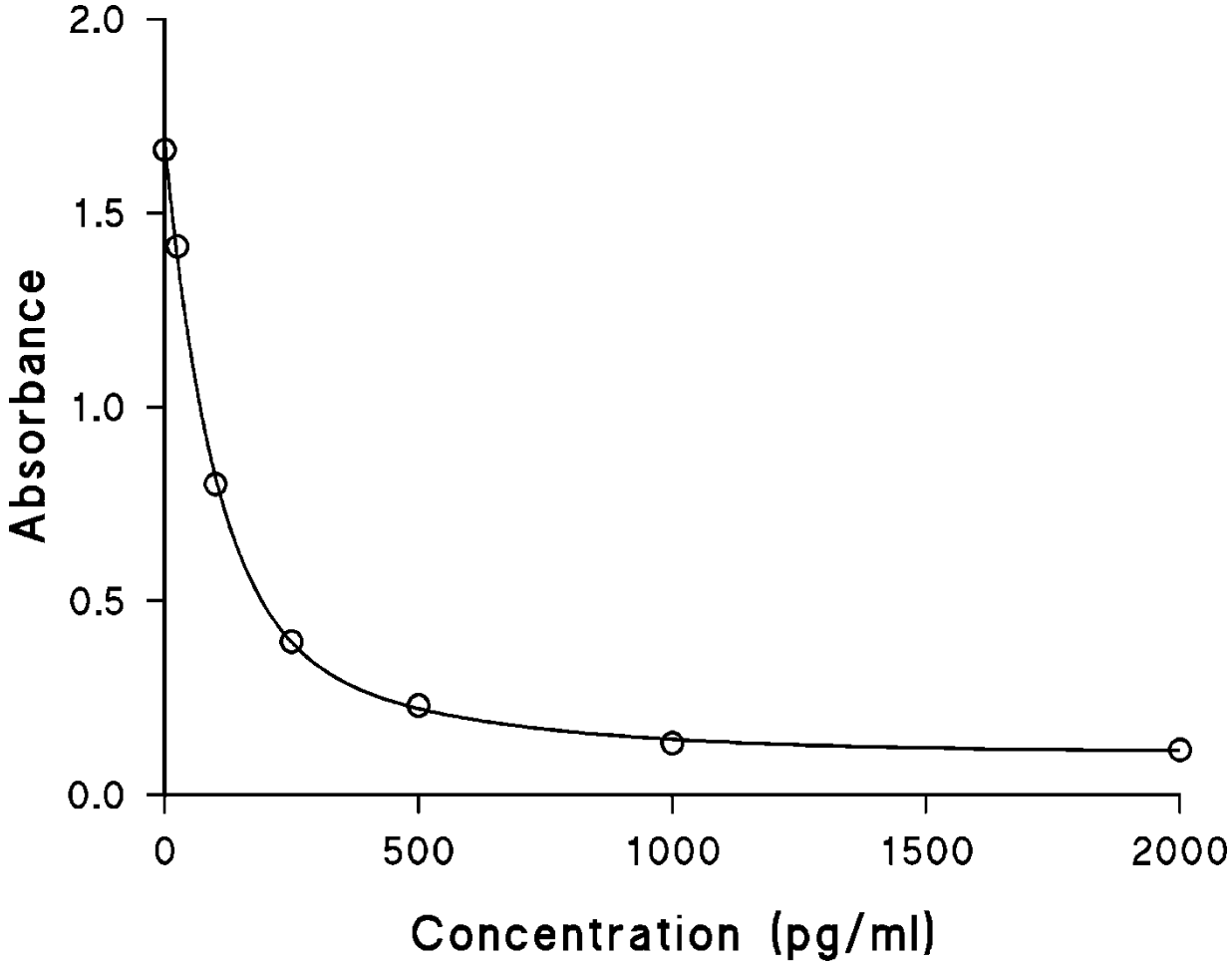

Figure 6: Standard curve used to calculate serum estrogen concentrations in samples.

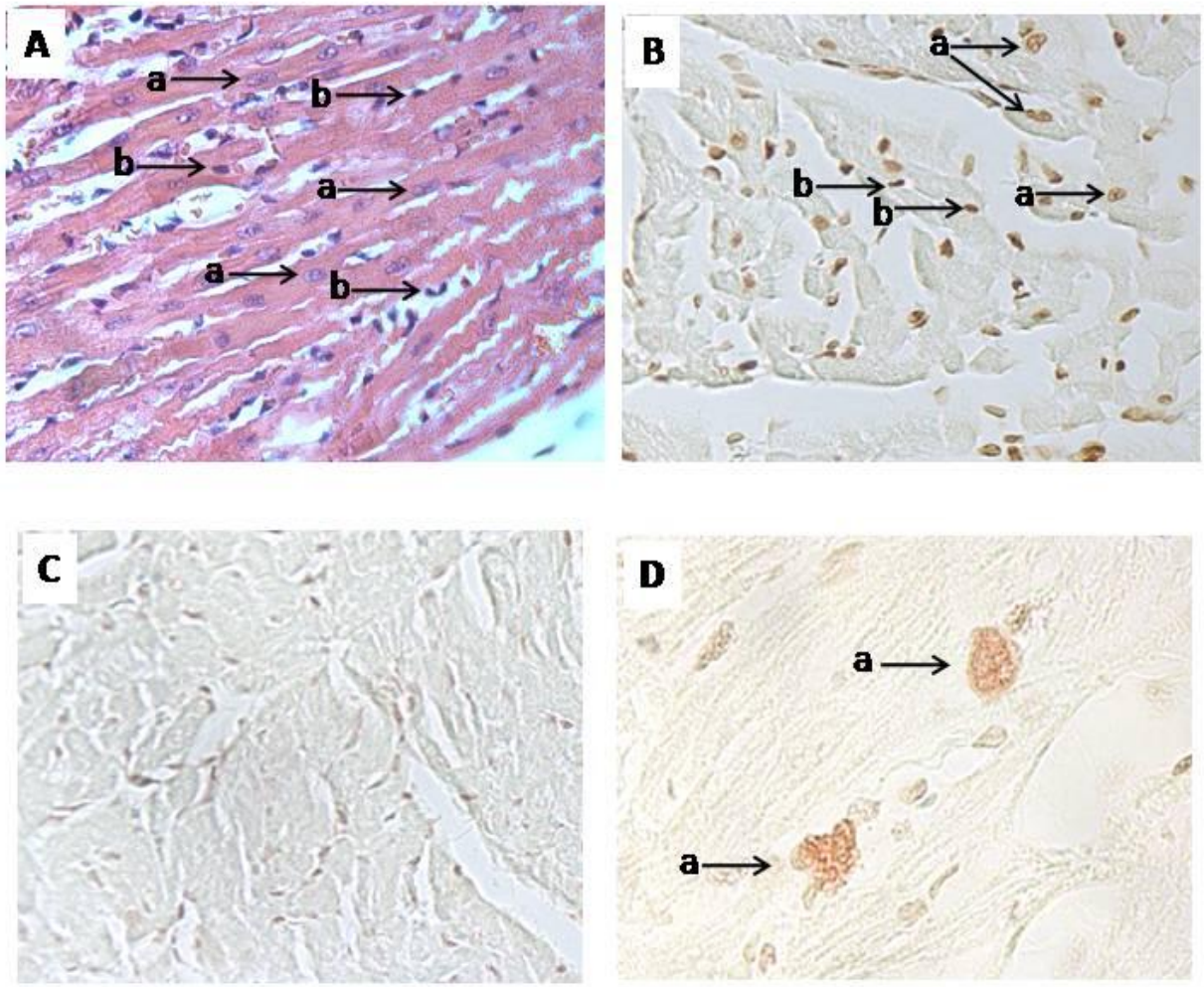

Figure 7: Representative examples of cardiac tissues sections stained with haemotoxylin and eosin (A), and TUNEL (B, C and D).

A, H & E stained section, a indicates cardiomyocyte nuclei, b indicates fibroblast or other nuclei; B, TUNEL positive tissue section indicating numerous apoptotic nuclei, a indicates cardiomyocyte nuclei, b indicates fibroblast or other nuclei; C, TUNEL negative tissue section with few visible nuclei; D, enlarged section of rat myocardium showing the presence of apoptotic cardiomyocyte nuclei (a) stained a speckled orange brown colour and fragmented around the edges.

Positive and negative control slides were stained to ensure that the TUNEL assay worked effectively and did not produce false results. Briefly, the positive control slides had an additional step in which the slides were incubated in DNAse enzyme which breaks down DNA and hence produces DNA fragments as found in apoptotic nuclei. Thus the biotinylated nucleotide mix in the presence of TDT enzyme binds to all or the majority of nuclei in the tissue section. Hence the positive control slides demonstrated a majority of brown apoptotic nuclei as shown in Figure 7B. On the other hand, the negative control slides did not receive the TDT enzyme, hence preventing the binding of the biotinylated nucleotide mix to any fragmented DNA. Thus the negative control slides demonstrated no brown apoptotic nuclei (Figure 7C).

2.7.1.2 Calculation of apoptotic nuclei

The extent of cardiomyocyte apoptosis per tissue section was expressed as a percentage. In order to calculate the percentage of cardiomyocyte apoptosis, the total number of cardiomyocyte nuclei needed to be quantified. The latter was done by staining tissue sections with haematoxylin and eosin (H&E) and counting the number of cardiomyocyte nuclei in ten evenly spaced fields, from apex to base, at 400x magnification using a computer based image acquisition and analysis system (Nikon ACT2U imaging software, Nikon Corporation 2004, version 1.0.0.117). The number of apoptotic nuclei stained using the TUNEL technique (described above) were counted and expressed as a percentage of the total number of H&E cardiomyocytes (Figure 7A) per tissue section (see formula below). The cardiomyocyte nuclei were easily identified from their characteristic large size, cigar shape and speckled pale blue appearance (Figure 7A, cells labelled a), compared to the fibroblast nuclei and the nuclei of other cardiac tissue cells which were small and stained a solid dark blue (Figure 7A, cells labelled b). In addition, the cardiomyocyte nuclei were mostly located centrally within the cytoplasm of the cardiomyocytes (Figure 7A, cells labelled a).

$$Apoptosis\ (\%) = \frac{Total\ TUNEL\ cardiomyocyte\ nuclei}{Total\ H\&E\ cardiomoycyte\ nuclei} \times 100$$

2.7.2 Van Gieson's staining - cardiomyocyte necrosis and fibrosis

In order to assess necrosis and fibrosis, sections were stained with Van Gieson's stain. Slides were viewed at 10X magnification and the amount of "pink" fibrosis was qualitatively analysed. Briefly a double-blinded observer assessed the percentage of "pink stained" fibrosis and necrosis (Figure 8A and C) in relation to the "green stained" myocardial tissue (Figure 8B and D) throughout the slide and a pathological score was assigned. The pathological scoring as modified from Teerlink et al *(*1994) was as follows: 0 indicated no necrosis/fibrosis, 1 and 2 indicated patchy necrosis/fibrosis in <20% and > 20% of the field respectively, 3 and 4 indicated diffuse subendocardial necrosis/fibrosis in <50% and >50% of the field respectively and 5 and 6 indicated full thickness necrosis/fibrosis in <50% and >50% of the field respectively.

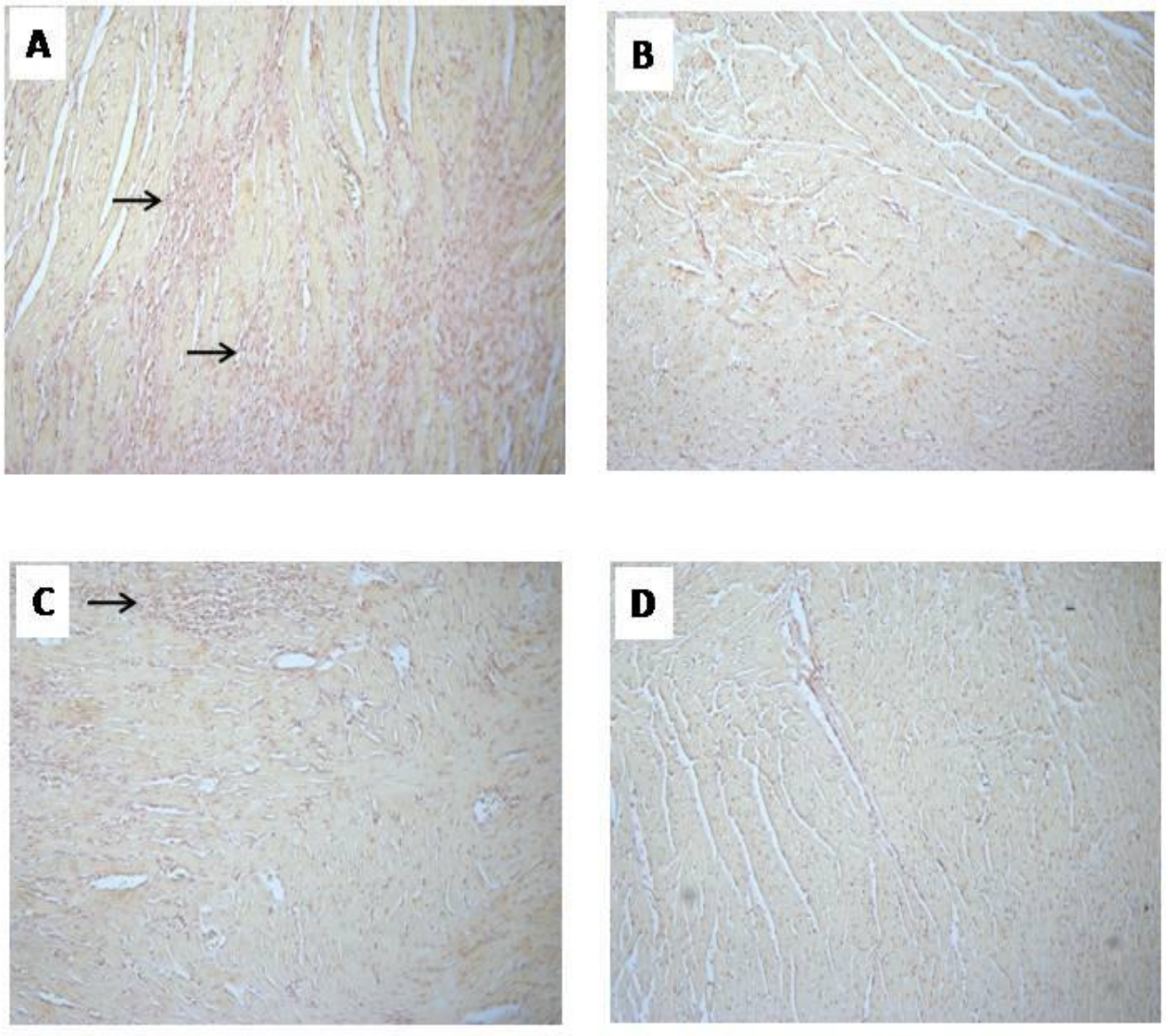

Figure 8: Representative myocardial tissue sections stained with Van Gieson's stain for necrosis and fibrosis.

A, tissue section from an ISO male, the arrow indicates large amounts of dispersed necrosis/fibrosis throughout the section; B, tissue section from a CON male with no necrosis/fibrosis; C, tissue section from an ISO female, the arrow indicates a small portion of necrosis/fibrosis; D, tissue section from a CON female indicating no necrosis/fibrosis; arrows, collagen stains pink and indicates nerosis/fibrosis; the cytoplasm of the cardiac tissue cells are stained light green.

2.8 Statistical analysis

A two-way (regimen and gender) ANOVA was used to determine the effects of 5 days of ISO or saline administration, gender effects and gender-regimen interaction on body weight, heart weight, left and right ventricular weights, and cardiomyocyte apoptosis. Tukey-Kramer *post hoc* analysis was performed following significant F values on ANOVA. A Kruskal-Wallis rank sum test determined differences between the groups for necrosis/fibrosis as this data was non-parametric. To determine the effects of 5 days of ISO or saline administration, as well as the effects of acute administration of ISO or saline, on cardiac function and geometry, a two-way (regimen and gender) repeated measures (baseline and after acute ISO or saline) ANOVA was performed. Tukey-Kramer *post hoc* analysis was performed following significant F values on ANOVA. An unpaired t-test was used to calculate the differences in estrogen concentrations. The statistical programs used were SAS software, version 9.1 (SAS institute Inc., Cary NC) and Instat (Graphpad Instat version 3.00, 32 bit for win95/NT, 1997). Data were expressed as x±sem and $P<0.05$ was considered significant.

Chapter 3

Results

3 Results

3.1 Body and heart weights

The body weights of the two groups of male rats were significantly greater ($p<0.001$) than those of the two groups of female rats (Table 3). Consequently, the heart, left ventricular and right ventricular weights were greater in the two groups of male rats compared to the weight of the two groups of female rats (Table 3). Hence, comparisons of heart, left ventricular and right ventricular weight were also made after these weights had been adjusted to 100g body weight. Five days of isoproterenol administration had no effect on the body weights of either the male or the female rats (Table 3). In the male rats, 5 days of isoproterenol resulted in increased ($p<0.001$) heart, left ventricular and right ventricular weight, an effect not observed in the female rats. However, when assessing heart and left ventricular weights adjusted to 100g body weight; increases ($p<0.01$) in heart and left ventricular weight were observed after 5 days of isoproterenol administration in both the male and the female rats (Table 3). An isoproterenol induced increase ($p<0.01$) in right ventricular weight adjusted to 100g body weight was observed in the male rats but only when compared to the female rats receiving saline (Table 3).

3.2 Echocardiography data

As a consequence of anaesthetic related deaths (1 rat from the ISO F group; 1 rat from the CON M group and 3 rats from the ISO M group), echocardiography data could not be obtained in these rats. Hence the samples sizes for the echocardiography data are: CON F= 14; ISO F=14; CON M=12; ISO M=11.

Table 3: Effects of 5 days of isoproterenol administration on body, heart, left and right ventricular weights in male and female rats

	CON F (n=14)	**ISO F (n=15)**	**CON M (n=13)**	**ISO M (n=14)**
Body weight (g)	233.79±16.92	228.33±23.05	317.00±27.67*	312.60±37.99*
Heart weight (g)	0.66±0.04	0.74±0.07	0.90±0.10*	1.02±0.11*†
Heart weight/ 100g body weight	0.28±0.02	0.33±0.04‡	0.28±0.03	0.33±0.02‡
LV weight (g)	0.52±0.05	0.59±0.05	0.70±0.10*	0.79±0.10*†
LV weight/ 100g body weight	0.22±0.02	0.26±0.04‡	0.22±0.02	0.25±0.01‡
RV weight (g)	0.13±0.02	0.15±0.02	0.19±0.03*	0.23±0.05*†
RV weight/ 100g body weight	0.06±0.01	0.06±0.01	0.06±0.01	0.07±0.02**

*P <0.001 vs. CON F and ISO F; **P<0.01 vs. CON F; ‡ P<0.01 vs. CON M and CON F; †P<0.001 vs. CON M

3.2.1 Heart rate

The two-way repeated measures ANOVA demonstrated a gender effect ($p<0.05$), a regimen effect ($p<0.0001$), a time effect ($p<0.0001$), a time-regimen interaction ($p<0.0001$), a gender-regimen interaction ($p<0.05$) and a time-regimen-gender interaction ($p=0.004$). On post-hoc analysis, a gender difference was noted, with both groups of male rats having higher heart rates ($p<0.05$) than the two groups of female rats at baseline (B) and after saline administration (Figure 9). Five days of isoproterenol administration had no effect on heart rates in either the male or the female rats ($p=0.995$, comparison of heart rates at baseline, B; Figure 9). However, acute administration of isoproterenol; but not saline, increased heart rate in both the male ($p<0.005$) and the female ($p<0.0001$) rats (after, A; Figure 9). Moreover, the increment in heart rate observed in response to acute isoproterenol in the female rats was greater than that observed in the male rats (increase in heart rate in females: 144.2±5.5 bts/min; males: 103.1±18.2bts/min; $p<0.05$).

3.2.2 Left ventricular endocardial fractional shortening

On two-way repeated measures ANOVA, a time effect ($p<0.0001$), a regimen effect ($p<0.0001$) and a time-regimen interaction ($p<0.0001$) were demonstrated. Post-hoc analysis, revealed an effect of acute administration of isoproterenol in both the male and the female rats (Figure 10). No gender related differences were observed in endocardial fractional shortening (Figure 10). Similarly, 5 days of isoproterenol administration had no effect on endocardial fractional shortening in either the male or the female rats (Figure 10; at baseline, B). Importantly, however acute isoproterenol administration induced an increase ($p<0.0001$) in endocardial fractional shortening in both male and female rats (Figure 10; after, A), consistent with a positive inotropic response. Acute saline administration, as expected, had no effect on endocardial fractional shortening. The positive inotropic effect was similar between

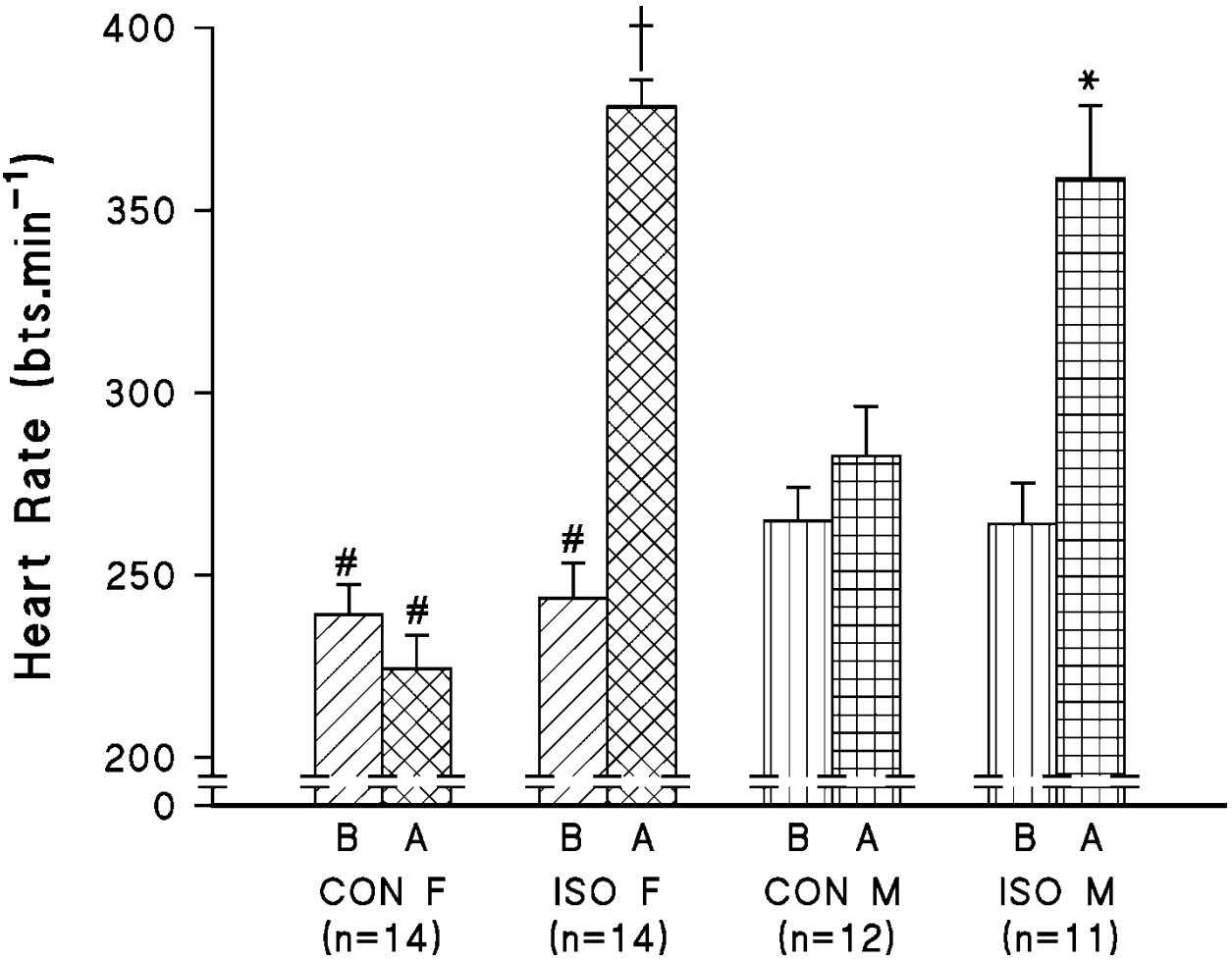

Figure 9: Effects of gender, 5 days of isoproterenol or saline administration and acute administration of isoproterenol or saline on heart rates in male and female rats.

B=before (baseline) an injection of saline (CON F and CON M, hatched bars) or isoproterenol (ISO F and ISO M, hatched bars); A=after an injection of saline (CON F and CON M, cross-hatched bars) or isoproterenol (ISO F and ISO M, cross-hatched bars); # $p<0.05$ vs corresponding male group; * $p<0.005$, ISO M after isoproterenol injection vs. ISO M before, CON M (before and after), CON F (before and after), and ISO F before; † $p<0.0001$, ISO F after isoproterenol injection vs. ISO F before, CON M (before and after), CON F (before and after), and ISO M before.

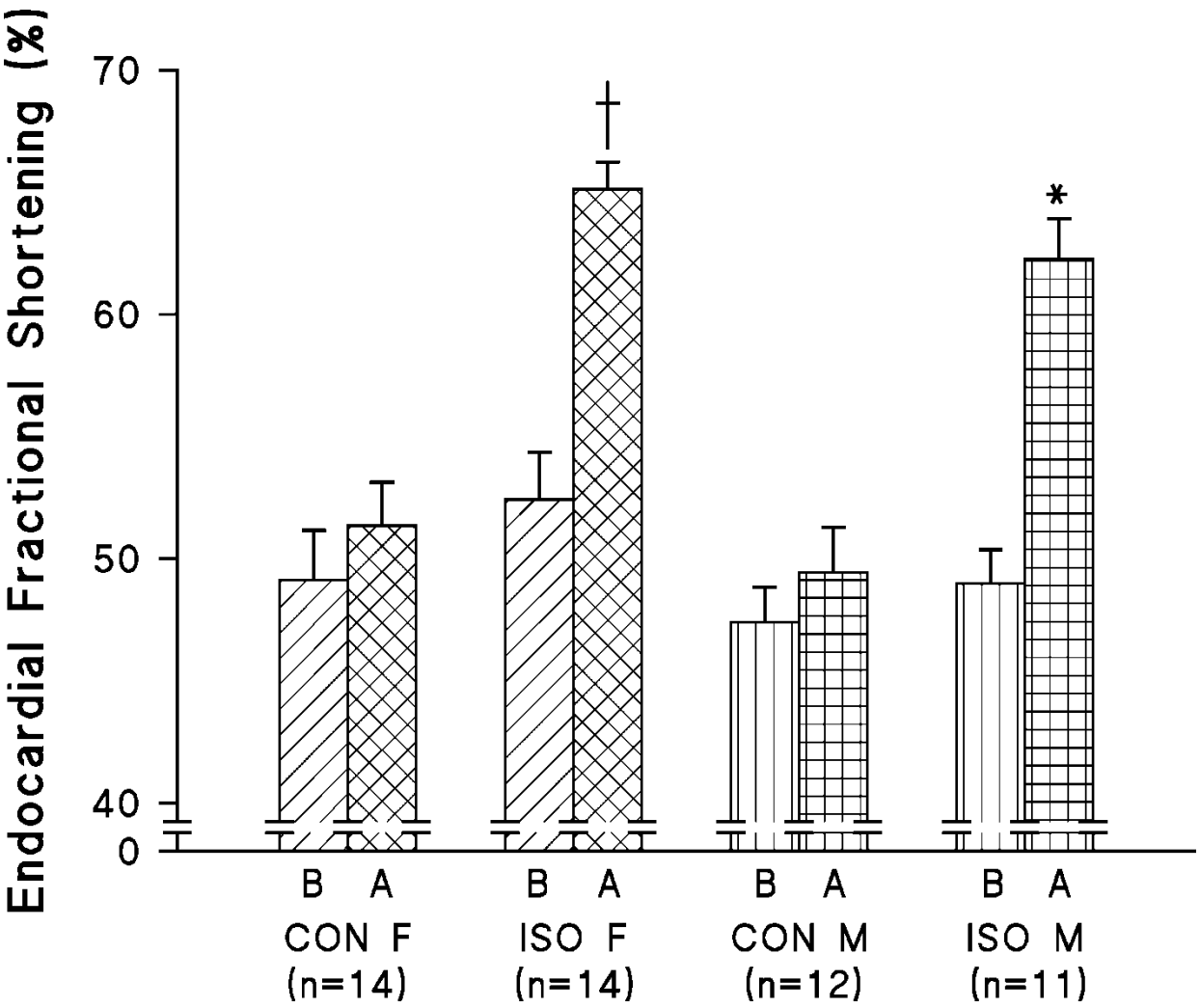

Figure 10: Effects of gender, 5 days of isoproterenol or saline administration and acute administration of isoproterenol or saline on endocardial fractional shortening in male and female rats.

B=before (baseline) an injection of saline (CON F and CON M, hatched bars) or isoproterenol (ISO F and ISO M, hatched bars); A=after an injection of saline (CON F and CON M, cross-hatched bars) or isoproterenol (ISO F and ISO M, cross-hatched bars); * $p<0.0001$, ISO M after isoproterenol injection vs. ISO M before, CON M (before and after), CON F (before and after), and ISO F before; † $p<0.0001$, ISO F after isoproterenol injection vs. ISO F before, CON M (before and after), CON F (before and after), and ISO M before.

the male and the female rats (change in FSend in males: 13.3±2.0%; females: 12.7±2.2%; $p>0.05$).

3.2.3 Left ventricular midwall fractional shortening

Two-way repeated measures ANOVA of left ventricular midwall fractional shortening data revealed a time effect ($p=0.01$), a regimen effect ($p<0.05$) and a time-regimen interaction ($p=0.01$). At baseline no gender differences in left ventricular midwall fractional shortening were noted (Figure 11; at baseline, B). Moreover, 5 days of isoproterenol administration had no effect on left ventricular midwall fractional shortening in male or female rats (Figure 11; at baseline, B). Acute isoproterenol produced significant increments in left ventricular midwall fractional shortening in male and female rats (Figure 11; after, A), which are consistent with the positive inotropic effects of isoproterenol. As expected, acute saline administration did not alter left ventricular midwall fractional shortening in male or in female rats (Figure 11; after A). The increments in left ventricular midwall fractional shortening were no different in the male compared to the female rats (change in FSmid in males: 3.9±1.6%; females: 4.1±2.0%; $p>0.05$).

3.2.4 Left ventricular end diastolic diameter, chamber area and relative wall thickness

Analysis of left ventricular end diastolic diameters revealed a regimen effect ($p<0.0001$), a gender effect ($p<0.001$), a time effect ($p<0.0001$) and a time-regimen interaction ($p<0.0001$). At baseline significant gender differences were noted, with the male rats having greater ($p<0.0001$) left ventricular end diastolic diameters compared to the female rats (Figure 12). These gender differences in left ventricular end diastolic diameter could be attributed to the fact that the male rats were larger and hence had heavier heart weights compared to the female rats (Table 3). Five days of isoproterenol administration had no effect on left ventricular end diastolic diameters in either male or female rats; however acute

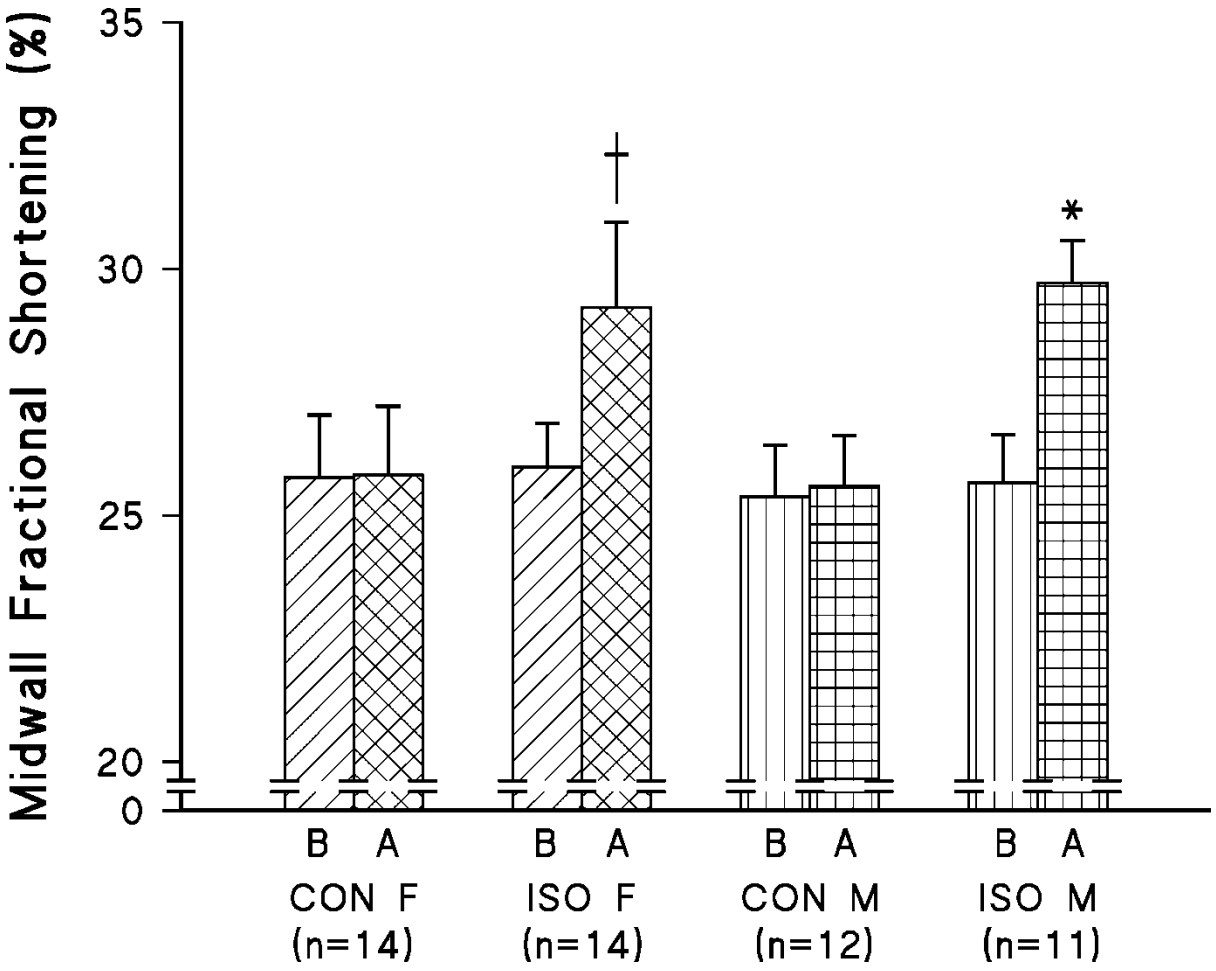

Figure 11: Effects of gender, 5 days of isoproterenol or saline administration and acute administration of isoproterenol or saline on left ventricular midwall fractional shortening in male and female rats.

B=before (baseline) an injection of saline (CON F and CON M, hatched bars) or isoproterenol (ISO F and ISO M, hatched bars); A=after an injection of saline (CON F and CON M, cross-hatched bars) or isoproterenol (ISO F and ISO M, cross-hatched bars); * $p<0.05$, ISO M after isoproterenol injection vs. ISO M before, CON M (before and after), CON F (before and after), and ISO F before; † $p<0.05$, ISO F after isoproterenol injection vs. ISO F before, CON M (before and after), CON F (before and after), and ISO M before.

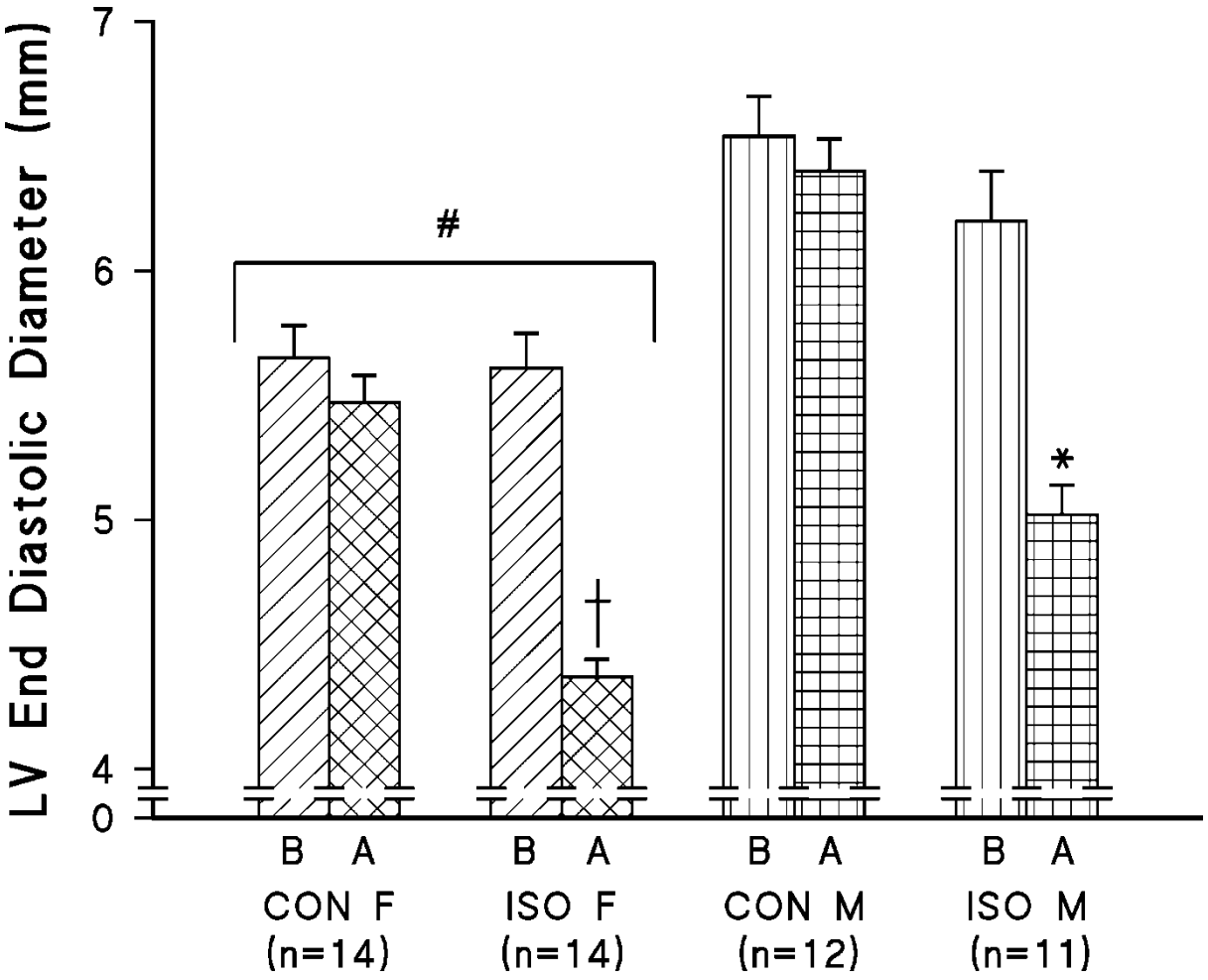

Figure 12: Effects of gender, 5 days of isoproterenol or saline administration and acute administration of isoproterenol or saline on left ventricular end diastolic diameter in male and female rats.

B=before (baseline) an injection of saline (CON F and CON M, hatched bars) or isoproterenol (ISO F and ISO M, hatched bars); A=after an injection of saline (CON F and CON M, cross-hatched bars) or isoproterenol (ISO F and ISO M, cross-hatched bars); # $p<0.001$ vs. males; * $p<0.0001$, ISO M after isoproterenol injection vs. ISO M before, CON M (before and after), CON F (before and after), and ISO F before; † $p<0.0001$, ISO F after isoproterenol injection vs. ISO F before, CON M (before and after), CON F (before and after), and ISO M before.

isoproterenol, but not saline, resulted in a decrease ($p<0.0001$) in left ventricular end diastolic diameter indicative of a positive inotropic effect (Figure 12). The decrease in end diastolic diameter observed in the male rats was no different from that observed in the female rats (change in EDD, males: -1.17±0.22mm; females: -1.24±0.13mm).

Consistent with the greater end diastolic diameters observed in the male rats compared to the female rats, left ventricular chamber diastolic areas were larger ($p<0.05$) in the male rats compared to the female rats (Figure 13 upper panel). However, even after left ventricular chamber areas had been adjusted to 100g body weight, the male rats had larger ($p<0.05$) left ventricular chamber diastolic areas than the female rats (Figure 13 lower panel). Hence, the increased chamber dimensions observed in the male rats are probably more likely due to a greater degree of dilatation (eccentricity) in male rats compared to female rats. Indeed, the relative wall thickness was decreased ($p<0.05$) in the male rats compared to the female rats (Figure 14).

Five days of isoproterenol had no effect of LV diastolic chamber area ($p<0.0001$); but in response to acute isoproterenol similar positive inotropic effects ($p<0.0001$) were observed in male and female rats (Figure 13). Five days of isoproterenol, however resulted in increases in relative wall thickness in both male and female rats (Figure 14), consistent with the isoproterenol-induced increments in LV weight/100g body weight (Table 3). In addition, increases in relative wall thickness were observed in response to acute isoproterenol in both the male and the female rats (Figure 14).

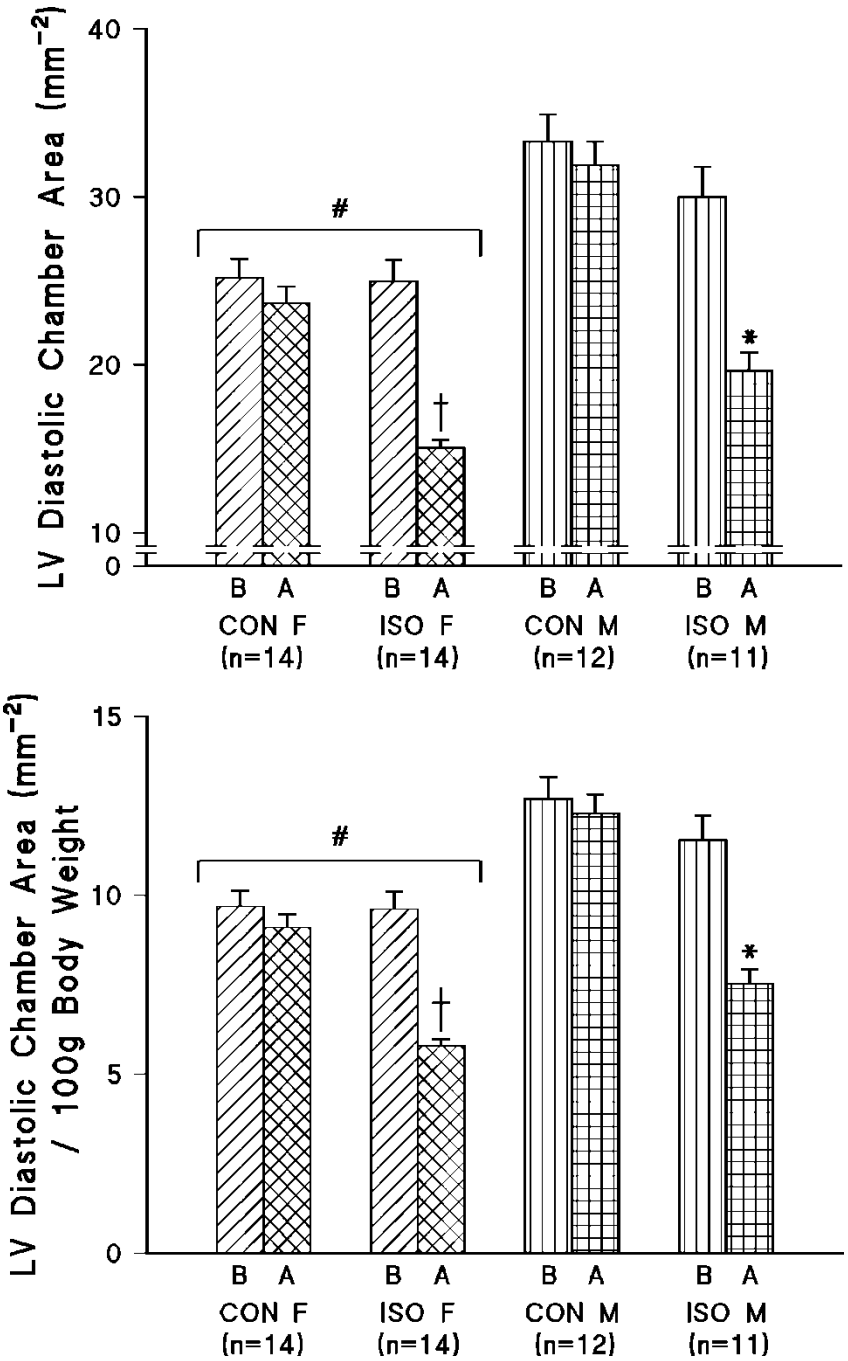

Figure 13: Effects of gender, 5 days of isoproterenol or saline administration and acute administration of isoproterenol or saline on left ventricular diastolic chamber area in male and female rats.

A=after an injection of saline (CON F and CON M, cross-hatched bars) or isoproterenol (ISO F and ISO M, cross-hatched bars); # $p<0.05$ vs. males; * $p<0.0001$, ISO M after isoproterenol injection vs. ISO M before, CON M (before and after), CON F (before and after), and ISO F before; † $p<0.0001$, ISO F after isoproterenol injection vs. ISO F before, CON M (before and after), CON F (before and after), and ISO M before.

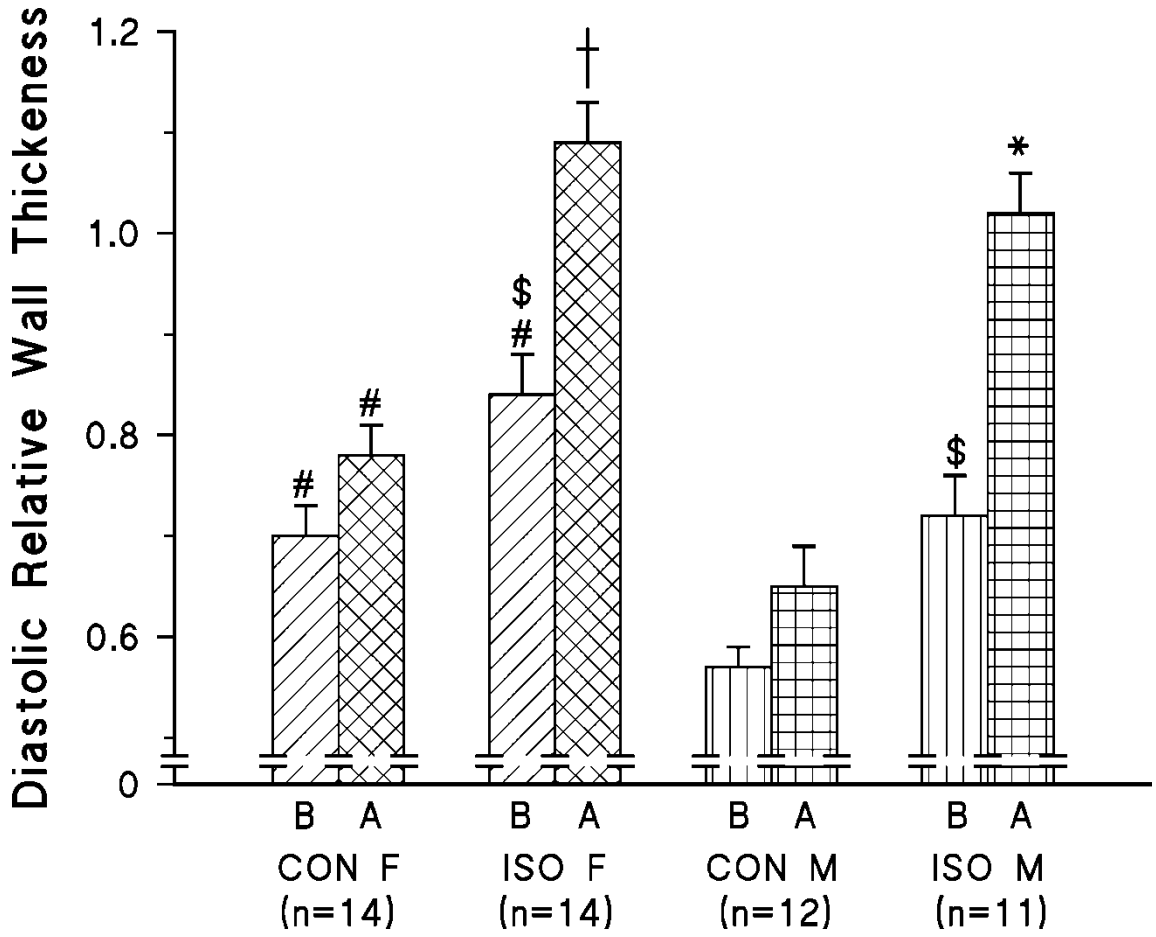

Figure 14: Effects of gender, 5 days of isoproterenol or saline administration and acute administration of isoproterenol or saline on left ventricular diastolic relative wall thickness in male and female rats.

B=before (baseline) an injection of saline (CON F and CON M, hatched bars) or isoproterenol (ISO F and ISO M, hatched bars); A=after an injection of saline (CON F and CON M, cross-hatched bars) or isoproterenol (ISO F and ISO M, cross-hatched bars); # $p<0.05$ vs. corresponding male group; * $p<0.001$, ISO M after isoproterenol injection vs. ISO M before, CON M (before and after), CON F (before and after), and ISO F before; † $p<0.001$, ISO F after isoproterenol injection vs. ISO F before, CON M (before and after), CON F (before and after), and ISO M before; $ $p<0.05$ vs. gender-matched CON group.

3.2.5 Left ventricular end systolic diameter

Similar to left ventricular end diastolic diameters, two-way repeated measures ANOVA of left ventricular systolic diameters revealed a regimen effect ($p<0.0001$), a gender effect ($p<0.001$), a time effect ($p<0.0001$) and a time-regimen interaction ($p<0.0001$). At baseline significant gender differences were noted, with the male rats having greater ($p<0.0001$) left ventricular end systolic diameters compared to the female rats (Figure 15). Five days of isoproterenol administration had no effect on left ventricular end systolic diameters in either male or female rats; however, consistent with a positive inotropic effect, acute isoproterenol administration decreased the left ventricular end systolic diameters in both male and female rats (Figure 15).

3.3 ELISA estrogen levels

The mean estrogen concentration in the female rats was greater than the mean estrogen concentration measured throughout the estrus cycle in rats (Yang et al 2004) (Figure 16). Hence, the rats were indeed terminated during proestrus (peak estrogen concentrations). As expected female rats had higher ($p<0.01$) estrogen concentrations compared to the males rats (Figure 16). Importantly, 5 days of isoproterenol administration had no effect on plasma estrogen concentrations (ISO F: 23.00±3.47; CON F: 19.31±3.66; ISO M: 7.04±1.41; CON M: 7.14±0.53pg.ml^{-1}).

3.4 Cardiomyocyte apoptosis

On two-way ANOVA of cardiomyocyte apoptosis data, a regimen effect ($p=0.0002$) and a regimen-gender interaction ($p=0.013$) were noted. The latter is important as it demonstrates that the isoproterenol induced cardiomyocyte apoptosis was gender specific. Indeed, on *post hoc* analysis, five days of isoproterenol administration induced cardiomyocyte apoptosis in the male rats, an effect which was not evident in the female rats

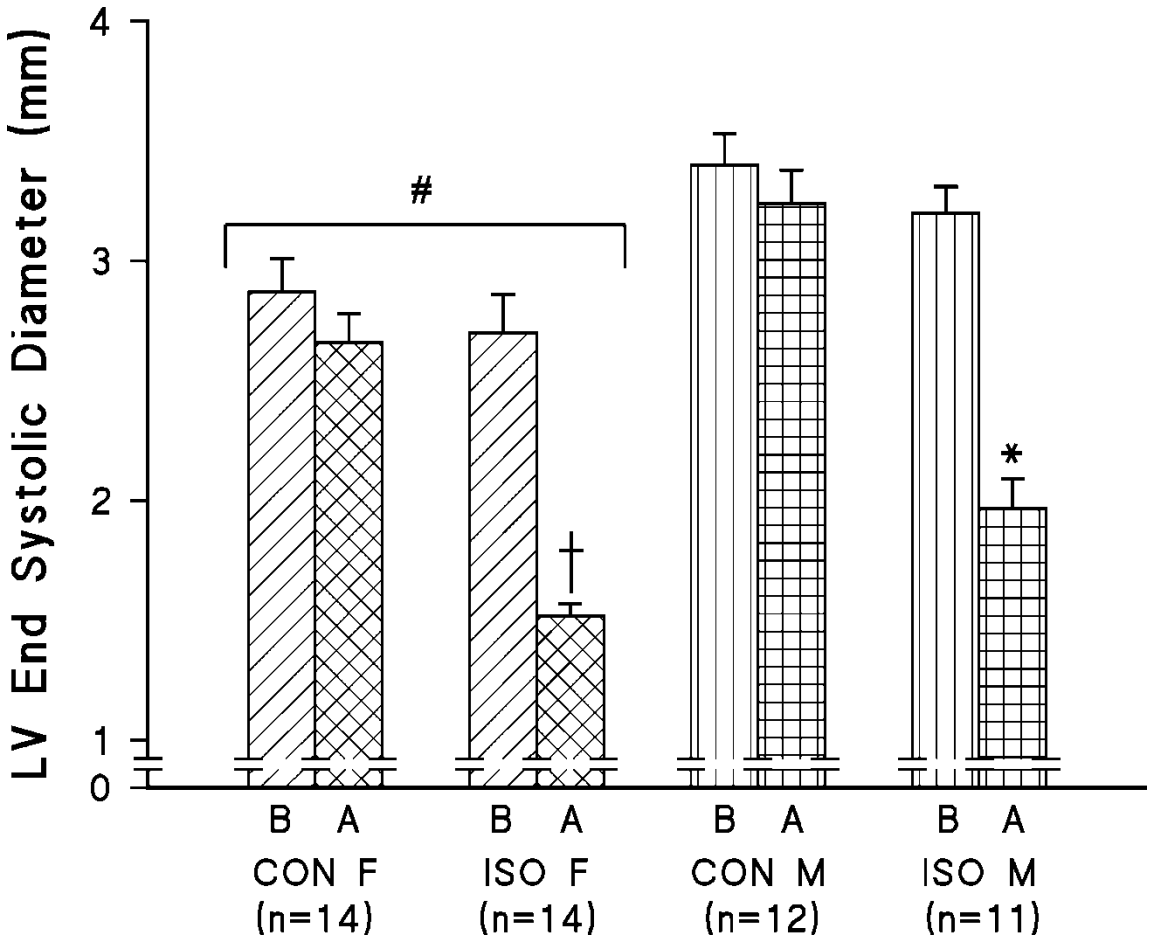

Figure 15: Effects of gender, 5 days of isoproterenol or saline administration and acute administration of isoproterenol or saline on left ventricular end systolic diameter in male and female rats.

B=before (baseline) an injection of saline (CON F and CON M, hatched bars) or isoproterenol (ISO F and ISO M, hatched bars); A=after an injection of saline (CON F and CON M, cross-hatched bars) or isoproterenol (ISO F and ISO M, cross-hatched bars); # $p<0.001$ vs. males; * $p<0.0001$, ISO M after isoproterenol injection vs. ISO M before, CON M (before and after), CON F (before and after), and ISO F before; † $p<0.0001$, ISO F after isoproterenol injection vs. ISO F before, CON M (before and after), CON F (before and after), and ISO M before.

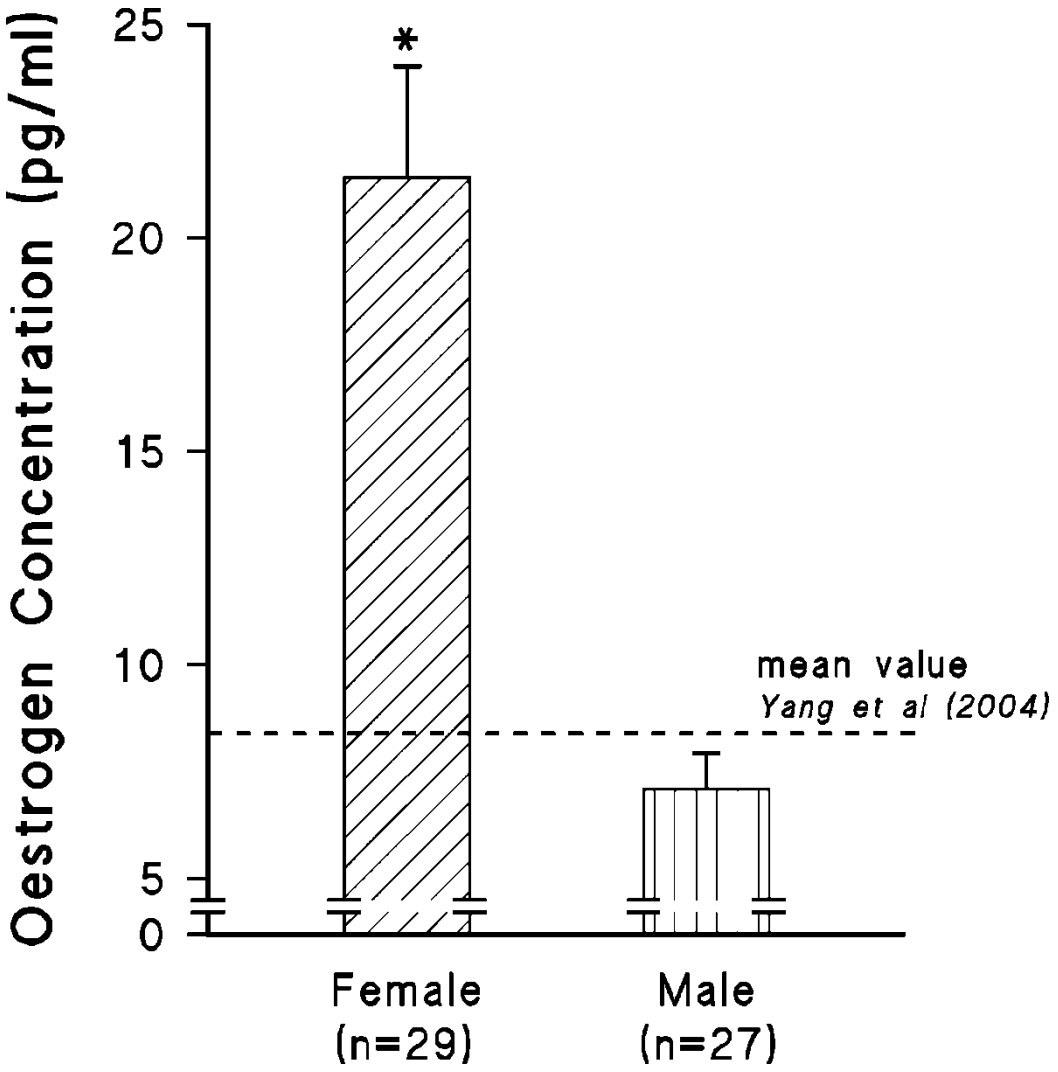

Figure 16: Plasma estrogen concentrations in female rats and in male rats.

* $p < 0.01$ vs. male rats; mean values throughout the estrus cycle as measured by Yang et al (2004) are shown for comparison.

(ISO M vs, ISO F, p=0.039) (Figure 17). In addition, the percentage cardiomyocyte apoptosis was increased in the male rats receiving isoproterenol in comparison to both the male rats receiving saline (p=0.0002) and the female rats receiving saline (p=0.0018) (Figure 17).

3.5 Cardiomyocyte necrosis and fibrosis

Non-parametric ANOVA revealed a p value of 0.0028 between groups. On *post hoc* analysis (Kruskal-Wallis test) five days of isoproterenol administration significantly increased (p=0.015) necrosis and fibrosis in male rats, an effect not observed in the female rats (Figure 16). Five days of saline administration had no effect on cardiomyocyte necrosis and fibrosis in either the male or the female rats (Figure 18).

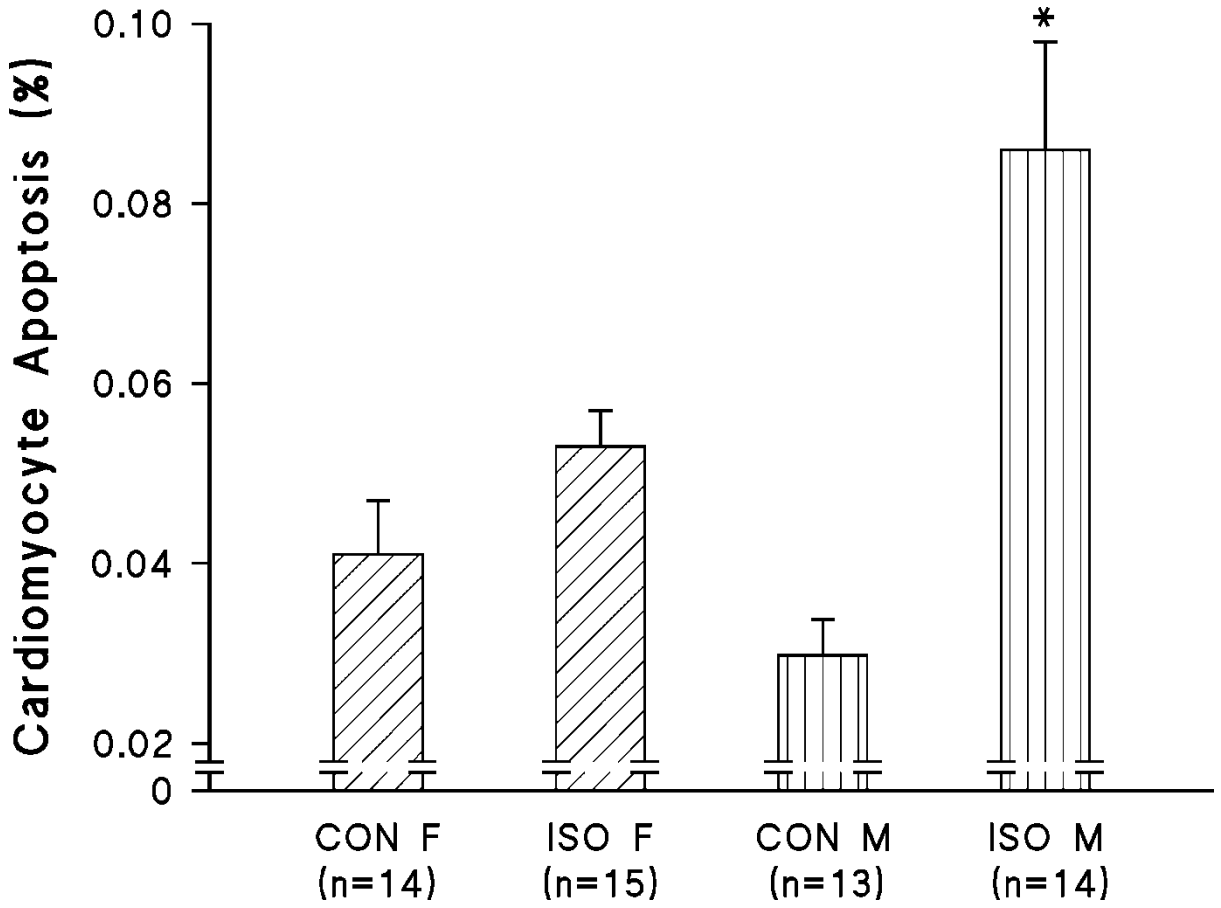

Figure 17: Effects of five days of isoproterenol or saline administration on percentage cardiomyocyte apoptosis in male and female rats.

* $p<0.05$ vs. CON M; CON F and ISO F.

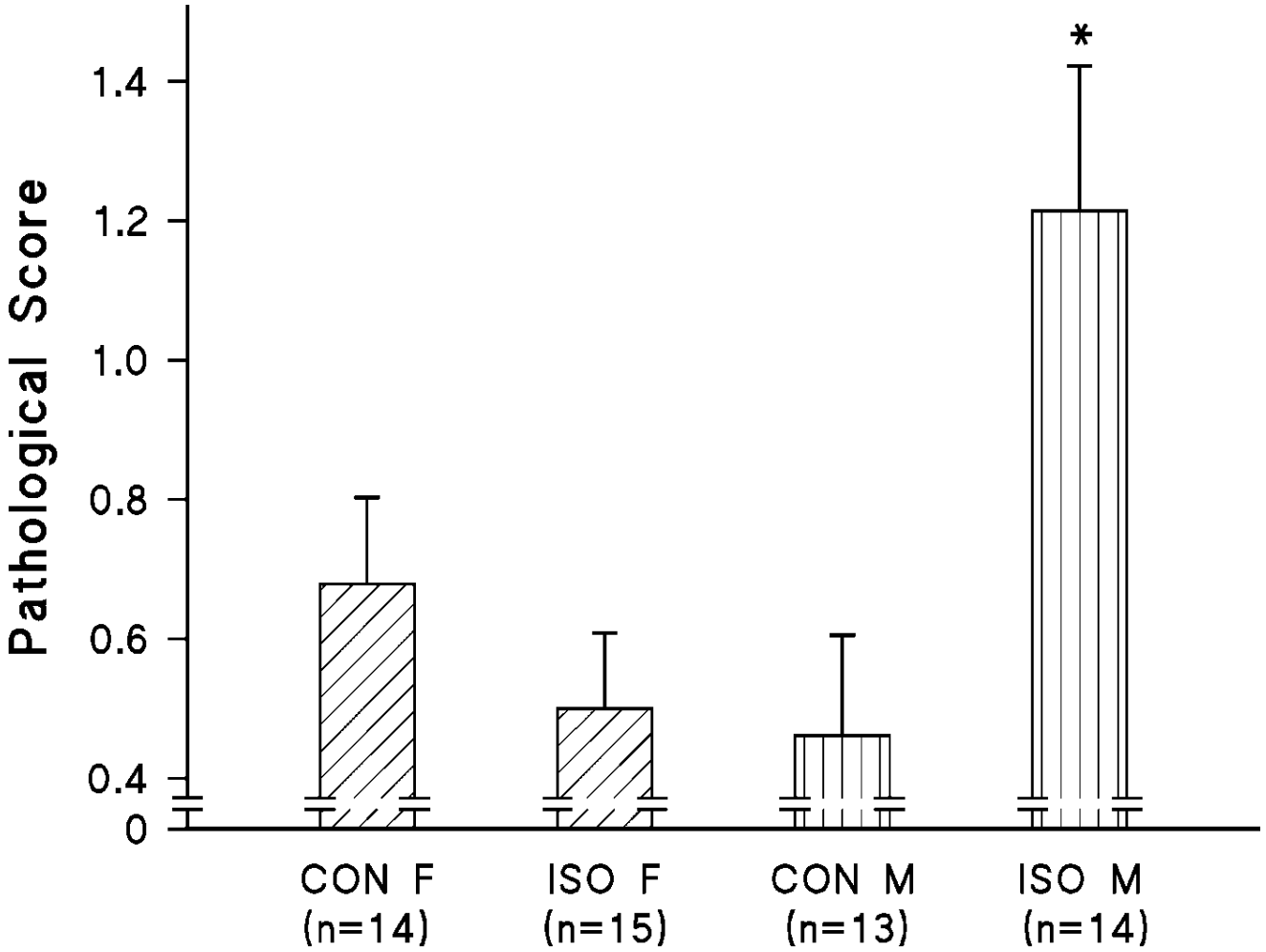

Figure 18: Effects of five days of isoproterenol or saline administration on pathological score (necrosis) in male and female rats.

* $p<0.01$ vs. CON M; ISO F and CON F.

Chapter 4

Discussion

4 Discussion

4.1 Introduction

The main findings of my study were that 5 days of β-adrenergic receptor activation, at a dose which produced positive inotropic and chronotropic effects, induced cardiomyocyte apoptosis and increased myocardial pathological score in male rats but not in female rats. The gender differences in cardiomyocyte apoptosis and pathological score were not accompanied by differences in cardiac responses to β-adrenergic activation. Heart rates however were lower in the female compared to the male rats, an effect which was noted in both the female rats receiving 5 days of saline as well as the female rats receiving the β-adrenergic agonist for 5 days. Five days of β-adrenergic agonist administration resulted in modest cardiac hypertrophy in both male and female rats, after the correction for differences in body weight. Compared to male rats, female rats had concentric hypertrophy as demonstrated by increased diastolic relative wall thickness (RWT) and decreased chamber area. Importantly the data were collected when the female rats were in proestrus, thus ensuring that the data was collected in the female rats when they were at their peak estrogen concentration.

4.2 Uniqueness of current study

This is the first study *in vivo* where the heart of male rats has been compared to that of female rats, in the absence of any manipulation of the sex steroids. Previous *in vivo* studies have assessed the impact of ovariectomy in the presence or absence of exogenous estrogen administration. Importantly in these studies the exogenous estrogen was administered at supraphysiological doses and hence these studies fail to address the possible impact of endogenous concentrations of estrogen. In this regard van Eickels et al (2003) reported that exogenous estrogen administration to ovariectomised female mice resulted in a reduction in LAD coronary artery induced infarct size and cardiomyocyte apoptosis. Importantly the concentrations of estrogen in ovariectomised mice after replacement with exogenous estrogen

was 81pg.ml^{-1} (van Eickels et al 2003) as compared to the peak estrogen concentrations of 23pg.ml^{-1} reported in the current study (female rats terminated during proestrus, peak estrogen concentrations) as well as 8 pg.ml^{-1}on average as demonstrated by Jang et al (2004). Hence, although excessively high concentrations of estrogen have been reported to be cardioprotective; the current study is the first to demonstrate the cardioprotective effects of endogenous estrogen concentrations *in vivo.* Our data showing lower heart rates in female rats compared to male rats, as well as enhanced β-adrenergic receptor induced cardiomyocyte apoptosis and pathological score in male rats but not female rats, suggest that even endogenous concentrations of oestrogen are cardioprotective; however the possible role of the hormone progesterone cannot be ignored. In this regard a study of the effects of gender on aging induced cardiomyocyte apoptosis in normal human hearts, demonstrated that cardiomyocyte apoptosis was three fold greater in males compared to females (Mallat et al 2001). Furthermore, Mendelsohn and Karas (2005) showed that myocardial contractility is increased in healthy women as compared to age-matched healthy men.

In addition, this is the first study, to our knowledge, to show *in vivo* a gender discrepancy in β-adrenergic receptor induced cardiomyocyte apoptosis. These data corroborate the findings of Gao et al (2003), in that male transgenic mice with β-adrenergic receptor overexpression had lower survival rates compared to female transgenic mice. Although it has previously been shown that β-adrenergic receptor activation induces cardiomyocyte apoptosis, an effect which is ameliorated by β-adrenergic blockade, these studies were collected in cultured cardiomyocytes (Communal et al 1998 and Saito et al 2000) and the impact of gender was not assessed.

4.3 Benefits of *in vivo* compared to *ex vivo* studies

This is the first study to our knowledge, to evaluate gender differences in an *in vivo* animal model. The advantage of an *in vivo* model is that the effects of β-adrenergic receptor stimulation are determined throughout the whole physiological system and not in an isolated environment. In this regard, Kim et al (2006) demonstrated that exogenous estrogen attenuated hypoxic re-oxygenation induced cardiomyocyte apoptosis and reactive oxygen species production in cultured cardiomyocytes. The latter study was a short term study whereby hypoxic re-oxygenation lasted 18 hours and the TUNEL technique was used to assess cardiomyocyte apoptosis; two attributes which are similar to the present study. However, there are certain limitations namely; Kim et al (2006) used neonatal cultured cardiomyocytes which are immature in nature, and cell culture studies fail to assess the effects of other normal physiological systems that impact on the myocardium. To elaborate, cultured cells represent an isolated environment which therefore lacks the influence of the neurohormonal system to maintain normal physiological homeostasis.

4.4 Comparisons with previous studies

4.4.1 Degree of apoptosis

In the short term study performed by Kim et al 2006 the concentration of cardiomyocyte apoptosis as detected by TUNEL was extremely high, 3% TUNEL positive cells in cultured cells (Kim et al 2006), as compared with the present study in which 5 days ISO administration only induced 0.06% apoptosis. The significantly greater percentages of apoptosis observed *in vitro* compared to *in vivo* were confirmed by a study which assessed the effects of exogenous estrogen administration on cardiomyocyte apoptosis in both *in vivo* and *in vitro* animal models (Patten et al 2004). Myocardial infarctions were induced by LAD coronary artery ligation *in vivo* and cardiomyocyte apoptosis was evaluated after 6, 24 and 72 hours (short term study). LAD coronary artery ligation increased the amount of

cardiomyocyte apoptosis with TUNEL values of 2%, 1.3% and 0.8% corresponding to 6, 24 and 72 hours in placebo treated mice. However, in mice treated with exogenous estrogen, LAD coronary artery ligation induced cardiomyocyte apoptosis was attenuated at 24 and 72 hours with TUNEL values of 0.5% and 0.3% respectively. Moreover, in the *in vitro* model (cultured cardiomyocytes, apoptosis induced by antracycline and daunorubicin) the percentage cardiomyocyte apoptosis determined using TUNEL was 31.5% within 24 hours, an effect which was attenuated with exogenous estrogen administration (TUNEL value of 26.1%) (Patten et al 2004). Importantly, in the *in vivo* model the highest percentage of cardiomyocyte apoptosis was 2% as compared to 31.5% in cultured cardiomyocytes; hence confirming that the percentage cardiomyocyte apoptosis is far greater in *in vitro* models.

It may be argued that although gender differences were observed in the current study, the percentage apoptosis was small. Isolated cardiomyocytes exposed to ISO for an acute period of 48 hours produced a 40% increase in apoptosis (Iwai-Kanai et al 1999). However, in the present *in vivo* study a maximum 0.1% increase in apoptosis was observed after five days of ISO administration. Importantly, *in vitro* cultured cardiomyocytes are exposed to an isolated environment with no external influences and could therefore produce an exaggerated response to β-adrenergic receptor stimulation. An *in vivo* model, on the other hand, such as in the present study, presents a realistic physiological outcome. Furthermore, Colucci (1997) explained that a 0.2% increase in cardiomyocyte apoptosis per day, if sustained for a year, could result in half the functioning cardiomyocytes being destroyed and consequently induce severe myocardial damage. Therefore, a 40% increase in apoptosis after 48 hours does not represent a realistic clinical picture; whereas the present study demonstrates a more realistic increase in cardiomyocyte apoptosis (0.1% after 5 days) which if sustained for a longer time period could induce severe myocardial remodelling. Indeed, Veliotes et al (2005) revealed

that long term exposure to ISO (five months) induced changes in left ventricular geometry, cardiac dilatation and pump dysfunction.

4.4.2 Degree of necrosis

In conjunction with the gender differences in ISO induced apoptosis, the current study also revealed a gender difference in pathological scores, which are an index of necrosis and replacement fibrosis. Short term administration of ISO significantly increased the pathological score in male rats, an effect not demonstrated in the female rats. In accordance with the present study's results that estrogen possibly prevents ISO-induced necrosis, Booth et al 2001 demonstrated that 17β-estradiol administration decreased LAD coronary artery ligation induced necrosis in ovarectomised female rabbits. However, unlike in the present study, no gender comparisons were made. Hence, the study of Booth et al (2001) only revealed that exogenous estrogen administration inhibits LAD coronary artery ligation induced necrosis. Similarly, Jeanes et al (2008) revealed that ovariectomized female rats receiving exogenous 17β-estradiol were protected against ischaemic-reperfusion induced myocardial damage by significantly decreasing myocardial necrosis again highlighting the importance of estrogen and its plausible cardioprotective effects. It is important to note however, that the current study is the first study to our knowledge to evaluate the impact of gender on β-adrenergic receptor induced necrosis and replacement fibrosis.

4.4.3 Degree of hypertrophy

Before the correction for differences in body weight, the present results revealed hypertrophy in ISO treated male rats and not in the ISO treated female rats. However, after both heart weight and left ventricular weight had been corrected for 100g body weight, a modest increase in heart and left ventricular weight (hypertrophy) was noted in both genders in response to 5 days of ISO administration. The female rats were noted to have a greater degree of concentric hypertrophy compared to male rats as demonstrated by an increased

relative wall thickness and chamber dimensions. However, as with heart and left ventricular weights per 100g body weight, no gender differences in the response to ISO were noted. Previous research has revealed that females present with a greater degree of concentric hypertrophy in response to pressure overload and hypertension induced MI as compared to males (Douglas et al 1998; Jain et al 2002). Moreover, human studies have demonstrated that postmenopausal women respond to aortic stenosis and systolic hypertension by concentric hypertrophic remodelling as opposed to men who present with eccentric cardiac hypertrophy (Aurigemma et al 1995). In accordance with the latter statement and the current study, Podesser et al (2006) revealed that in a pressure overload model, female rats responded by developing concentric hypertrophy as compared to males who developed eccentric remodelling.

It is possible that 5 days ISO administration was too short a time to detect a gender difference in the response to ISO. Indeed, Skavdahl et al (2004) revealed that hypertrophy was more pronounced in male mice as compared to age matched female mice after TAC induced pressure overload. However, the animal model used by Skavdahl et al (2004) was different from the present study. Indeed, TAC induced pressure overload is an extremely aggressive model of myocardial insult and thus could have resulted in a gender difference in cardiac hypertrophy over a short period of time (2 weeks). It has also been noted that during the aging process myocardial mass is maintained better in women compared to men (Mendelsohn and Karas, 2005). In accordance with the latter statement in the study performed by van Eickels et al (2001) pressure-overload induced myocardial hypertrophy was significantly reduced in ovariectomised female mice receiving exogenous estrogen as compared to placebo treated ovariectomised mice. Although the study of van Eickels et al (2001) was not a gender study it does emphasize the cardioprotective effects of estrogen and the role that estrogen plays in decreasing myocardial hypertrophy.

4.4.4 Gender differences in heart rate

An interesting finding of our study was that male rats had higher heart rates compared to female rats at baseline measurements. It is well established that heart rate and contractility are controlled by the SNS, more specifically β-adrenergic receptors (Dorn 2002). The latter point was demonstrated by Thawornkaiwong et al (2003) whereby estrogen and progesterone decreased the expression of β-adrenergic receptors in ovariectomized female mice. In addition it was also noted that long term ovarian hormone deprivation produced significant up-regulation of β-adrenergic receptors. The latter study could offer a plausible explanation as to why female rats had lower baseline heart rates in the current study. It is possible that estrogen down-regulated myocardial β-adrenergic receptor activity and thus induced lower heart rates in female rats as compared to male rats. Indeed, in a study in which rat hearts and ventricular myocytes were treated with estrogen and ISO, estrogen administration was found to prevent the ISO-induced increase in heart rates, diastolic pressure and ischemic induced arrhythmias (Li et al 2000). An important note regarding the study of Li et al (2000) is that the dose of estrogen used was in the normal physiological range of estrogen concentrations, which coincides with the present study in which female rats were examined during normal estrus cycle and presented with lower heart rates as compared to males. Interestingly, Skafar et al (1997) showed that estrogen has a direct effect on vascular smooth muscle cell (VSMC) function, more specifically contractility and growth. Research reveals that estrogen decreases voltage-dependent T and L-type calcium channel currents within the VSMC and thus hyperpolarizes the resting membrane potential which consequently attenuates myocardial and vascular contractility (Skafar et al 1997). In addition, estrogen has also been shown to attenuate angiotensin II and noradrenaline induced contractility, two mediators which induce the release of calcium from intracellular storage sites and hence increase contractility (Skafar et al 1997). Hence, it is likely that the lower heart rates measured in the female compared to the male rats, are due to the high estrogen concentrations in the females during proestrus.

Nevertheless, the increment in heart rate observed in response to acute isoproterenol in the female rats was greater than that observed in the male rats. An explanation for the increased acute response in the females despite possible down regulation of β-adrenergic receptors in response to 5 days of ISO administration is unclear.

4.5 Different models of CVS disease

The β-adrenergic receptor activation model is of significant clinical value as heart failure is accompanied by over-activation of the sympathetic nervous system and increased plasma concentrations of noradrenaline (Tan et al 2004). In comparison, previous *in vivo* models which have demonstrated gender differences do not closely resemble the slow progression of heart failure. In this regard both TAC and LAD are aggressive models of acute pressure-overload, and acute hypoxia and MI respectively. Hence these data are difficult to translate into possible clinical impacts. To elaborate van Eickels et al (2001) induced pressure overload hypertrophy by TAC within a period of 4-8 weeks. Generally, hypertrophy, cardiac remodelling and heart failure are slow processes, thus the short term effects of TAC (i.e. 4-8 weeks) fail to mimic the natural progression of cardiac dysfunction. Moreover, the effects of acute MI may differ from the mechanisms responsible for chronic heart failure. Conversely, Veliotes et al (2005) demonstrated that low dose ISO for a period of 4.5 months induced pump dysfunction and increased left ventricular cavity volume; physiological changes that normally occur in chronic heart failure. Thus, although TAC and LAD have the potential to induce myocardial damage the mechanisms are aggressive and do not correlate with the natural progression of chronic heart failure unlike the usage of ISO which favours the natural progression of cardiovascular disease in both short term and long term use (Osadchii et al 2007 and Veliotes et al 2005). Another CVS model of disease is the over-expression of β-adrenergic receptors. Gao et al (2003) revealed that male transgenic mice with β_2-adrenergic receptor over-expression had lower survival rates as compared to female

transgenic mice, thus highlighting the possible cardioprotective effects of estrogen via β_2-adrenergic receptors. Although β_2-adrenergic receptor over-expression induced left ventricular dilatation and reduced left ventricular fractional shortening, the shortcoming of this model is that the animals are genetically manipulated and hence do not truly represent the natural progression of heart failure in humans.

4.6 Different manipulations of estrogen concentrations

In the present study estrogen concentrations were not manipulated as female animals were used to assess the effects of the natural estrus cycle. However, in the literature various models of estrogen manipulation have been assessed. One such example of estrogen manipulation is estrogen receptor knockout models (Pelzer et al 2005; Korte et al 2005). Indeed, Pelzer et al (2005) demonstrated that female estrogen β-receptor knockout mice had increased mortality rates, infarct sizes and biochemical markers of heart failure as compared to wild type mice receiving exogenous 17β-estradiol. The study revealed the cardioprotective effects of estrogen more specifically the role of the ER-β. Although receptor knockout models are useful in detecting the possible function of a receptor, they fail to assess normal physiological mechanisms as well as the effect of other receptors involved such as the other estrogen receptor, ER-α. In another study, Skavdahl et al (2004) analysed the effect of TAC in female ER-α and ER-β knockout mice. The results demonstrated that the ER-β knockout mice had a greater degree of hypertrophy compared to wild type female mice and this effect was not noted in the ER-α knockout female mice; thus revealing that estrogen is cardioprotective and could possibly protect the myocardium via ER-β.

The current study is the first study, to our knowledge, which assesses the effects of ISO induced cardiomyocyte apoptosis and necrosis in normal cycling female rats. Various studies have assessed the effects of estrogen; however these studies have manipulated estrogen and not identified the mechanisms of the endogenous estrus cycle. Indeed, van Eickels et al

(2003) assessed the effects of estrogen in LAD coronary artery ligation induced cardiomyocyte apoptosis in female mice. The results were favourable in that estrogen attenuated LAD coronary artery ligation induced apoptosis; however the mice were ovarectomised and received constant high levels of exogenous 17β-estradiol at concentrations of 81 $pg.ml^{-1}$. Conversely, in the present study female rats had decreased cardiomyocyte apoptosis and necrosis as compared to male rats and revealed peak estrogen concentrations of 23 $pg.ml^{-1}$. Moreover, Zhan et al (2008) investigated the effects of low, medium and high doses of exogenous estrogen in female mice post-MI (LAD coronary artery ligation). The aim of the study was to address the controversy regarding the correct and most physiological dose of estrogen that should be administered in animal models. The results demonstrated that low dose exogenous estrogen tended to be more cardioprotective, that is improved myocardial function and remodelling, as compared to medium and high doses of estrogen (Zhan et al 2008). In addition, moderate and high doses of estrogen increased myocardial fibrosis, hypertrophy, dysfunction and dilatation. The importance of the study of Zhan et al (2008) is that it emphasises that low doses but not medium or high doses of estrogen are cardioprotective. Hence the study of Zhan et al (2008) corroborates the present study, which shows that normal physiological concentrations of estrogen are cardioprotective and crucial for myocardial homeostasis.

It is well understood that human studies are controversial regarding HRT. One theory that has been explored is whether the dose of HRT is too high. Indeed, Varas-Lorenzo et al (2000) assessed the effects of high, medium and low dose HRT in healthy post menopausal women and the effective incidence of MI. The results demonstrated that medium doses produced the most favourable odds ratio as compared to high and low dose HRT. Thus, this study agrees with the results of Zhan et al (2008) that the dose of estrogen is crucial when assessing the cardioprotective mechanisms of estrogen.

4.7 Possible mechanisms of protective effects of estrogen

The present study reveals that a marked gender difference exists with female rats having less cardiomyocyte apoptosis and necrosis in response to ISO as compared to age matched male rats. It can be postulated that a relationship exists between estrogen receptors and the β-adrenergic receptors of the SNS. Indeed, Kam et al (2004) demonstrated that long term estrogen administration suppresses myocardial β_1-adrenoceptors in ovariectomized female rats which consequently decreases myocardial injury during ischaemic induced SNS hyper stimulation (Kam et al 2004). In the study of Kam et al (2004), ischemia was produced by LAD coronary artery ligation and female rats were either intact; ovariectomized receiving exogenous 17β-estradiol; or ovariectomized receiving placebo. In order to mimic SNS hyper-stimulation, ISO was administered during the ischemic period (LAD coronary artery ligation) and revealed that infarct sizes increased significantly in ovariectomized rats receiving placebo as compared to intact rats and ovariectomized rats receiving 17β-estradiol. In order to assess the effects of estrogen on β-adrenergic expression, Kam et al (2004) measured the mRNA expression and protein concentration of β-adrenergic receptor in the left ventricular tissue of the different groups. Interestingly, β-adrenergic receptor levels were up-regulated in ovariectomized rats and suppressed in intact and ovariectomized rats receiving exogenous 17β-estradiol, thus highlighting the possible cardioprotective mechanism of estrogen is by down regulation of β-adrenergic receptors. In a follow up study, Kam et al (2005) further investigated the relationship between estrogen and β-adrenergic receptors. Indeed in the latter study, Kam et al (2005) revealed that estrogen inhibits protein kinase A (PKA) activity which decreases calcium influx via calcium channels and consequently decreases myocardial contractility. The interactive effects of estrogen on β-adrenergic receptors support the results of the present study that natural estrogen attenuated ISO induced apoptosis and necrosis in female rats, but not in male rats.

In order to investigate the possible cardioprotective mechanisms of estrogen, Kim et al (2006) assessed the role that estrogen plays in preventing hypoxic re-oxygenation induced cardiomyocyte apoptosis. Cultured rat cardiomyocytes were treated with estrogen or placebo and chemicals that induced hypoxic re-oxygenation. The results demonstrated that estrogen treated cells had less cardiomyocyte apoptosis as assessed by annexin V staining and the TUNEL technique. In addition, the exact apoptotic pathway was assessed and revealed that estrogen blocked the activity of p38α kinase, a protein involved in pro-apoptotic pathways. The production of reactive oxygen species (ROS), was also measured and demonstrated that estrogen attenuated hypoxic re-oxygenation induced ROS which is a potent mediator of cell death. In agreement with Kim et al (2006), Patten et al (2004) also assessed plausible mechanisms for decreased apoptosis in cardiomyocytes treated with estrogen and revealed that estrogen increased the stimulation of the pro-survival pathway, serine-threonine, Akt and PI3 kinase dependent pathway. Thus both studies highlighted that estrogen prevents pro-apoptotic pathways as a plausible mechanism of cardioprotection.

4.8 Possible mechanisms for gender differences

Females have improved myocardial function and survival rates in both human and animal studies (Mendelsohn and Karas 2005; Guerra et al 1999; Zhai et al 2000; Skavdahl et al 2004; van Eickels et al 2001). A plausible reason for these gender differences, are gender differences in the cardiac expression of glycolytic and mitochondrial metabolic enzymes as well as the pro-survival effects of ER2-ER on cardiomyocytes which is controlled by ER-α and phosphatidylinositol 3-kinase-Akt-dependent pathways (Mendelsohn and Karas, 2005). Indeed, Guerra et al (1999) demonstrated that failing hearts in human females had less cardiomyocyte death as compared to those in human males. The authors postulated that estrogen improved the phosphorylation of insulin growth factor-1 (IGF-1) which increases the expression of anti-apoptotic gene products such as Bcl-2 and Bcl-x_1 and inhibits Bax, a

pro-apoptotic protein; thus decreasing the degree of apoptosis in females. Cavasin et al (2003) revealed that female mice post MI had less myocardial dysfunction and chronic remodelling as compared to male mice post MI. Moreover, in the latter study, non-castrated male mice receiving exogenous estrogen had improved myocardial function and decreased remodelling. The authors explained that estrogen was shown to increase nitric oxide production which induces rapid dilation of coronary arteries (Williams et al 1992) and furthermore, estrogen increases HDL concentrations, improves endothelial function and prevents inflammatory cell activation (Pelzer et al 1996), all of which could explain the cardioprotective effects of estrogen.

Indeed, Jovanovic et al (2000) assessed whether a gender difference exists in the response to metabolic stress. Single ventricular cardiomyocytes were used and underwent hypoxic re-oxygenation. Cardiomyocytes from either gender had increased calcium loading, a predictor of cellular damage, in response to hypoxic re-oxygenation. However, when pretreated with 17β-estradiol female cardiomyocytes had less hypoxic re-oxygenation induced calcium loading an effect not demonstrated in the male cardiomyocytes. Moreover, administration of tamoxifen, an estrogen receptor blocker, prevented the estrogen induced decrease in calcium loading thus demonstrating that the decrease in calcium was estrogen dependent. The authors explained that the gender difference could be as a result of estrogen receptors being more functional and present in higher concentrations in females as compared to in males (Jovanovic et al 2000).

4.9 Limitations

The present study has certain limitations which must be addressed. The study demonstrated ISO induced cardiomyocyte apoptosis, necrosis and replacement fibrosis in male rats, but not in the female rats. However, whether the effect was in fact as a result of the

presence of testosterone, rather than the absence of estrogen, in male rats was not elucidated. Hence further research must assess the effects of testosterone on ISO induced cardiomyocyte apoptosis and pathological score. Moreover, the effects of progesterone in the female rats were not assessed or measured. However in the present study we chose to focus on the effects of estrogen, rather than progesterone, as evidence from the literature points towards estrogen being the most likely cardioprotective female hormone. In addition certain limitations exist with the techniques used. TUNEL was used to quantify the percentage of cardiomyocyte apoptotic nuclei. Although it is a widely used technique for the detection of fragmented DNA, a biochemical feature of apoptosis, TUNEL is extremely sensitive and stains fragmented DNA of cells undergoing repair. Thus, over estimation of the total number of apoptotic nuclei could have occurred (Jugdutt and Idikio 2005). Moreover, the precise stage of apoptosis, early or late, could not be determined and necrotic cells were not easily distinguished from apoptotic cells. Alternative methods for evaluating apoptosis and the distinguished stages are caspase-3 activity and flow cytometry (Annexin V and propidium iodide). However, in order to assess caspase activity, the hearts would need to have been freeze clamped hence precluding the assessment of cardiac weights and apoptosis via TUNEL. Flow cytometry requires the use of isolated cardiomyocytes, preferably those grown in cell culture, as the isolation procedure (collagenase digestion) is likely to result in alterations in the cell membrane and thus the signs of early apoptosis (annexin V binding) would be masked by overall propidium iodide staining. Nevertheless, the purpose of the study was not to quantify accurately the degree and stage of apoptosis; but rather to assess *in vivo* the impact of gender on the degree of ISO induced cardiomyocyte apoptosis and necrosis. The estrogen ELISA assay that was used was a human assay, as rodent assays are currently not available. However the aim of the assay was to verify that female rats had

higher concentrations of estrogen as compared to male rats, rather than to quantify the exact concentrations of estrogen.

In future studies we plan to investigate ISO induced apoptosis and necrosis in ovariectomized compared to naturally cycling female rats to confirm the results observed in the present study. Also, to investigate the effects of pharmacologically blocking estrogen and assessing the effects in female rats both naturally cycling and ovariectomized receiving exogenous estrogen. Moreover, it is important to determine whether the presence of testosterone (rather than the absence of estrogen) is in fact cardiotoxic. In this regard, in future studies we plan to evaluate the effect of castration on ISO induced apoptosis and necrosis in male rats. It is also important to investigate the effects of long term ISO administration in male and female rats and elucidate whether estrogen attenuation of cardiomyocyte apoptosis and necrosis translates into protection of the female heart from ISO-induced cardiac dilatation and chamber dysfunction *in vivo*. In addition, the effects of the different stages of the estrus cycle on β-adrenergic induced apoptosis and necrosis can be assessed; however this must be done with caution as certain stages of the estrus cycle are extremely short and thus could be missed on examination (The four stages of the rat estrus cycle, namely proestrus, estrus, metestrus and diestrus last twelve, twelve, twenty one and sixty hours respectively, hence the estrus cycle lasts a total of 4 days and 19 hours (Marcondes et al 2002).

4.10 Clinical implications

It is well established that patients in heart failure and myocardial infarction have increased levels of noradrenaline (Tan et al 2004). In addition, increased stimulation of the SNS and even more specifically β-adrenergic activation is an early indication of myocardial damage (Communal et al 1998). Thus, it is crucial to evaluate the possible detrimental effects of prolonged β-adrenergic stimulation. In this regard, prolonged β-adrenergic stimulation

results in myocardial apoptosis and necrosis, which contribute to myocardial dysfunction and dilation, by the loss of viable functioning cardiomyocytes. Thus, investigating the mechanisms of the effects of activation of the β-adrenergic system is crucial. Moreover, research has established a relationship between β-adrenergic receptors and estrogen (Thawornkaiwong et al 2003; Kam et al 2003 and Vizgirda et al 2002), indicating that estrogen is cardioprotective. Further knowledge of the relationship between estrogen and β-adrenergic receptors, and the mechanisms of the protective effects of estrogen against β-adrenergic induced cardiac dysfunction, is crucial for the development of possible drug targets for cardioprotection.

4.11 Conclusion

Thus in conclusion, this is the first study, to our knowledge, that demonstrates a gender difference in the cardiac response to short term β-adrenergic stimulation *in vivo*. The present study demonstrates the possible cardioprotective effects of estrogen by decreasing β-adrenergic induced cardiomyocyte apoptosis and necrosis, although the possible effects of the hormone progesterone cannot be ignored. The latter is important as it sheds light on a plausible mechanism of how endogenous estrogen is cardioprotective and will allow for further research to fully elucidate how estrogen attenuates β-adrenergic induced myocardial damage.

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AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2007/28/'04

APPLICANT: C Mielke

SCHOOL: Physiology
DEPARTMENT:
LOCATION: Medical School

PROJECT TITLE: **The effect of gender on short term beta-adrenergic receptor- induced cardiomyocyte apopsis**

Number and Species

Wistar rats 30 male 30 female

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20070327. This approval remains valid until 20090327

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Signed: _____ (Chairperson, AESC) Date: 18/07/07

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 21/05/07

cc: Supervisor: #
Director: CAS

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