SEX- AND AGE-SPECIFIC ACUTE BENEFICIAL EFFECTS OF AN ESTROGEN RECEPTOR AGONIST ADDED TO A CARDIOPLEGIC SOLUTION IN ADULT AND AGING MOUSE HEARTS

by

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Dedication

This thesis is dedicated to:

My parents, Govinda Ghimire and Bhagawati Ghimire

Thank you so much for believing on me and supporting me throughout. You are world to me. My every step towards success is always dedicated to you.

And

My husband, Dinesh Thapa.

Words cannot express how important you are to me. Thank you for your constant support, encouragement, love and care.

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Abstract

This study investigated effects of G1, a G-protein estrogen receptor (GPER) agonist, in a Langendorff-perfused mouse heart model of cardioplegia in adult and aged mice of both sexes. The recovery of cardiac function using St. Thomas' Hospital cardioplegic solution No. 2 (STH2), prior to ischemia/reperfusion insult was better in adult female hearts compared to adult males. By contrast, hearts from old mice of both sexes recovered similarly or recovery was even better in males. However, the extent of recovery with STH2 was sub-optimal. When G1 was incorporated in STH2 results showed enhanced cardiac recovery in adult female mouse hearts but not in males. This protection with G1 was, however, observed in old mouse hearts from both sexes. The effect of G1 was also blocked by the GPER antagonist, G15. Together these findings suggest that addition of G1 in STH2 is a useful strategy to improve outcomes of cardiac surgery.

List of Abbreviations and Symbols Used

ºC	Degree Celsius
+dP/dt	Rate of pressure rise
-dP/dt	Rate of pressure decay
µm	Micrometer
µM	Micromolar
Akt	Protein kinase B
ANOVA	Analysis of variance
ARRIVE	Animal Research: Reporting of *In Vivo* Experiments
ATP	Adenosine triphosphate
bpm	Beats per minute
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CCAC	Canadian Council on Animal Care
CO_2	Carbon dioxide
cTnI	Cardiac troponin I
CVD	Cardiovascular Disease
DMSO	Dimethyl sulfoxide
EDP	End diastolic pressure
ELISA	Enzyme-linked immunosorbent assay
ERs	Estrogen receptors
ERK	Extracellular signal-regulated kinases
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
g	Gram
GPER	G-protein estrogen receptor

GPR30	G-protein coupled orphan receptor
H^+	Hydrogen ion
HCl	Hydrogen chloride
HCO_3^-	Bicarbonate ion
HR	Heart rate
I/R	Ischemia/Reperfusion
K^+	Potassium ion
K_{ATP}	Adenosine triphosphate sensitive potassium channel
KCl	Potassium chloride
kg	kilogram
KH_2PO_4	Potassium biphosphate
LV	Left ventricular
LVDP	Left ventricular developed pressure
Mg^{2+}	Magnesium ion
MEK	Mitogen activated protein kinase
mg	milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
mmHg	Millimeter of mercury
mos	Months
mPTP	Mitochondrial permeability transition pore
N_2	Nitrogen
Na^+	Sodium ion

NaCl	Sodium chloride
NCX	Sodium-calcium exchanger
ng	Nanogram
$NaHCO_3$	Sodium bicarbonate
nM	Nanomolar
NOS	Nitric oxide synthase
O_2	Oxygen
PI3K	Phosphatidylinositol 3-kinase
pO_2	Partial pressure of oxygen
RM ANOVA	Repeated measures analysis of variance
ROS	Reactive oxygen species
RPP	Rate pressure product
sec	Second
SEM	Standard error of the mean
SR	Sarcoplasmic reticulum
STH1	St. Thomas' Hospital cardioplegic solution No. 1
STH2	St. Thomas' Hospital cardioplegic solution No. 2
TTC	Triphenyltetrazolium chloride
WHO	World Health Organization
wks	Weeks

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CHAPTER 1: INTRODUCTION

1.1 Overview

Cardiovascular disease (CVD) is the leading cause of death throughout the world and its prevalence increases with age in both sexes (Cardiovascular diseases, 2017; Mosca *et al.*, 2011). Many CVDs require surgical intervention as a part of treatment, although variable outcomes are reported at different ages and between the sexes. This could partially arise due to structural and functional remodelling of the heart as a person ages, or could be due to sex-specific differences in heart function. Cardiac surgeries are performed on hearts arrested with a "cardioplegic solution". This solution is also known as cardioplegia, where "cardio" means heart and "plegia" means paralysis. This solution protects the heart from ischemia/reperfusion (I/R) injury that occurs during cardiac surgery (Bhakri *et al.*, 2014). However, variable outcomes have been reported with existing clinical cardioplegic solutions. Many pharmacological strategies have been implemented to enhance the properties of these solutions such as adding potentially protective drugs and modifying other parameters of existing cardioplegic solutions (*e.g.* ionic composition and temperature) (Ascione *et al.,* 2002; Powell *et al.,* 1995; Fogelson *et al.,* 2000). Still outcomes for cardiac surgery are not optimal, especially in older adults (Ascione *et al.,* 2002; Hausenloy *et al.,* 2012).

The sex steroid hormone estrogen plays a cardioprotective role and can reduce I/R injury (Murphy and Steenbergen, 2007). The traditional nuclear estrogen receptors (ERs), estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), are thought to play a role in these beneficial effects of estrogen (Wang *et al.,* 2006; Mendelsohn and Karas,

1999). There are also G-protein estrogen receptors (GPERs) present in ventricular myocytes that initiate acute, non-genomic responses when activated (Meyer *et al.,* 2011). The GPER agonist G1 is protective in animal models of I/R injury when the heart is exposed to a brief period of global I/R (Deschamps and Murphy, 2009). However, whether G1 can enhance the properties of cardioplegic solutions to protect the heart exposed to longer periods of ischemia is not known. Thus, the objective of this thesis is to determine whether G1 enhances the protective properties of traditional cardioplegic solutions. The effects of incorporating G1 in cardioplegic solution in hearts from aged and adult mice of both sexes are examined in this thesis.

1.2 CVD and its prevalence

CVDs are disorders that affect the normal functioning of the heart and involve a range of conditions that adversely affect the heart and vasculature. Arrhythmias, congenital heart disease, ischemic heart disease and valvular heart disease are all different types of CVDs. More people die due to CVDs than any other cause globally. According to the World Health Organization (WHO), about 17.7 million people died in 2015 from CVDs (Cardiovascular diseases, 2017). This represents 31% of all global deaths. Though CVD is the leading cause of death in both men and women, the burden and prevalence of the disease differs between the sexes. Throughout the life course, CVD mortality rates are higher in males than in females. However, the prevalence of CVD is higher in the elderly female population due to the longer life expectancy of women compared to men (Mosca *et al.,* 2011; Maas and Appelman, 2010). In addition, in those with CVD, women are at higher risk of poor outcomes compared to men (Feldman, 2016). Endogenous estrogen is

thought to play an important role in cardioprotection in women. Bairey *et al.* (2003) concluded that young women with estrogen deficiency had more than a seven-fold increase in coronary artery disease risk compared to age-matched women with normal estrogen levels. This suggests that older, post-menopausal women could be at higher risk of developing CVD due to declining estrogen levels. There is also experimental evidence to support this. For example, in hearts from aged and ovariectomized mice, high Ca^{2+} levels in ischemia promote contractile dysfunction and cell death (Ross and Howlett, 2012). This suggests that low estrogen levels alter myocyte Ca^{2+} homeostasis and promote I/R injury. Overall, the incidence of CVDs in men and women increases with age, which shows that the older population is at higher risk than the younger population. In consequence, older individuals are more likely to undergo cardiac surgeries. The next section will review the impact of age on the heart, with a focus on changes that may increase the susceptibility of the heart to CVDs.

1.3 Factors affecting heart health

Heart health is affected by many factors that cause structural and functional remodelling of the heart. Among them, advanced age is one of the major risk factors that makes the heart more susceptible to CVDs. It is also important to note that age-dependent remodelling of the heart varies between the sexes.

1.3.1 Age-associated changes in the heart

Advanced age is associated with prominent structural and functional changes in the heart. With advancing age, there are pronounced changes in both the atria and

ventricles as shown in **Figure 1.1**. Atrial hypertrophy and dilatation commonly occur in older adults, possibly due to increased diastolic filling pressures (Boyd *et al*., 2011). Myocyte loss and changes in loading promote atrial and ventricular fibrosis in elderly people (Mirza *et al.*, 2012; Dzeshka *et al.,* 2015). Increased left ventricular (LV) mass and wall thickness are also major changes that are observed as people age (Chen *et al.,* 2016; Gebhard *et al.,* 2013; Eng *et al.,* 2016). This happens as a result of increased fibrosis and myocyte hypertrophy. Also, the number of pacemaker cells in the sinoatrial node declines with age in humans (Lakatta, 1993; Mirza *et al*., 2012). These macroscopic and microscopic changes in the heart affect its function. Older people have depressed systolic and diastolic function. Specifically, stroke volume and ejection fraction decline with age and LV filling in diastole is slowed (Claessons *et al.*, 2007; Boyd *et al.*, 2011; Horn and Trafford, 2016; Hamdani *et al.*, 2013). Calcification of aortic valve leaflets also occurs with aging (New and Aikawa, 2011). This obstructs LV outflow and increases the chance of developing heart failure (New and Aikawa, 2011). A decrease in maximal heart rate (HR), increase in action potential duration and slower conduction are other major functional changes that occur with age (Mirza *et al.,* 2012; Ferrara *et al.,* 2014). Taken together, these data suggest that aging causes cardiac remodelling, which increase the risk for development of CVDs. There is emerging evidence that these age-dependent changes in the heart differ between the sexes, as discussed in the next section.

1.3.2 Sex-specific changes in the heart with age

There is evidence that men are at higher risk of developing CVD than women, although, a woman's risk increases dramatically after menopause (Mosca *et al.,* 2011; Maas and Appelman, 2010). This suggests that estrogen plays an important role in

cardioprotection in women. Apart from hormonal differences, there are other changes in the heart that differ between the sexes with age. Increased LV mass and LV wall thickness are more prominent in older women than in men (Chen *et al.,* 2016; Gebhard *et al.*, 2013; Eng *et al.,* 2016; Hees *et al.,* 2002). Reduction in the number of ventricular myocytes and hypertrophy of the remaining cells are more common in older men than in women (Olivetti *et al.*, 1991). Also, the number of fibroblasts in the atria and ventricles are higher in older men (Liu *et al.,* 2013). There is a higher risk of systolic heart failure (due to aortic valve calcification) and atrial fibrillation (due to atrial hypertrophy and fibrosis) in older men than in women (New & Aikawa, 2011; Milin *et al.*, 2014; Pancholy *et al.*, 2014). However, diastolic heart failure is more prevalent in older women than in men (Dunlay & Roger, 2012; Greiten *et al.*, 2014). These findings suggest that age-related structural and functional changes in the heart vary between the sexes.

1.4 I/R injury in cardiac surgery

Many individuals with CVDs go through cardiac surgery as a part of treatment. During cardiac surgery, blood flow to the myocardium is interrupted by applying a clamp across the ascending aorta, which causes an imbalance between oxygen (O_2) supply and demand resulting in tissue damage. This phase is called ischemia. After the ischemic insult, blood flow to the heart is restored. Paradoxically, this further induces injury by increasing intracellular calcium (Ca^{2+}) levels, promoting higher levels of reactive O_2 species and causing neutrophil accumulation in the myocardium. This post-ischemic phase, when blood re-enters the heart, is called reperfusion. Hence, cardiac injury occurs

both during ischemia and in reperfusion in the setting of cardiac surgery (Maeda and Ruel, 2015; Frank *et al.,* 2012).

During ischemia, there is depletion of high energy phosphate, an increase in anaerobic metabolism and intracellular acidosis (Frank *et al.,* 2012; Bhakri *et al.*, 2014). Intracellular acidosis causes the loss of transmembrane ionic homeostasis, which leads to increased sodium (Na^+) levels in cardiomyocytes through the Na^+/H^+ exchanger and the Na^+/HCO_3^- symporter (Frank *et al.,* 2012). This increase in Na^+ influx causes increased intracellular Ca^{2+} through Na^+/ Ca^{2+} exchange. This, in turn, causes enhanced Ca^{2+} induced Ca^{2+} release from the sarcoplasmic reticulum (SR) resulting in Ca^{2+} overload (Kalogeris *et al.,* 2012). Also, the superoxide free radicals produced during ischemia act as a neutrophil chemo-attractant causing membrane disruption (Bhakri *et al.*, 2014; Piper *et al.* 1999; Allen *et al.,* 1993).

In reperfusion, further damage to myocardium occurs as intracellular Ca^{2+} rises due to increased cell membrane permeability (Bhakri *et al.*, 2014). Also, Ca^{2+} reuptake into SR is impaired (Kalogeris *et al.,* 2012). This elevated intracellular Ca^{2+} results in myocardial contracture during early reperfusion, which can promote cell damage or death (Bhakri *et al.*, 2014). Structural integrity of the endothelium is disrupted due to increased neutrophil-endothelial interactions. Also, due to the loss of free radical scavenging enzymes, the cells are subjected to oxidant injury in reperfusion (Bhakri *et al.*, 2014). Hence, a series of events that occur during both ischemia and reperfusion phases of cardiac surgery can induce tissue stiffening, damage and/or cell death. This results in poor cardiac outcomes after surgery. Therefore, cardioprotective measures are taken to

minimize I/R injury during surgeries. In particular, cardioprotective solutions known as cardioplegic solutions are used to minimize damage, as discussed below.

1.5 Cardioprotection with cardioplegic solutions

As I/R injury occurs during cardiac surgery, different strategies have been developed to protect the heart from this damage. During cardiac surgery, a surgeon infuses the coronary circulation with a cardioplegic solution which arrests the heart and provides cardioprotection throughout the surgical intervention. The various cardioplegic solutions used clinically provide protection via different mechanisms. They rapidly inhibit contractions, reduce metabolic rate and decrease O_2 demand by the myocardium. Cardioplegia typically contains high potassium (K^+), which depolarizes the cells inactivating Na^+ channels and abolishing the action potential. This causes inhibition of Ca^{2+} influx from the extracellular space and inhibits Ca^{2+} release from the SR, thus preventing contraction and promoting cardioplegic arrest (Fallouh *et al.,* 2009). Another common chemical agent in cardioplegia is magnesium (Mg^{2+}). It acts as an enzymatic co-factor for reactions involving energy metabolism, hence preventing the loss of adenosine triphosphate (ATP) during ischemia (Shakerinia, Ali and Sullivan, 1996). Also, Mg^{2+} has been shown to have Ca^{2+} antagonist effects, which protects cells from deleterious effects of Ca^{2+} overload (Iseri, and French, 1984). In addition to these chemicals, cardioplegia can also be supplemented with other agents that help to limit damage induced by I/R, as discussed in more detail in section (**1.7)**.

Cardioplegic solutions are either delivered alone, as a clear crystalloid solution, or they are mixed with blood in various ratios (Nardi *et al.,* 2018; Govindapillai *et al.,*

2013). Other variables like the temperature (hypothermia or normothermia) and dosing intervals (single or multiple doses) of cardioplegia can also be altered to provide better cardioprotection (Bhakri *et al.*, 2014; Durandy, 2015). A study by Ascione and colleagues (2002) showed that, in patients undergoing aortic valve surgery, myocardial injury and ischemic stress were reduced in patients treated with cold blood cardioplegia (6-8ºC) when compared to those treated with warm blood cardioplegia (34ºC). In contrast, Sirvinskas et al. (2005) conducted a randomized study comparing the effects of warm blood (36ºC), tepid blood (28-30ºC) and cold crystalloid (4ºC) cardioplegia in patients undergoing coronary artery bypass graft surgery. They concluded that warm blood cardioplegia provided better myocardial protection compared to other solutions (Sirvinskas *et al.,* 2005). So, it is still unclear whether warmer or cold cardioplegia is better clinically. Therefore, different strategies are employed to enhance the protective properties of cardioplegia to improve cardiac outcomes after surgery.

There are many clinically available cardioplegic solutions which are particularly beneficial to specific groups of people. For example, the use of del Nido cardioplegia (high K^+ and high Mg^{2+}) has found to be especially beneficial in pediatric patients (O' Brien *et al.,* 2009). Other clinically available cardioplegic solutions are Custodial (low Na^+ and Ca^{2+}; high histidine based), Buckberg (dextrose-based), Celsior (low potassium and glutathione based) and Calafiore (high potassium and magnesium based) cardioplegia (Edelman *et al.,* 2013; Mick *et al.,* 2015; Pereda *et al.,* 2007; Comentale *et al.,* 2018). A traditional crystalloid cardioplegic solution that is widely used in clinical practice is St. Thomas' Hospital cardioplegic solution No. 2 (STH2). In the early 1970s, Hearse and colleagues developed a crystalloid cardioplegic solution and called it St. Thomas'

Hospital cardioplegic solution No. 1 (STH1) (NaCl 144.0 mM, KCl 20.0 mM, $MgCl_2$ 16.0 mM, $CaCl_2$ 2.4 mM, Procaine-HCl 1mM, pH 5.5-7) (Hearse, Stewart and Braimbridge, 1976; Chambers *et al.,* 1996). Later, based on many clinical and preclinical experimental studies and validation, a refined formulation was developed. This new solution was shown to be better than STH1 in terms of myocardial protection and antiarrhythmic effects and was called STH2 (NaCl 110.0 mM, $NaHCO_3$ 10.0 mM, KCl 16.0 mM, $MgCl_2$ 16.0 mM, $CaCl_2$ 1.2 mM, pH 7.8) (Chambers *et al.,* 1989; Ledingham *et al.*, 1987). The lower Ca^{2+} level in STH2 cardioplegic solution was thought to be the most important factor for providing better protection compared to STH1. Since then, STH2 has become the most widely used crystalloid cardioplegic solution in cardiac surgery.

1.6 Use of cardioplegia in pre-clinical models of heart perfusion

The use of pre-clinical models to test the effectiveness of cardioplegic solutions is a crucial step before tailoring these solutions for use in the human population. Many studies have been done in animal models including sheep, rabbits, dogs, rats and mice to validate the effectiveness of modified or existing cardioplegic solutions. These models are also important to understand the extent of I/R injury that happens in different age groups and sexes. Many studies have used Langendorff-perfused heart models as described below.

1.6.1 The Langendorff perfusion- retrograde heart perfusion

In 1895, Oscar Langendorff, a German physiologist, established a technique to retrogradely perfuse isolated mammalian hearts to explore heart biology. Later, this method was widely used in pre-clinical studies to test pharmacological compounds, induce I/R injury and assess cardiac performance. This technique is known as 'retrograde heart perfusion' because the heart is perfused by cannulating the ascending aorta, where the buffer solution flows in a direction that is opposite to normal physiologic flow (Bell *et al.,* 2011). This retrograde flow down the aorta closes the aortic valve leaflets under pressure and prevents perfusion fluid from entering the left ventricle. Then, the coronary arteries get perfused with buffer entering the vasculature through the left and right coronary ostia at the aortic root. After passing through the coronary vasculature, the perfusate is drawn off in the right atrium via the coronary sinus (Bell *et al.,* 2011; Skrzypiec-Spring *et al.,* 2007).

Retrograde heart perfusion can be done using either constant pressure or constant flow techniques. In constant pressure mode, constant hydrostatic pressure is maintained by positioning a reservoir, with buffer solution, at a known distance above the perfusion cannula. Another method of maintaining constant pressure is by monitoring the sealed pressurized chamber (pressurized with carbogen (95% O_2 and 5% CO_2)) with the buffer solution, where the pressure at the aorta is measured by transducer placed just above it (Bell *et al.,* 2011). By contrast, in constant flow mode, a peristaltic roller-pump is used to maintain a consistent coronary flow. This method is primarily used to study coronary vascular tone, smooth muscle and endothelial function. In this latter mode of perfusion, along with the constant flow rate, perfusion pressure can be measured using a pressure transducer. This allows calculation of coronary vascular resistance during constant flow

(Bell *et al.,* 2011). Hence, different modes of perfusion are selected based on the study objectives.

With the Langendorff-perfused heart model, many cardiac parameters can be measured. Coronary flow rate, myocardial contractility (LV systolic and diastolic function), HR, temperature and electrocardiogram can be recorded. The major advantages of using this method of heart perfusion are: reproducibility, lower cost and most importantly, isolation of the heart from other organ systems that might influence the heart's performance. Thus, this isolation technique makes it easy to study the effect of drugs on the heart without effects on other bodily systems. However, isolating the heart (*ex-vivo*) can also be seen as a disadvantage of this model, as it takes it away from the clinical relevance. However, this model is widely used in pre-clinical studies for drug development or to study cardiac function in response to different interventions.

1.6.2 Cardioplegia in animal heart models

Several existing, modified or new cardioplegic solutions have been studied in various animal heart models. Minasian *et al.* (2013) studied the effectiveness of new Krebs-Henseleit buffer-based cardioplegic solution against STH2 in Langendorff-perfused adult male rat hearts. They suggested that, at normothermic (37ºC) and moderate hypothermic (22ºC) temperatures, Krebs-Henseleit buffer-based cardioplegia might be superior to STH2 in protecting male hearts after I/R insult (Minasian *et al.*, 2013). Other studies used del Nido (24 KCl, 7$MgCl_2$) cardioplegic solution in a rat heart model to determine whether it was better than standard cardioplegic solution (18 KCl, 5 $MgCl_2$) (Govindapillai *et al.,* 2013; Govindapillai *et al.*, 2016). In these studies, the dosing intervals (single bolus dose vs. multiple doses) were also compared to determine the most

effective protocol for the cardioplegia delivery. Results showed that del Nido solution was better than standard solution in protecting old male rat hearts and a single bolus dose administered prior to ischemia was found to be more beneficial than multi doses (Govindapillai *et al.,* 2013; Govindapillai *et al.*, 2016).

Another study used a cardiopulmonary bypass model to compare tepid (28ºC) modified full blood cardioplegia with cold crystalloid cardioplegia in male piglet hearts (Munch *et al.,* 2015). They showed no significant variations in cardiac markers (troponin I, myoglobin) and cardiac output after coming off bypass between tepid blood and cold crystalloid cardioplegia (Munch *et al.,* 2015). Brown *et al.* (1993) also looked at the variability in recovery of cardiac functions after 60 min of ischemia using cold crystalloid, cold blood and warm blood cardioplegic solutions in an adult dog cardiopulmonary bypass model (sex not specified). They concluded that warm blood cardioplegia had important advantages over the two other widely used clinical hypothermic protection techniques, being cold blood and cold crystalloid. In contrast, Baretti *et al.,* (2002) demonstrated in a pig cardiopulmonary bypass model that no biochemical or functional disturbances were seen with cold blood cardioplegia in adult male pig hearts, but tepid cardioplegia was associated with arrhythmias. A study done in adult male rat hearts using cold vs. warm Calafiore blood cardioplegia showed only mild differences in cardiac function between the two groups (Boning *et al.,* 2014). Therefore, despite similar modes of delivery of cardioplegia, differences were observed in various experimental models. This suggests that the available cardioplegic solutions do not show consistent results.

Most of these studies have used young adult male animals to determine the effectiveness of cardioplegic solutions by modifying either the composition or other parameters (like temperature and dosing intervals) of cardioplegia. Along with these manipulations of cardioplegic solutions, another important strategy to potentially enhance the protective properties of cardioplegia could be adding pharmacologic agents to these solutions.

1.7 Incorporating pharmacologic agents in cardioplegia

Despite advances in enhancing the benefits of cardioplegic solutions as described above, myocardial protection in high risk patients is still sub-optimal (Hausenloy *et al.,* 2012). Therefore, other strategies, like incorporating pharmacologically active adjuncts, are now being used to improve outcomes following cardiac surgery. Agents that block the sodium channels, reduce oxidative stress or close the mitochondrial permeability transition pore (mPTP) have been used to enhance the protective effects of cardioplegia. Some of the work using these potentially beneficial agents is discussed below.

1.7.1 Inhibition of either NCX or Na+ channels in myocardial protection

Myocardial I/R injury occurs when Ca^{2+} accumulates in the cardiomyocyte causing hypercontracture and mitochondrial dysfunction (Steenbergen *et al.*, 1990; Allen *et al.*, 1993; Baines, 2009). Thus, inhibition of either NCX or Na^{+} channels has been used as an appropriate strategy to reduce Ca^{2+} overload and prevent ischemic damage. Clinical studies done in paediatric patients undergoing cardiac surgery have shown that adding lidocaine (a Na^{+} channel blocker) to cardioplegic solutions is protective for paediatric

hearts (O'Brien *et al.,* 2009). O'Brien and colleagues also looked at the young adult male rat cardiomyocytes to study the effect of lidocaine incorporation in cardioplegia. In rat cardiomyocytes as well, lower intracellular Ca^{2+} and fewer spontaneous contractions were observed when treated with lidocaine-incorporated cardioplegia compared to standard cardioplegia (O'Brien *et al.,* 2009). Another study that added SEA0400 (an NCX inhibitor) to a blood-based cardioplegic solution showed attenuation of myocardial injury and improvement of LV function in Langendorff-perfused young male rat hearts (Egar *et al.,* 2014). SEA0400 also reduced the accumulation of intracellular Ca^{2+} during ischemic arrest in cardiomyocytes from male hearts (Egar *et al.,* 2014). These experiments suggest that inhibiting either Na^+ channels or the NCX is an effective approach to limit I/R injury in cardiac surgery. However, whether these agents are beneficial in both sexes and in older hearts have not been investigated.

1.7.2 Inhibition of the Na^+/H^+ exchanger in myocardial protection

Intracellular acidosis in ischemia promotes Na^+/H^+ exchange to remove H^+ in exchange for Na^+, which causes Na^+ accumulation and activates NCX. Activation of NCX brings Ca^{2+} ions into the cell and removes Na^+ ions, resulting in Ca^{2+} overload and I/R injury. Based on this rationale, inhibition of Na^+/H^+ exchange was thought to be a good strategy for cardioprotection. Indeed, studies have shown that inhibition of the Na^+/H^+ exchanger reduces Ca^{2+} overload and attenuates damage in ischemia (Karmazyn, 1999). In addition, Na^+/H^+ exchange inhibitors (dimethyl amiloride, HOE 694, cariporide) used in Langendorff-perfused hearts from young adult rats and rabbits improve recovery of left ventricular developed pressure (LVDP) and reduce myocardial creatine kinase release when added in cardioplegia (Koike *et al.,* 1996; Choy *et al.,* 1997;

Kevelaitis *et al.,* 2005). Although, Na^+/H^+ exchanger inhibitors are cardioprotective in cardioplegia, most pre-clinical studies are limited to young adult animals, without specifying their sex. Thus, further studies are required to see if results can be extrapolated to older animals of both sexes.

1.7.3 Modulation of K^+ channel in myocardial protection

A common protective mechanism that is employed during cardiac surgery using cardioplegia is to hyperpolarize the membrane to inhibit action potentials and prevent contractions. Increasing the K^+ current through K^+ channels will hyperpolarize the membrane, limiting depolarization and reducing Ca^{2+} accumulation (Wu *et al.*, 2013). A study in the isolated adult male rat hearts shows improved recovery of LVDP with cardioplegia supplemented with rottlerin, Ca^{2+} activated large conductance K^+ channels opener, compared to hearts perfused with cardioplegia alone (Clements *et al.,* 2011). Wu and colleagues (2013) also found that incorporation of the K^+ channel opener zacopride in cardioplegia improved LVDP and led to smaller infarcts in adult male rat hearts. However, one study showed age and sex-specific results with K^+ channel modulators, where older hearts had minimal protection, particularly in old females (McCully *et al.,* 2006). These observations suggest that the protective effects of K^+ channel modulators are limited to specific populations, although additional work would be beneficial.

1.7.4 Other pharmacologic agents used in myocardial protection

In addition to the above studies, other potential therapeutic agents that may enhance the protective properties of cardioplegic solutions have been investigated. For example, beta-blockers, like esmolol, when added to cardioplegia improved cardiac function in a

pig cardiopulmonary bypass model (Dahle *et al.,* 2015). Trescher *et. al.* (2015) concluded that adding the nitric oxide donor, S-nitroso-human serum albumin to cardioplegia improved post-ischemic myocardial perfusion and enhanced contractile function in Langendorff-perfused adult male rat hearts.

Hence, many pharmacologic agents could, in theory, be beneficial in enhancing the protective properties of cardioplegic solutions. However, virtually all the studies with potential protective agents have not investigated age and sex-specific differences that might occur. In this list of potentially advantageous pharmacologic agents, another drug that directly targets a membrane receptor present in the heart and may be beneficial in cardioplegia is G1, which is an acute GPER agonist. The case for the use of GPER agonists as adjunct agents in cardioplegia is argued below.

1.8 Targeting GPER for cardioprotection

Several animal studies have shown that estrogen has protective effects on cardiovascular function in the setting of ischemia (Wang *et al.,* 2006; Deschamps and Murphy, 2009; Bopassa *et al.*, 2010; Murphy and Steenbergen, 2007). Even in human studies it has been shown that premenopausal women have a lower risk of developing CVD than their male counterparts, although this advantage disappears after menopause (Barrett-Connor,1997). Two clinical trials, the Women's Health Initiative (WHI) and the Heart and Estrogen/Progestin Replacement Study, reported that estrogen replacement therapy did not reduce the incidence of coronary heart disease in postmenopausal women (Anderson *et al.,* 2004; Hulley *et al.,* 1998). On the other hand, the Kronos Early Estrogen Prevention Study concluded that estrogen/progesterone treatment starting soon

after the menopause (at least 6 mos but not more than 36 mos after the onset of menopause) was safe and improved markers of CVD risk (Harman *et al.,* 2005; Harman *et al.,* 2014). They argued that early initiation of hormone therapy delays CVD in women. By contrast, the average age of the women in the WHI trial was 63 years, which was 12 years after the average age of menopause. This time difference in initiating hormone therapy could account for the lack of efficacy of estrogen in the WHI study (Wharton *et al.,* 2013). Further animal studies using estrogen or targeting ERs would be helpful to understand the underlying mechanism of estrogen's cardioprotection before tailoring it for use in humans.

1.8.1 Estrogen and ERs

Estrogen is a sex hormone primarily produced in the ovaries in females and testes in males. It is also synthesized by the aromatase enzyme in peripheral tissues in both sexes, (*e.g.* adipose, liver and vascular endothelium) at comparatively lower levels (Barakat *et al.,* 2016; Lee *et al.*, 2012). Estrogen plays an important role not only in the reproductive system but also in the immune, nervous and cardiovascular systems (Filardo and Thomas, 2012). These biological functions of estrogen are mediated by ERs. There are primarily three ERs, namely, ERα, ERβ and GPER. ERα and ERβ are the two isoforms of nuclear ERs that mediate genomic responses. More recently, GPER has been identified. GPER can directly bind estrogen and is known to mediate rapid, non-genomic actions of estrogen (Albanito *et al.,* 2007; Sirianni *et al.,* 2008; Noel *et al.,* 2009). However, the localization of GPER is still in debate. Revankar *et al.,* (2005) showed that GPER is primarily localized to the endoplasmic reticulum. By contrast, Filardo and colleagues (2007) reported the presence of GPER in the plasma membrane. Despite

uncertainty about its localization, GPER has been shown to initiate cellular signalling upon activation by estrogen.

GPER was formerly known as the G-protein coupled orphan receptor (GPR30), as it was not clear whether estrogen was the ligand that bound to this G-protein coupled receptor. Filardo *et al.* (2000) identified estrogen as a ligand for GPR30. They showed that estrogen stimulated SKBR3 breast cancer cells, which did not express ERα and ERβ, by activating extracellular signal-regulated kinases-1 (ERK-1) and ERK-2 mitogen activated protein kinases. Hence, after identification of the ligand for GPR30 (by then called, GPER) and its distribution in the body, further studies were done to determine the potential roles of GPER in different organ systems (Prossnitz and Barton, 2011). To investigate the functional significance of GPER using *in vivo* and *in vitro* models, Bologa and colleagues (2006) identified the first highly specific (Ki = 11 nM) GPER agonist, G1. This agonist exhibited little to no binding affinity for ERα and ERβ. Thus, G1 has been widely used in experimental animal studies to explore the effects of GPER activation.

1.8.2 G1 in cardioprotection

The highly specific GPER agonist, G1, has been extensively used in cardiovascular studies for its cardioprotective properties. As GPER is present in both human and animal hearts, studies were conducted using G1 to determine whether it had cardioprotective effects (Patel *et al.,* 2010). In 2009, Deschamps and Murphy demonstrated that GPER was present in adult male and female rat hearts in similar amounts (Deschamps and Murphy, 2009). They also concluded that G1 improved functional recovery and reduced infarct size in isolated rat hearts subjected to 20 min of global ischemia and 120 min reperfusion (Deschamps and Murphy, 2009). Also, in

H9C2 myocardial cells subjected to I/R injury, G1 reduced apoptosis and significantly increased superoxide dismutase and ATP levels (Li *et al.,* 2015). Another study using isolated Langendorff-perfused mouse hearts reported better functional recovery with G1 following I/R (Bopassa *et al.*, 2010) compared to control. Hence, treating hearts with G1 was found to be cardioprotective in the setting of global ischemia.

To elucidate the effects of G1 through activation of GPER, GPER antagonists, like G15 and G36 have been used in different animal models (Francesco *et al.,* 2013; Evans *et al.,* 2016; Ashton *et al.,* 2015). Studies have reported that different underlying mechanisms are associated with the cardioprotective properties of G1. Bopassa *et al.* (2010) have shown that G1 exhibited cardioprotective effects by inhibiting Ca^{2+}-induced mPTP opening. The mPTP opening occurs in response to Ca^{2+} overload, oxidative stress and ATP depletion (Bopassa *et al.,* 2010). These conditions occur when the heart is subjected to I/R injury. Bopassa and colleagues showed that isolated mitochondria from G1 treated young male mouse hearts had faster Ca^{2+} uptake rate and required increased number of Ca^{2+} pulses for mPTP opening compared to control hearts. They also suggested that the inhibition of mPTP opening by G1 might be through activation of the ERK signalling pathway, as the effects of G1 were blocked by PD-98059 (ERK pathway inhibitor) (Bopassa *et al.,* 2010). However, another study showed that G1-mediated cardioprotection was due to activation of phosphatidylinositol 3-kinase (PI3K) signalling pathway and suggested that ERK activation may not contribute to G1-induced protection (Deschamps and Murphy, 2009). They demonstrated that inhibiting MEK (mitogen activated protein kinase) which is upstream of ERK, did not affect G1-mediated cardioprotection (Deschamps and Murphy, 2009). GPER activation is also associated

with intracellular Ca^{2+} mobilization (Revankar *et al.,* 2005). Ullrich *et al.* (2008) showed that estrogen inhibited L-type Ca^{2+} current in ventricular myocytes from mouse hearts, independent of ERα and ERβ activation. This suggests that G1 via GPER activation could also inhibit L-type Ca^{2+} current and prevent Ca^{2+} overload via Ca^{2+}-induced Ca^{2+} release from SR (Ullrich *et al.,* 2008). However, studies done so far have been limited to a specific age group and sex, largely young adult males. Therefore, further investigation is required to determine the age- and sex-specific effects of G1 in animal models. The work reported in this thesis will investigate the impact of G1 in a Langendorff-perfused mouse heart model of cardioplegia in young adult and aged mice of both sexes.

1.9 Objectives and Hypothesis

The existing literature suggests that the GPER agonist, G1, has beneficial effects in the setting of acute I/R in the mouse heart. However, whether G1 is beneficial in aged hearts and in hearts exposed to the longer ischemic periods experienced in cardiovascular surgery has not yet been investigated. In addition, existing cardioplegic solutions used during cardiovascular surgeries provide sub-optimal cardioprotection that may differ at different ages and between the sexes (Ascione *et al.,* 2002). Therefore, the specific objectives of this study were:

1. To determine male-female differences in the recovery of hearts from variably aged mice after hearts are exposed to I/R injury.
2. To determine age-specific differences in the recovery of hearts from both sexes after hearts are exposed to I/R injury.

3. To enhance the protective properties of a traditional (classic) cardioplegic solution to maximize recovery in hearts from variably aged mice of both sexes.

The hypothesis to be tested in this study is: “The GPER agonist, G1, enhances the protective properties of traditional cardioplegic solutions across a wide age range in both sexes.”

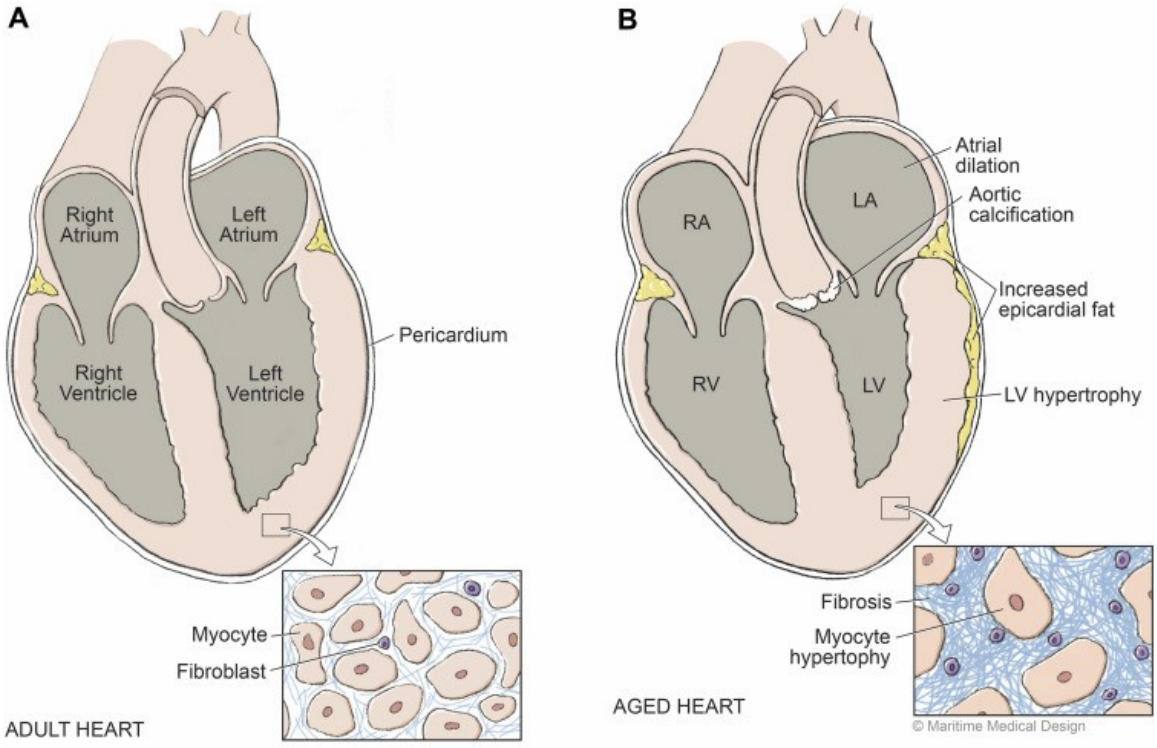


Figure 1.1 Age-dependent changes in the structure of the heart. (A) Adult heart. **(B)** Aged heart. There are significant numbers of structural changes observed in the aged heart compared to adult heart. Epicardial fat deposition, atrial hypertrophy and dilatation, ventricular hypertrophy and calcification of the aortic valve are the major structural changes seen in the aged compared to adult heart. At the cellular level, distinct loss of ventricular myocytes and hypertrophy of the remaining cells are observed. Also, there is an increase in the number of fibroblasts with age and marked increase in fibrosis. This was taken from Keller and Howlett (2016) with permission from the illustrator Monique Guilderson.

CHAPTER 2: METHODS

2.1 Animals

All animal care and handling procedures were approved by the Dalhousie Committee on Laboratory Animals and followed the guidelines provided by the Canadian Council on Animal Care (CCAC, Ottawa, ON: Vol 1, 2nd edition, 1993; revised March 2017). All studies that involved animals were reported according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (Kilkenny *et al.,* 2010). Male and female C57BL/6 mice were obtained from Charles River Laboratories (St. Constant, QC, Canada) and The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in micro-isolator cages in the Carleton Animal Care Animal Facility at Dalhousie University. These mice were allowed a minimum of one week to acclimatize on a light/dark cycle (07:00 - 19:00/19:00 - 07:00). Animals were given free access to food (Prolab Rat/Mouse/Hamster 3000, LabDiet, St. Louis, MO, USA) and water. At least 30 min prior to each experiment, a mouse was transported in a clean cage to the laboratory.

2.2 Isolation of the mouse heart

To isolate the mouse heart, first the mouse was anaesthetized with an intraperitoneal injection of sodium pentobarbital (220 mg/kg) co-administered with heparin (3000 U/kg) to prevent blood clotting. Corneal reflexes and pedal withdrawal were tested before any incision was made. Mouse forelimbs were secured with the help of clamps. The skin was first swabbed with ethanol and then a longitudinal incision was made in the chest to expose the thoracic region. The diaphragm was cut to separate it from the ribs. Then lateral cuts were made on either side of the rib cage parallel to the

sternum and this was folded towards head to expose the heart. The pericardium and connective tissues were removed to clearly locate the aorta. A suture was loosely tied around the aorta and a precise cut was made across the arch of the aorta. Immediately, a cannula, previously primed with perfusate, was inserted and the aorta was tightly secured with a silk suture (4-0; American Cyanamid Company, NY, USA). Care was taken not to insert the cannula too deep in the aorta, which could disrupt the aortic valve and interfere with coronary perfusion. The cannulated heart was then isolated from the body and mounted in the Langendorff setup.

2.3 The Langendorff heart perfusion

2.3.1 Setup

A Langendorff constant pressure setup (ADInstruments Inc., Colorado, USA) was used for retrograde perfusion of the mouse heart via the aorta. As shown in **Figure 2.1**, this setup consisted of a water-jacketed reservoir, bubble traps, tubing and a jacketed heart chamber. The setup also featured a thermal circulating water-bath, pressure transducers and an aortic cannula. The perfusate in the reservoir was bubbled with carbogen (95% O_2 and 5% CO_2) and was allowed to flow through a bubble trap, which was then routed towards the aortic cannula. A circulating water-bath was used to maintain the temperature of the system at 37ºC by circulating warm water throughout the water-jacketed system. Perfusate flow pressure was measured with the aortic pressure transducer. Perfusate flow pressure was adjusted as required by controlling the carbogen pressure in the reservoir. Developed pressure in the left ventricle was measured with a LV pressure transducer connected to a custom made ventricular balloon. A temperature

probe was connected near the aortic cannula to record myocardial temperature directly from the cannulated heart.

2.3.2 Perfusion and stabilization of the mouse heart

Following successful cannulation of the aorta, the heart was perfused with Krebs-Henseleit buffer solution **(Table 2.1)**, which was equilibrated with 95% O_2 and 5% CO_2, at a constant pressure of 80 ± 1 mmHg. The left atrium was removed to insert the custom made balloon, prepared from GLAD® Cling Wrap (The Clorox Company of Canada LTD., Brampton, ON, Canada), into the left ventricle through the mitral valve. The balloon was then inflated with degassed distilled water to yield a minimum LV pressure of 10.2 ± 0.5 mmHg. The myocardial temperature was maintained at 36.3 ± 0.2ºC. The heart was allowed to stabilize for about 5 mins before baseline measurements were recorded.

2.4 Perfusion protocol

The overall experimental protocol is illustrated in **Figure 2.2**. Hearts were perfused with Krebs-Henseleit buffer for 15 min and baseline measurements of LVDP, HR, rate of pressure rise (+dP/dt) and rate of pressure decay (-dP/dt) were recorded. After the baseline perfusion, the heart was perfused with STH2 cardioplegia **(Table 2.2)** at 6-9ºC, which arrested the heart immediately. Hypothermia was maintained by using an ice cold water bath and tubing jacketed with ice cold water. Maintenance of hypothermia was important, as it is associated with lower O_2 demand and reduced basal energy requirement of the myocyte (Ascione *et al.,* 2002). During cardioplegic perfusion, the aortic pressure was maintained at 80 mmHg by altering the flow rate of the solution using

a peristaltic pump. The cardioplegic perfusion lasted for 6 min to model the approach used clinically (Whittington *et al.,* 2016; Liu *et al.,* 2008). Following cardioplegic arrest, the heart was subjected to 90 min of global ischemia where it was submerged in cardioplegia at room temperature (23 - 24ºC) as done clinically (Kim *et al.,* 2014). A single initial dose of cardioplegia was preferred over multiple doses because studies have shown that a single dose of cardioplegia is sufficient to protect the heart if ischemia does not exceed 90 min (Sorabella *et al.,* 2014; Whittington *et al.,* 2016; Timek *et al.,* 2016). Subsequently, the heart was reperfused with Krebs-Henseleit buffer (~37 ºC) and parameters including LVDP, HR, +dp/dt and –dp/dt were measured to determine the extent of recovery. Myocardial temperature was recorded throughout the experiment with the help of a temperature probe at the myocardial surface. The coronary effluent was collected during baseline (10 min and 15 min), after cardioplegic perfusion (21 min) and at several times after reperfusion (121 min, 131 min and 141 min) to determine the concentration of cardiac troponin I (cTnI) released, as described in section 2.8. These coronary effluent samples were immediately frozen in liquid nitrogen and then stored at -80ºC until analysis.

2.5 Cardioplegia preparation and delivery

STH2 cardioplegia was prepared in our laboratory. The chemical composition of this crystalloid cardioplegia was matched with standard cardioplegia used in clinical practice as shown in **Table 2.2** (Ledingham, Braimbridge, Hearse, 1987)**.** The solution was cooled in an ice bath (~ 3-4ºC) and then pH was maintained at 7.8 to counterbalance anaerobic metabolism caused by ischemia (Brzozowski, 1995). Drugs (G1 / G15 / G36)

were added to the cardioplegic solution as required. This hypothermic solution was then delivered to heart with the help of a peristaltic pump (ADInstruments Inc., Colorado, USA), which pumped the solution through a Millex®-HV 0.45 µm filter unit that was changed after each experiment (Merck Millipore ltd., Co. Cork, Ireland). The solution was then passed, via a bubble trap, to the aortic cannula. The aortic pressure was maintained at 80 mmHg by controlling the flow rate of the cardioplegic solution using the peristaltic pump.

2.6 Administration of G1 (or G15 / G36)

A stock solution of G1 (Cayman Chemical Company, Michigan, USA) was prepared by dissolving 1 mg of G1 in 1 ml of anhydrous DMSO to obtain a final concentration of 2.4 mM. Similarly, stock solutions of G15 and G36 (Cayman Chemical Company, Michigan, USA) were prepared by adding 1 ml of anhydrous DMSO to 1 mg of G15/G36 to obtain 2.7 mM (G15) and 2.4 mM (G36) stock solutions. These solutions were dispensed in multiple aliquots and stored at -20ºC until use. G1 and/or G15/G36 were incorporated in cardioplegia and delivered to the heart during the 6 min of cardioplegia perfusion. A final concentration of 0.05% DMSO was maintained in each experimental group including control experiments to control for solvent effects. Three different concentrations of G1 (110 nM, 500 nM and 1 µM) were used to determine the effective concentration for functional recovery of hearts (Deschamps and Murphy, 2009; Bopassa *et al.,* 2010; Skrzypczak *et al.,* 2013). For the antagonist experiments, a 1 µM concentration of G15 or G36 was used with 500 nM G1 (Evans *et al.,* 2016).

2.7 Parameters measured during experiments

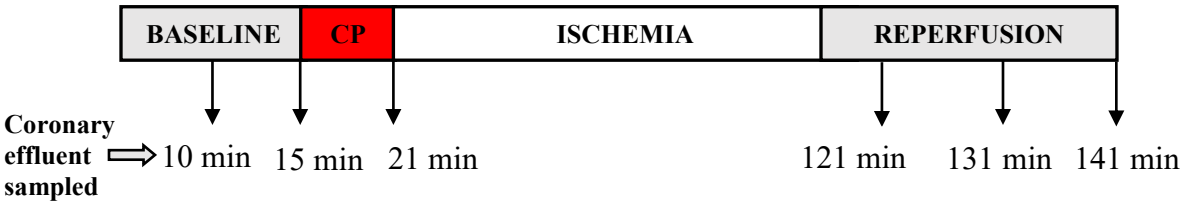
Different cardiac functional parameters were measured and analysed using LabChart Pro v.8 software (ADInstruments Inc., Colorado, USA). **Figure 2.3** shows a representative experimental recording of pressure as a function of time. The values for each of the cardiac parameters measured were averaged over a 10 sec recording period at different time-points. The parameters measured were as follows:

- **LVDP:** LVDP was calculated by subtracting the end diastolic pressure (EDP; minimum pressure maintained by inflating LV balloon) from the systolic pressure (maximum pressure obtained). It was expressed in mm Hg.
- **HR:** The number of contractions of the heart per minute was calculated from the LV pressure waves. It was expressed as beats per minute, where a beat refers to one complete cycle (systole and diastole) of the heart.
- **+dP/dt and -dP/dt:** +dP/dt and –dP/dt were calculated by measuring the steepest slope during the upstroke and the downstroke of the LV pressure recording. They were expressed in mm Hg/sec.
- **Rate pressure product (RPP):** RPP was calculated by multiplying LVDP and HR, which was then divided by the respective heart weight. It was used to determine myocardial work load and expressed in mm Hg.bpm/mg.
- **Contracture:** This was measured by calculating the rise of EDP during reperfusion from the initial level seen in baseline perfusion of the heart. It was expressed in mm Hg.
- **Coronary flow rate:** The coronary flow rate was measured by collecting effluent samples in a 5 ml graduated cylinder during baseline and reperfusion

periods of the experiment. The volume of flow per minute was calculated and expressed in ml/min.

2.8 Measuring release of cTnI in the coronary effluent

The amount of cTnI released in the coronary effluent samples was used as an index of myocardial damage during ischemia and reperfusion of the heart (Chocron *et al.,* 1996). Coronary effluent samples were collected at different time points throughout the experiment, as shown below:



These samples were immediately frozen in liquid nitrogen after they were collected. Then they were stored at -80ºC in the freezer until they were analyzed. Effluent samples from each time point were taken from both male and female hearts to measure cTnI release throughout the experiment, as shown in **Figure 2.4**. It is evident from **Figure 2.4** that cTnI release reached a plateau between 131 and 141 min. Therefore, samples from the 141 min time-point in reperfusion were used from each heart (adult male and female; old male and female) for detailed analysis. These samples were analyzed using a high sensitivity mouse cTnI ELISA kit (Life Diagnostics, Inc., West Chester, PA), following the procedures outlined in the kit.

2.9 Measurement of infarct area

2.9.1 Staining viable tissue with Triphenyltetrazolium chloride (TTC)

The extent of myocardial infarction caused by ischemia and reperfusion was measured by staining the hearts with TTC (Sigma-Aldrich, Okaville, ON, Canada). TTC is reduced to triphenyltetrazolium formazan in the mitochondria of viable cells, imparting a deep red color, whereas the non-viable cells remain unstained (pale pink/white area), as shown in **Figure 2.5.**

Following reperfusion, the balloon was removed from the LV and the cannula, with the heart tied on, was taken off the Langendorff apparatus. This cannula was connected to a 10 ml syringe filled with 1% TTC solution (0.1 g TTC in 10 ml Krebs-Henseleit buffer solution). Then the heart was perfused with TTC solution manually at an approximate flow rate of 5 ml/min. After that, the heart was detached from the cannula and placed in a beaker containing the excess perfusate that dripped from the heart during perfusion. The heart was then incubated in a C24 benchtop incubator shaker (New Brunswick Scientific CO., Classis, series, Enfield, CT, USA) with the TTC solution at 37^{o}C for 45 minutes. Following incubation, the heart was preserved in 10% formalin solution (Sigma-Aldrich, Okaville, ON, Canada) and stored for at least 48 hours for fixation.

2.9.2 Analysing the infarct area

The hearts stored in formalin were evenly sliced (1 mm in thickness) with razor blades by placing the hearts in a heart slicer matrix (Zivic Instruments, Pittsburgh, PA, USA). Images from both sides of each slice were taken using a light microscope (Stemi 2000 C, Carl Zeiss Microscopy, Thornwood, NY, USA). The infarct area was then

quantified by computerized planimetry with Adobe Photoshop 8 CS (Adobe System Incorporated, USA) and ImageJ 1.50i (National Institutes of Health, USA). From every heart, both sides of each of the slices were then analyzed. The total infarct area was calculated and expressed as a percentage of the total area of the heart.

2.10 Experimental groups

The animals were divided into different experimental groups considering two factors: the age and the sex of the animal. Animals between 6-9 mos were considered adult and those between 22-28 mos were considered old. **Table 2.3** shows the number of animals used in the different experimental groups, considering these factors. Additionally, the mean body weight for adult female and male mice were 29.6 ± 0.8 g and 31.5 ± 1.5 g respectively. Whereas, for the old female and male mice the weights were 36.8 ± 1.2 g and 35.2 ± 1.5 g respectively.

2.11 Statistics

All the data were analyzed and plotted using SigmaPlot 11.0 (Systat Software, Inc., Point Richmond, CA, USA). To determine the statistical significance between different experimental groups, functional parameters such as LVDP, +dP/dt, -dP/dt, HR and RPP were evaluated using two-way repeated measures ANOVA with a Holm-Sidak post-hoc test. Infarct area, coronary flow rate and cTnI release between two groups (control vs. G1) were analysed using unpaired t-tests (also known as Student's t-test). One-way ANOVA was used, when control, G1 and G1+G15 treated hearts were

compared for cTnI release and coronary flow rate. All the data are presented as the mean ± SEM and differences were reported as significant if $p < 0.05$.

Table 2.1 Chemical composition of Krebs-Henseleit buffer solution.

Chemical	Concentration (mM)
NaCl	91.3
KCl	4.7
$NaHCO_3$	25
$Mg.SO_4$	1.2
KH_2PO_4	1.2
Glucose	11
Na-pyruvate	0.79
$CaCl_2$	1.8
pH of the solution = 7.4 (Oxygenated with 95% O_2 + 5% CO_2)	

Table 2.2 Chemical composition of St Thomas' II cardioplegia.

Chemical	Concentration (mM)
NaCl	110
$NaHCO_3$	10
KCl	16
$MgCl_2$	16
$CaCl_2$	1.2
pH to 7.8 with HCl	

Table 2.3 Total number of mice used in different experimental groups.

Experimental Groups	Adult (6-9 mos)		Old (22-28 mos)	
	Female (n)	*Male (n)*	*Female (n)*	*Male (n)*
Control (STH2 + DMSO)	6	6	5	6
Treatment I (STH2 + 110 nM G1)	4	3	-	-
Treatment II (STH2 + 500 nM G1)	6	5	5	6
Treatment III (STH2 + 1 μM G1)	3	-	-	-
Treatment IV (STH2 + 500 nM G1 + 1 μM G36)	3	-	-	-
Treatment V (STH2 + 500 nM G1 + 1 μM G15)	5	-	-	-

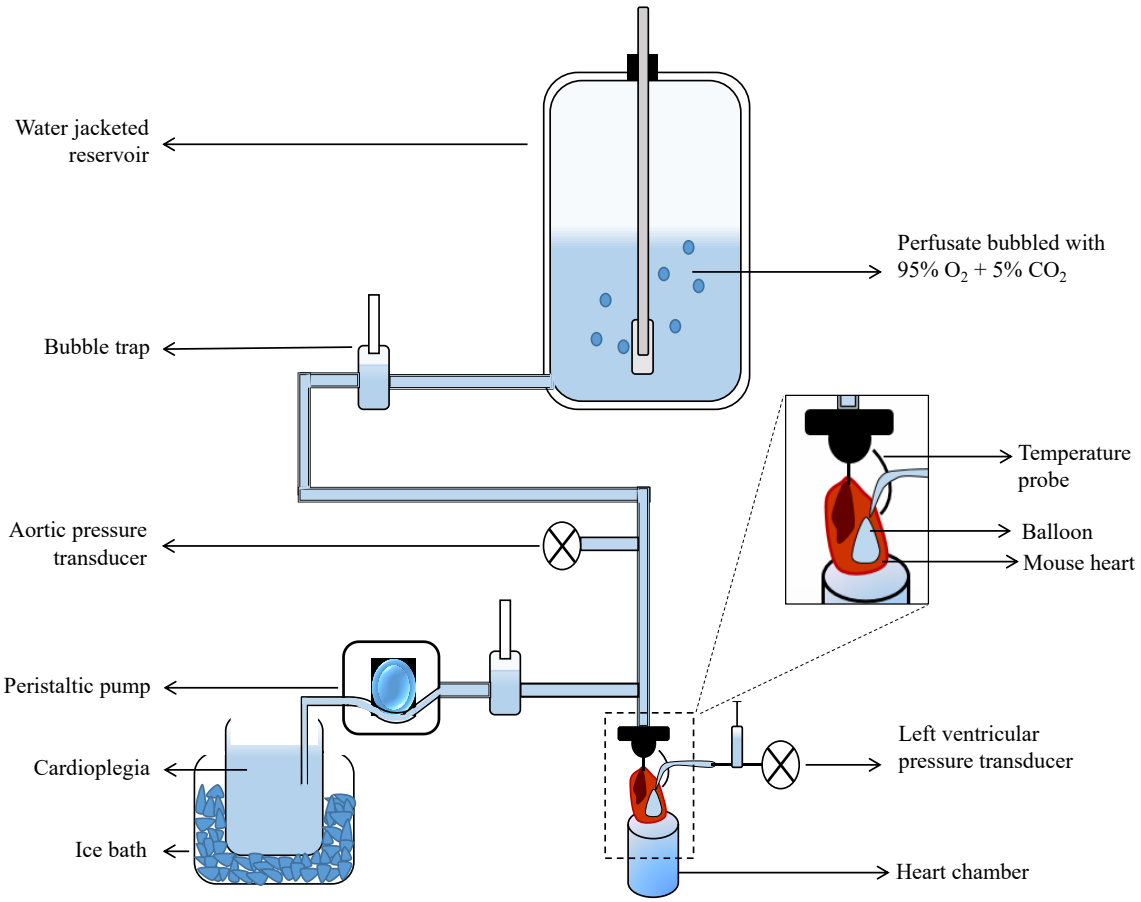


Figure 2.1 Schematic diagram of the Langendorff retrograde heart perfusion system.

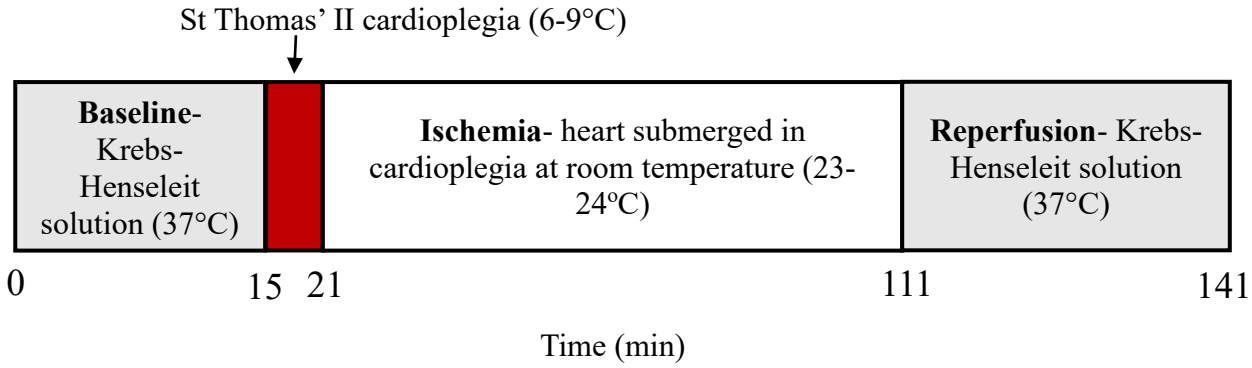


Figure 2.2 General experimental timeline. The mouse heart was first perfused with Krebs Henseleit buffer solution at 37°C for 15 min. Then the perfusion was switched to St Thomas' II cardioplegia (6-9°C) for 6 min. Following cardioplegic perfusion, the heart was subjected to 90 min of ischemia, when no solution perfused the heart and the heart remained submerged in cardioplegia at room temperature (23-24°C). Subsequently, the heart was reperfused with Krebs Henseleit solution for 30 min and recovery of heart function was recorded.

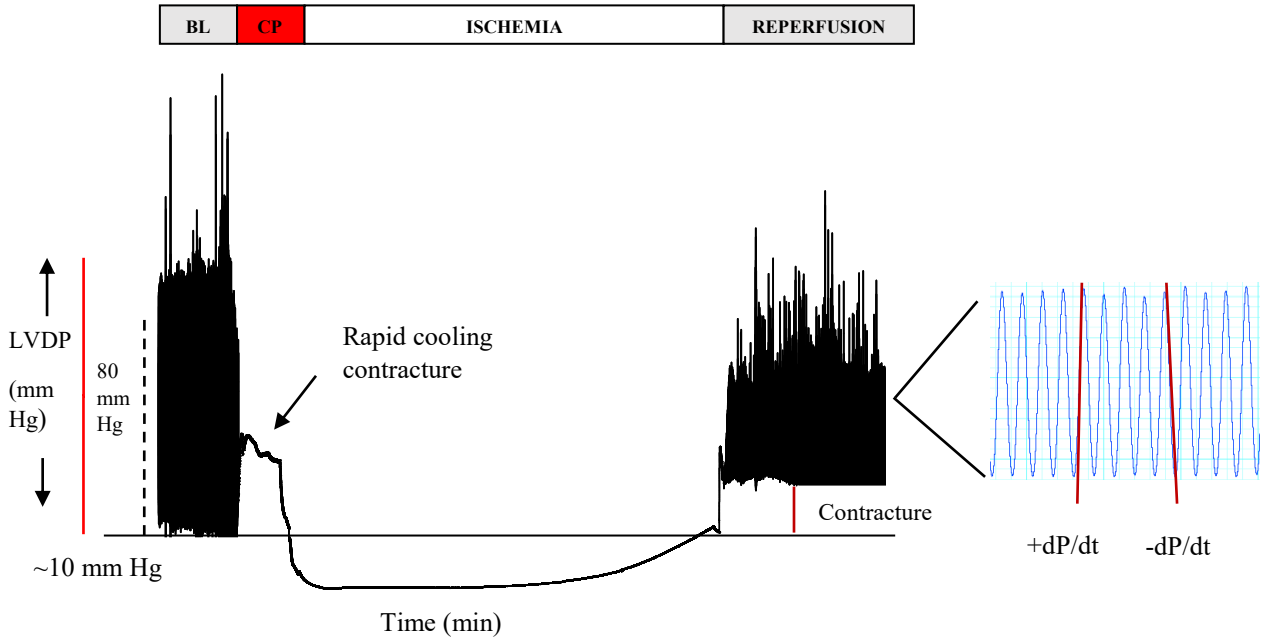
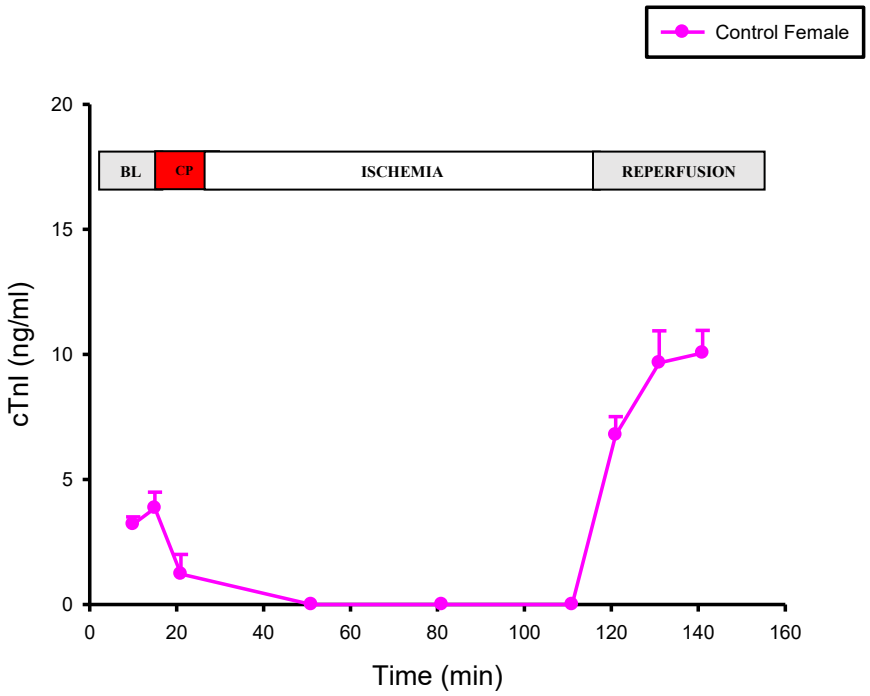


Figure 2.3 Representative experimental recording. In this trace, the baseline section (BL) showed normal cardiac function during initial perfusion of the heart. When the heart was perfused with hypothermic St. Thomas' II cardioplegia (CP), all the contractile activity ceased and a rapid cooling contracture was seen (arrow). During ischemia the heart relaxed. Subsequently, when the heart was reperfused, recovery of contractile function was observed. Here, LVDP was the total height of pressure developed (i.e systolic pressure –EDP); the steepest slope of the upstroke and downstroke of the pressure curve provided measures of +dP/dt and -dP/dt, respectively, and the rise of EDP level in reperfusion was used to measure contracture.

A.



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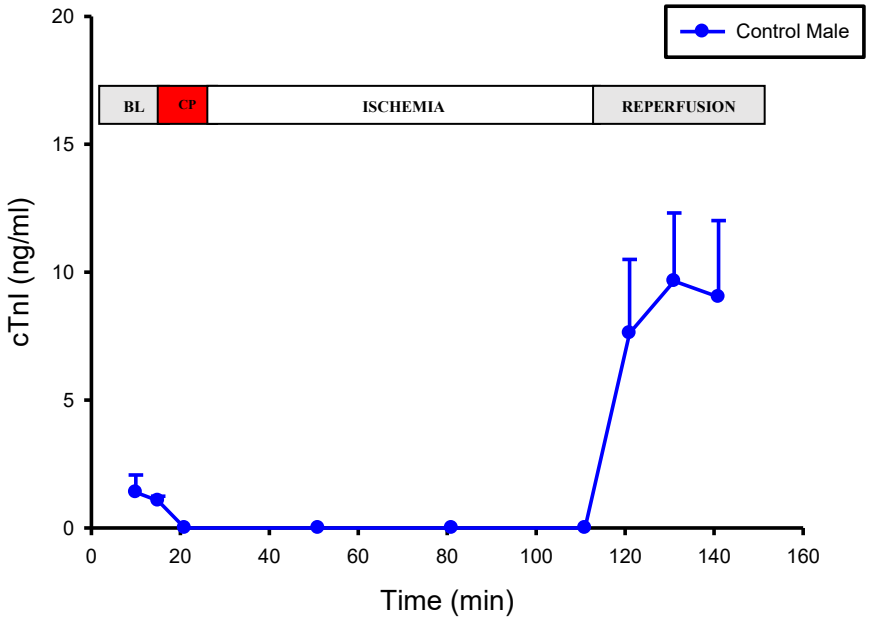


Figure 2.4 Concentration of cTnI release at different time points in male and female control hearts. cTnI release was calculated from the coronary effluent samples collected at different time points during the experiment from both female **(A)** and male **(B)** control hearts. The concentration of cTnI release was plotted against different time points to determine the optimal time for sampling in reperfusion. Control female n=4; control male n=3.

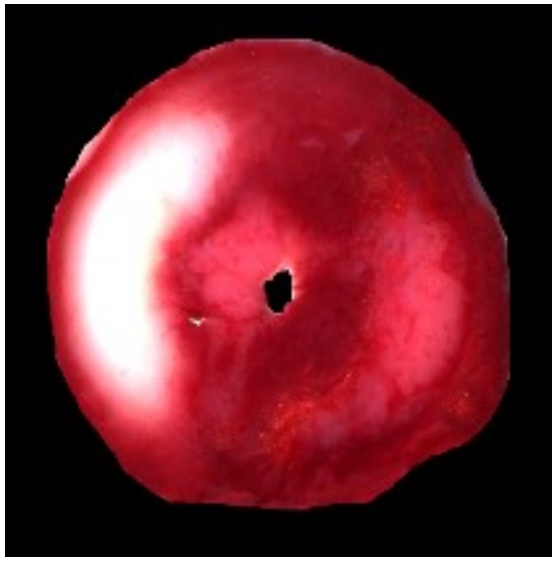


Figure 2.5 Area of infarction in the mouse myocardium. After the completion of an experimental protocol, each heart was perfused with TTC solution (TTC + Kreb's) which stained the viable tissue deep red while the non-viable (infarcted) tissue remained unstained (pale pink/white area).

CHAPTER 3: RESULTS

3.1 Development of a murine Langendorff-perfused heart model of cardioplegia

The first series of experiments was designed to develop a model of cardioplegia in the *ex vivo* mouse heart that mimicked conditions experienced in cardiac surgery. This involved modification of the existing Langendorff perfusion apparatus to closely control temperature, so that the heart temperature could be reduced rapidly to between 6-9ºC during cardioplegia (Timek *et al.,* 2016). With the help of a temperature probe, myocardial temperature was recorded throughout the experimental protocol. As shown in **Figure 3.1,** myocardium temperature was maintained at 36.3 ± 0.2ºC during baseline perfusion with Krebs-Henseleit buffer to mimic physiological temperature. Following baseline perfusion, hearts were then perfused with hypothermic cardioplegic solution (with or without drugs) for 6 min. This caused a rapid decrease in myocardial temperature to 6.5 ± 0.3ºC by the end of hypothermic perfusion. After that, hearts were subjected to 90 min of global ischemia, by submerging the hearts in cardioplegic solution to maintain the myocardium at room temperature (23-24ºC). Subsequently, hearts were reperfused with Krebs-Henseleit buffer solution for 30 min, which raised the myocardial temperature to 36.4 ± 0.1ºC. This protocol provided a reproducible mouse model of cardioplegia that showed changes in temperature similar to those experienced during cardiac surgery.

3.2 Sex differences in response to I/R in Langendorff-perfused adult mouse hearts

3.2.1 Recovery of LVDP, HR, +dP/dt, -dP/dt, RPP and contracture in adult mouse hearts

To determine whether there were sex differences in the extent of recovery of cardiac functional parameters, hearts from adult female and male mice were exposed to I/R injury. These hearts were perfused with STH2 cardioplegia (vehicle control) before ischemia. **Figure 3.2** shows representative pressure recordings from adult female and male mouse hearts at baseline and at the end of reperfusion. As shown, the contractile function recovered better in adult females than in males during reperfusion.

Next, the mean (± SEM) data were plotted as a function of time throughout the exposure to cardioplegia and reperfusion. Cardiac parameters (LVDP, +dP/dt, -dP/dt, HR and RPP) were normalized to baseline data and expressed as a percentage of mean baseline values. LVDP recovered significantly better in reperfusion in adult female hearts (50.6 ± 12.4 %) when compared to adult male hearts (31.6 ± 12.3%) as shown in **Figure 3.3 (A)**. By contrast, HR recovered completely in reperfusion regardless of sex **(Figure 3.3 B).** Similarly, recovery of +dP/dt and –dP/dt was also better in female hearts (+dP/dt = 51.7 ± 13.4%; -dP/dt = 53.9 ± 13.0% at 141 min of reperfusion) than in male hearts (+dP/dt = 33.48 ± 13.2%; -dP/dt = 34.3 ± 13.3% at 141 min of reperfusion) **(Figure 3.4)**. Also, the recovery of RPP was worse in male hearts (29.6 ± 12.1%) than in female hearts (51.9 ± 15.2%) at the end of reperfusion **(Figure 3.5)**. However, the level of contracture in reperfusion was similar in adult male and female hearts (37.7 ± 5.4 mm Hg vs 33.1 ± 7.0 mm Hg) **(Figure 3.6).** Taken together these data show that adult female hearts exhibit better recovery than adult male hearts with STH2 cardioplegia alone. However, this recovery of cardiac parameters was still suboptimal.

3.2.2 Recovery of coronary flow in adult mouse hearts

To evaluate the rate of perfusion of myocardium, the coronary flow rate was measured in mouse hearts. There was no difference in coronary flow rate when measured at baseline and in reperfusion between adult male and female mouse hearts. The values of mean flow for adult male and female hearts at baseline were 2.8 ± 0.3 ml/min and 2.9 ± 0.3 ml/min, respectively. Similarly, values of coronary flow rate for male and female hearts in reperfusion were 3.0 ± 0.3 ml/min and 2.6 ± 0.4 ml/min respectively as shown in **Figure 3.7**.

3.2.3 Differences in infarct area and concentration of cTnI released in adult mouse hearts

To determine the extent of myocardial injury in hearts subjected to I/R, the infarct area and the concentration of cTnI released were calculated. The percentage of infarct area did not differ between adult male (41.6 ± 12.1%) and adult female (33.7 ± 9.2%) mouse hearts **(Figure 3.8)**. Similarly, there was no difference in the concentration of cTnI released in effluent samples collected in reperfusion between adult male (14.4 ± 1.1 ng/ml) and female (9.4 ± 2.0 ng/ml) mouse hearts **(Figure 3.9)**.

In summary, the results of these studies of sex differences in adult mouse hearts show that female hearts had better recovery of contractile function (LVDP, +dP/dt, -dP/dt and RPP) than male hearts in reperfusion. However, there were no differences in the level of contracture, HR and coronary flow rate between both sexes. Also, no significant differences were seen in the concentration of cTnI released and the percentage of infarct area in the hearts from both sexes.

3.3 Sex differences in response to I/R in Langendorff-perfused mouse hearts from older mice

3.3.1 Recovery of LVDP, HR, +dP/dt, -dP/dt, RPP and contracture in old mouse hearts

To investigate sex differences in the recovery of cardiac parameters in reperfusion as a function of age, hearts from old male and female mice were exposed to I/R. **Figure 3.10** shows representative recordings of contractile function from old female **(A)** and old male **(B)** mouse hearts in baseline and reperfusion. In contrast to results in young mice, the heart from the old male appeared to recover better than the heart from the old female.

Normalized mean data show that the recovery of LVDP was better in old male hearts (66.5 ± 7.9%) than in old female hearts (54.9 ± 5.6%) at 141 min reperfusion **(Figure 3.11 A)**. HR recovered completely in reperfusion, although recovery was significantly higher in old female hearts (110.9 ± 6.5%) than in old male hearts (90.4 ± 6.8%) at 141 min reperfusion **(Figure 3.11 B)**. However, when +dP/dt, –dP/dt and RPP were measured, old male and female hearts exhibited similar recovery **(Figure 3.12, Figure 3.13).** Similarly, the amplitude of contracture throughout the experiment did not differ between the sexes (30.4 ± 4.1 mm Hg vs.32.0 ± 4.2 mm Hg at 141 min reperfusion) **(Figure 3.14).** Overall, in contrast to the results in young adult animals, the recovery of contractile function was similar in both sexes and males actually recovered somewhat better than females in the older group.

3.3.2 Recovery of coronary flow rate in old mouse hearts

The coronary flow rate in old male mouse hearts was significantly higher in baseline compared to old female mouse hearts. The values for coronary flow rate in old male and female mouse hearts were 2.8 ± 0.2 ml/min and 2.2 ± 0.06 ml/min, in baseline.

However, in reperfusion there was no difference in coronary flow rate between old male and female hearts. The values for old male and female hearts were 3.1 ± 0.3 ml/min and 2.3 ± 0.2 ml/min respectively, in reperfusion. **(Figure 3.15)**.

3.3.3 Differences in infarct area and concentration of cTnI released in old mouse hearts

The percentage of infarct area did not differ in between old male (15.5 ± 1.7%) and old female (27.3 ± 5.0%) mouse hearts **(Figure 3.16)**. Similarly, there was no sex difference in the concentration of cTnI released in effluent samples collected in reperfusion, (old male = 12.9 ± 1.2 ng/ml and old female = 12.7 ± 1.5 ng/ml) **(Figure 3.17).**

Overall, the recovery of contractile function was either similar in both sexes, or old males recovered more completely than old females. Recovery of LVDP and overall coronary flow rate were significantly better in old males than females. By contrast, other cardiac parameters (+dP/dt, -dP/dt, RPP, contracture, infarct area and cTnI concentration) recovered similarly in both sexes.

3.4 Age-specific differences in response to I/R in Langendorff-perfused adult and old female mouse hearts.

3.4.1 Recovery of LVDP, HR, +dP/dt, -dP/dt, RPP and contracture in adult and old female mouse hearts

After looking at sex-specific responses, the next objective was to explore age-specific responses of hearts towards I/R injury. To evaluate age-specific differences in the recovery of contractile function in female mouse hearts, adult and old hearts were

subjected to I/R injury. **Figure 3.18** shows representative recordings of contractile function in adult **(A)** and old **(B)** female mouse hearts at baseline and in reperfusion. When mean normalized LVDP was compared, there was no significant difference in recovery in adult (50.6 ± 12.4%) and old (54.9 ± 5.6%) female hearts in reperfusion, as shown in **Figure 3.19 (A)**. However, HR in old female hearts was significantly higher than in adult females at one time point in reperfusion (113.2 ± 4.9% vs. 95.9 ± 8.3) **(Figure 3.19 B)**. Furthermore, +dP/dt, –dP/dt and RPP recovered similarly in adult and old female hearts in reperfusion (+dP/dt, 51.7 ± 13.4% vs. 58.6 ± 6.0%; -dP/dt, 53.9 ± 13.0% vs. 59.0 ± 6.5%; RPP, 51.9 ± 15.2% vs. 60.9 ± 6.2%) **(Figure 3.20, Figure 3.21)**. Also, there were no differences in contracture in reperfusion when adult and old female hearts were compared (33.1 ± 7.0 mm Hg vs. 32.0 ± 4.2 mm Hg) **(Figure 3.22)**.

3.4.2 Recovery of coronary flow rate in adult and old female mouse hearts

There was no difference in coronary flow rate at baseline and reperfusion between adult and old female mouse hearts. The values for adult and old female hearts in baseline were 2.9 ± 0.3 ml/min and 2.2 ± 0.06 ml/min, respectively. Similarly, in reperfusion, the values for adult and old female hearts were 2.6 ± 0.4 ml/min and 2.3 ± 0.2 ml/min, respectively **(Figure 3.23)**.

3.4.3 Infarct area and concentration of cTnI released in adult and old female mouse hearts

Infarct area was not significantly different in between adult and old female mouse hearts (33.7 ± 9.2% vs. 27.3 ± 5.0%) **(Figure 3.24 A)**. Also, when the concentration of cTnI released in effluent samples collected in reperfusion was compared, no significant

difference was observed between adult and old female mouse hearts (9.4 ± 2.0 ng/ml vs. 12.7 ± 1.5 ng/ml) **(Figure 3.24 B)**.

In summary, contractile function in adult and old female mouse hearts recovered similarly in reperfusion, except HR which was higher at one time point in old females. Other cardiac parameters including infarct area, coronary flow rate and cTnI concentration, were also similar in both groups.

3.5 Age-specific differences in response to I/R in Langendorff-perfused adult and old male mouse hearts.

3.5.1 Recovery of LVDP, HR, +dP/dt, -dP/dt, RPP and contracture in adult and old male mouse hearts

To evaluate age-specific differences in male mouse hearts, the extent of recovery of different cardiac parameters was compared in adult and old male mouse hearts exposed to I/R. **Figure 3.25** shows representative recordings of contractile function in baseline and reperfusion from adult and old male mouse hearts. Mean data shows that the recovery of LVDP in reperfusion was significantly better in old male (67.2 ± 7.3%) compared to adult male (31.5 ± 12.3%) mouse hearts **(Figure 3.26 A)**. By contrast, HR recovered completely in reperfusion, regardless of age **(Figure 3.26 B)**. Also, +dP/dt, –dP/dt and RPP recovered significantly better in old male compared to adult male hearts in reperfusion (+dP/dt, 66.6 ± 6.6% vs. 33.4 ± 13.2%; -dP/dt, 65.2 ± 7.0% vs. 34.3 ± 13.3%; RPP, 64.2 ± 9.6% vs. 29.6 ± 12.1%) **(Figure 3.27, Figure 3.28)**. However, the level of

contracture in reperfusion did not differ significantly between adult (37.7 ± 5.4 mm Hg) and old (30.4 ± 4.1 mm Hg) male mouse hearts **(Figure 29)**.

3.5.2 Recovery of coronary flow rate in adult and old male mouse hearts

There was no difference in the coronary flow rate when measured at baseline and in reperfusion between adult and old male mouse hearts. The values for adult and old male hearts in baseline were 2.8 ± 0.3 ml/min and 2.8 ± 0.2 ml/min respectively. Also, coronary flow rates, in reperfusion, were 3.0 ± 0.3 ml/min and 3.1 ± 0.3 ml/min in adult and old male hearts, respectively **(Figure 3.30)**.

3.5.3 Infarct area and concentration of cTnI released in adult and old male mouse hearts

The infarct area was significantly smaller in old male mouse hearts (15.5 ± 1.7%) compared to adult male hearts (41.6 ± 12.1%) **(Figure 3.31 A)**. However, the concentration of cTnI released in effluent samples collected in reperfusion was not different between adult (14.4 ± 1.1 ng/ml) and old (12.9 ± 1.2 ng/ml) male mouse hearts **(Figure 3.31 B)**.

Taken together, these results indicate that old male hearts recovered better than adult male hearts. Cardiac parameters like LVDP, +dP/dt, -dP/dt and RPP recovered significantly better in old male hearts compared to adult male hearts. Infarct area was also significantly reduced in old male hearts. However, coronary flow rate, contracture and cTnI concentration were not significantly different between the two age groups.

3.6 Recovery of cardiac parameters with G1 in adult female mouse hearts

3.6.1 Effect of G1 on the recovery of LVDP, HR, +dP/dt, -dP/dt, RPP and contracture in adult female mouse hearts

After evaluating the extent of recovery with cardioplegia in adult female mice, it was clear that cardioplegia alone was not sufficient enough to protect the heart from I/R injury. Therefore, experiments that incorporated G1 in cardioplegia at different concentrations were conducted to determine whether this addition was beneficial. Firstly, 110 nM G1 was added in STH2 cardioplegia and functional parameters were measured. At this concentration of G1, the recovery of cardiac parameters was only slightly better than in hearts treated with vehicle alone (DMSO) **(Figure 3.32, Figure 3.33)**. Therefore, a higher concentration of G1 (500 nM) was used. With this concentration of G1, cardiac parameters recovered significantly more completely than the vehicle treated hearts (LVDP recovered to 76.8 ± 4.5% for G1 vs. 50.6 ± 12.4% for control at 141 min reperfusion). However, HR recovered completely in reperfusion regardless of drug **(Figure 3.32)**. +dP/dt and –dP/dt also recovered significantly better with 500 nM G1. The values for recovery of +dP/dt were 51.7 ± 13.4% vs. 78.2 ± 5.4% and for –dP/dt were 53.9 ± 13.0% vs. 81.8 ± 4.6% for control and G1, respectively at 141 min reperfusion **(Figure 3.33)**. In some studies, a higher concentration of G1 (1 μM) was used to determine whether increasing concentration could enhance the recovery (not illustrated). However, no further improvement in recovery was observed when 1 μM G1 was incorporated into cardioplegia in adult female hearts (LVDP = 74.1 ± 14.7%, +dP/dt = 75 ± 15.2%, -dP/dt = 78 ± 16.3%). Therefore, all subsequent experiments used 500 nM G1.

To verify that the beneficial effects of G1 were mediated through effects at GPER receptors, the GPER antagonist G15 was used. G15 (1 μM) was added to cardioplegia along with G1 (500 nM). This antagonist blocked the effects of G1 and reduced the level of recovery of LVDP to 59 ± 1.8% at 141 min reperfusion **(Figure 3.34 A)**. Also, the recoveries of +dP/dt and –dP/dt were blocked by G15, where +dP/dt recovered to 62.2 ± 2.1% and –dP/dt recovered to 63.8 ± 3.2% at the end of reperfusion **(Figure 3.35)**. However, HR was unaffected by G15 **(Figure 3.34 B)**. The recovery of RPP was also blocked by G15 and was reduced from 79.9 ± 8.4% (with 500 nM G1) to 63.0 ± 7.4% **(Figure 3.36)**. The level of contracture was also measured in the presence and absence of G1. There was no significant reduction in contracture in the 500 nM G1 group (21.5 ± 3.5 mm Hg) compared to control (33.1 ± 7 mm Hg) and G1+G15 (31.4 ± 3.6 mm Hg) groups **(Figure 3.37).** Other experiments were also done using another GPER antagonist, G36 (data not shown). G36 also reduced the effects of G1, although this reduction was not statistically significant.

3.6.2 Effect of G1 on coronary flow rate in adult female mouse hearts

To evaluate the rate of perfusion of the myocardium, coronary flow rate was measured in mouse hearts. When baseline values were compared, control (2.9 ± 0.3 ml/min) and G1-treated hearts (2.1 ± 0.1 ml/min) had similar coronary flow rates, however G1+G15 treated hearts (2.04 ± 0.04 ml/min) showed significantly lower flow rate compared to control hearts **(Figure 3.38 A)**. Nevertheless, in reperfusion, though G1 treated hearts (3.4 ± 0.4 ml/min) showed slightly improved flow rate, no significant difference was seen between control, G1 or G1+G15 treated hearts **(Figure 3.38 B).**

3.6.3 Effect of G1 on infarct area in adult female mouse hearts

To determine the extent of myocardial injury in hearts subjected to I/R, the infarct area was calculated. **Figure 3.39 (A)** shows representative images of heart slices from adult female hearts in the absence or presence of G1. G1 did not affect infarct area in the adult female hearts. Vehicle treated hearts showed 33.7 ± 9.2% mean infarct area and G1 (500 nM) treated hearts showed 16.3 ± 3.3% mean infarct area. Although G1 reduced infarct area, this reduction was not statistically significant. However, infarct area was significantly lower in G1 treated hearts when compared to hearts treated with G1+G15 (28.1 ± 2.3%) **(Figure 3.39 B).**

3.6.4 Effect of G1 on the concentration of cTnI released in reperfusion

The effluents collected at the 141 min time point in reperfusion were analyzed for the measurement of cTnI release, as a marker of cardiac injury. The concentration of cTnI released in reperfusion was not affected by 500 nM G1 (7.1 ± 1.1 ng/ml) compared to control (9.4 ± 2.0 ng/ml). There was slight reduction of cTnI release in samples from G1 treated hearts; however, this was not statistically significant**.** However, the concentration of cTnI released was significantly higher in G1+G15 (15.0 ± 0.4 ng/ml) treated hearts compared to hearts treated with G1 **(Figure 3.40)**.

These results show that, in adult female mouse hearts, 500 nM G1 enhanced recovery of cardiac parameters in reperfusion. Contractile function was improved with G1 (500 nM) when compared to vehicle control in reperfusion. These effects of G1 were blocked by G15 (1 μM), which is a GPER antagonist. However, G1 did not affect the concentration of cTnI released, which remained similar to vehicle treated hearts.

3.7 Recovery of cardiac parameters with G1 in adult male mouse hearts

3.7.1 Effect of G1 on the recovery of LVDP, HR, +dP/dt, -dP/dt, RPP and contracture in adult male mouse hearts

Parallel experiments were conducted in adult male mouse hearts to determine the effect of G1 on recovery of cardiac functional parameters. Concentrations of 110 nM and 500 nM G1 were used to determine the effective dose for the optimal recovery in reperfusion. In contrast to results in adult females, neither 110 nM nor 500 nM G1 improved recovery of function in adult male hearts. As shown in **Figure 3.41 (A),** LVDP was not affected by G1 at any concentration. HR recovered completely regardless of drug treatment **(Figure 3.41 B)**. Additionally, G1 had no beneficial effects on recovery of +dP/dt, -dP/dt, contracture and RPP during reperfusion in adult male mouse hearts **(Figure 3.42, Figure 3.43 and Figure 3.44)**.

3.7.2 Effect of G1 on coronary flow rate in adult male mouse hearts

There was no difference in coronary flow rate in baseline as well as in reperfusion between control and G1-treated adult male mouse hearts. The coronary flow rate in reperfusion was 3.0 ± 0.3 ml/min and 3.7 ± 0.2 ml/min in control and G1-treated hearts respectively. Similarly, coronary flow rate was not different in baseline as well between control and G-treated hearts **(Figure 3.45)**.

3.7.3 Effect of G1 on infarct area in adult male mouse hearts

Figure 3.46 (A), shows representative images of heart slices from an adult male heart in the absence and presence of G1. As shown in **Figure 3.46 (B),** G1 did not affect

infarct area in adult male hearts compared to control hearts. The value for infarct area in G1-treated hearts was 31.5 ± 11.2% and for vehicle-treated hearts was 41.6 ± 12.1%.

3.7.4 Effect of G1 on the concentration of cTnI released in reperfusion

Effluents collected at the 141 min time point in reperfusion were assayed for the measurement of cTnI released. As shown in **Figure 3.47,** the concentration of cTnI released in reperfusion was not affected by 500 nM G1 (14.2 ± 1.9 ng/ml) compared to control (14.4 ± 1.1 ng/ml).

These observations demonstrate that, in contrast to adult females, adult male hearts did not benefit from G1 treatment. There was no improvement in contractile function in reperfusion with G1. Also, coronary flow rate and cTnI release were not affected by G1.

3.8 Recovery of cardiac parameters with G1 in old female mouse hearts

3.8.1 Effect of G1 on the recovery of LVDP, HR, +dP/dt, -dP/dt, RPP and contracture in old female mouse hearts

Next, the impacts of G1 on old female hearts were examined to study age-specific responses to the drug. Similar experiments were performed as in adult mouse hearts. The recovery of LVDP in reperfusion in old female mouse hearts was significantly and markedly improved by 500 nM G1 (90.0 ± 6.0%) when compared to vehicle treated hearts (54.9 ± 5.6%) **(Figure 3.48 A)**. HR recovered completely regardless of drug as shown in **Figure 3.48 (B)**. The values for recovery of +dP/dt were 58.6 ± 6.0% vs. 94.1 ± 6.2% and –dP/dt were 59.0 ± 6.5% vs. 96.0 ± 7.1% for control and 500 nM G1

respectively **(Figure 3.49).** Also, the RPP recovered significantly better with G1 (89.8 ± 8.1%) when compared to control (60.9 ± 6.2%) **(Figure 3.50).** Similarly, the level of contracture was significantly reduced by 500 nM G1 at 121 and 131 min time points in reperfusion compared to control **(Figure 3.51)**.

3.8.2 Effect of G1 on coronary flow rate in old female mouse hearts

The coronary flow rate was significantly improved in G1-treated old female hearts (3.4 ± 0.3 ml/min) compared to control hearts (2.3 ± 0.2 ml/min) in reperfusion. However, there was no difference in the flow rate at baseline between control and G-treated hearts **(Figure 3.52)**.

3.8.3 Effect of G1 on infarct area in old female mouse hearts

Figure 3.53 (A) shows representative images of heart slices from old female hearts in the absence or presence of G1. The percentage of infarct area was significantly lower in 500 nM G1 treated hearts (12.0 ± 0.9%) when compared to vehicle-treated control hearts (27.3 ± 5.0%), as shown in **Figure 3.53 (B).**

3.8.4 Effect of G1 on the concentration of cTnI released in reperfusion

The concentration of cTnI released in reperfusion was not affected by 500 nM G1 (12.0 ± 1.1 ng/ml) compared to control (12.7 ± 1.5 ng/ml) as shown in **Figure 3.54.** The effluent samples from the 141 min time point in reperfusion were used for analysis.

Together, these findings indicate that old female mouse hearts were protected from I/R injury by G1 (500 nM) treatment. Contractile function and coronary flow rates were significantly improved by G1 compared to control hearts. Also, G1 treated hearts

had much smaller infarcts when compared to vehicle treated hearts. However, G1 had no effect on the concentration of cTnI released in reperfusion.

3.9 Recovery of cardiac parameters with G1 in old male mouse hearts

3.9.1 Effect of G1 on the recovery of LVDP, HR, +dP/dt, -dP/dt, RPP and contracture in old male mouse hearts

The next series of experiments explored whether G1 was effective in improving cardiac function in old male mouse hearts. It was observed that the recovery of LVDP was better with 500 nM G1 (82.3 ± 9.8%) compared to control (67.2 ± 7.3%) as shown in **Figure 3.55 (A)**. HR, however, was not affected and recovered completely regardless of drug (**Figure 3.55 B).** Also, hearts treated with G1 showed significant recovery of +dP/dt and –dP/dt (+dP/dt = 83.1 ± 9.9%; -dP/dt = 83.5 ± 9.5%) when compared to control hearts (+dP/dt = 66.6 ± 6.6%; -dP/dt = 65.3 ± 7.0%) in reperfusion **(Figure 3.56)**. Similarly, the recovery of RPP was enhanced by G1 compared to control (81.0 ± 13.1% vs. 64.2 ± 9.6%) as shown in **Figure 3.57**. Hearts treated with G1 also showed a significant reduction of contracture in reperfusion compared to vehicle treated control hearts at the 121 and 131 min time points **(Figure 3.58)**.

3.9.2 Effect of G1 on coronary flow rate in old male mouse hearts

There was no difference in the coronary flow rate in baseline (2.8 ± 0.2 ml/min vs. 2.4 ± 0.1 ml/min) and in reperfusion (3.1 ± 0.3 ml/min vs. 3.2 ± 0.3 ml/min) between control and G1-treated old male hearts, as shown in **Figure 3.59**.

3.9.3 Effect of G1 on infarct area in old male mouse hearts

Figure 3.60 (A) shows examples of heart slices from old male hearts treated with G1 or in the absence of G1. Infarct area was reduced by 500 nM G1 treatment (9.5 ± 1.1%) when compared to vehicle-treated control hearts (15.5 ± 1.7%), as shown in **Figure 3.60 (B).**

3.9.4 Effect of G1 on the concentration of cTnI released in reperfusion

The concentration of cTnI released in reperfusion was not affected by 500 nM G1 (9.7 ± 2.4 ng/ml) compared to control (12.9 ± 1.2 ng/ml) as shown in **Figure 3.61** in old male hearts. The effluent samples were taken at the 141 min time point in reperfusion.

These observations strongly suggest that, in contrast to results in young males, G1 was beneficial in old male hearts. Contractile function, coronary flow rate and reduction in infarct area were significantly improved by G1 treatment in old males.

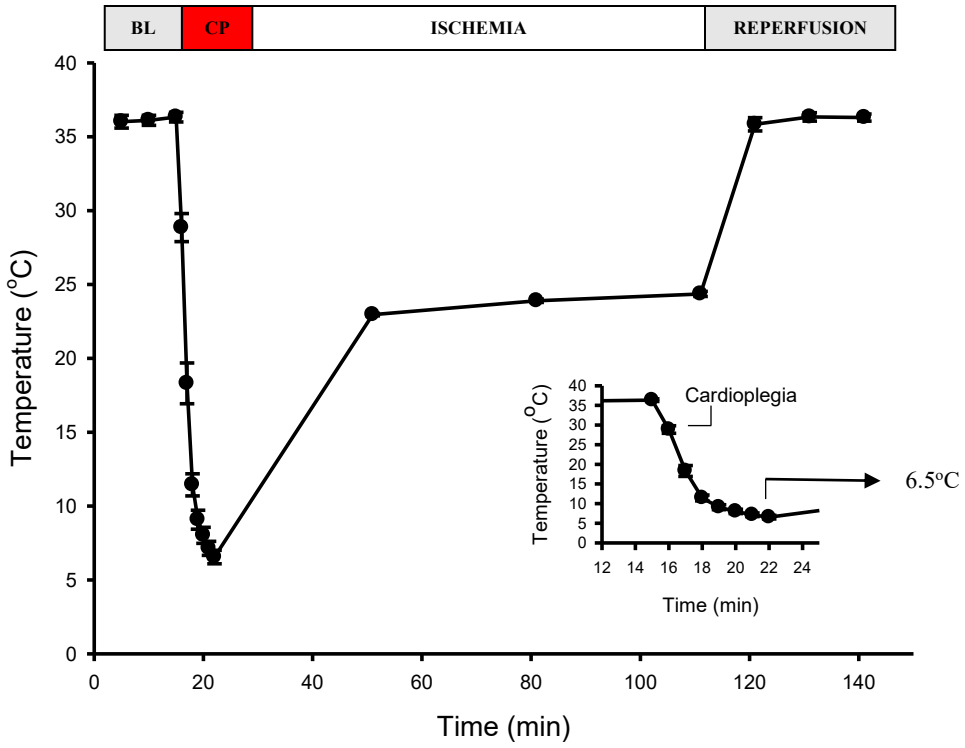


Figure 3.1 Temperature regulation throughout the experiment. During 10 min of baseline (BL) perfusion of the heart with Krebs-Henseleit buffer, the temperature of the myocardium was maintained around 37ºC. Following perfusion with hypothermic cardioplegia (CP), the myocardial temperature dropped to between 6-9ºC during 6 min of perfusion. Subsequently, during ischemia, the heart was submerged in cardioplegia at room temperature (23-24ºC) and temperature was maintained at that level. Later, hearts were reperfused with Krebs-Henseleit buffer and the temperature recovered to close to 37ºC. The inset graph is an enlarged view of the temperature drop during cardioplegic perfusion for 6 minutes.

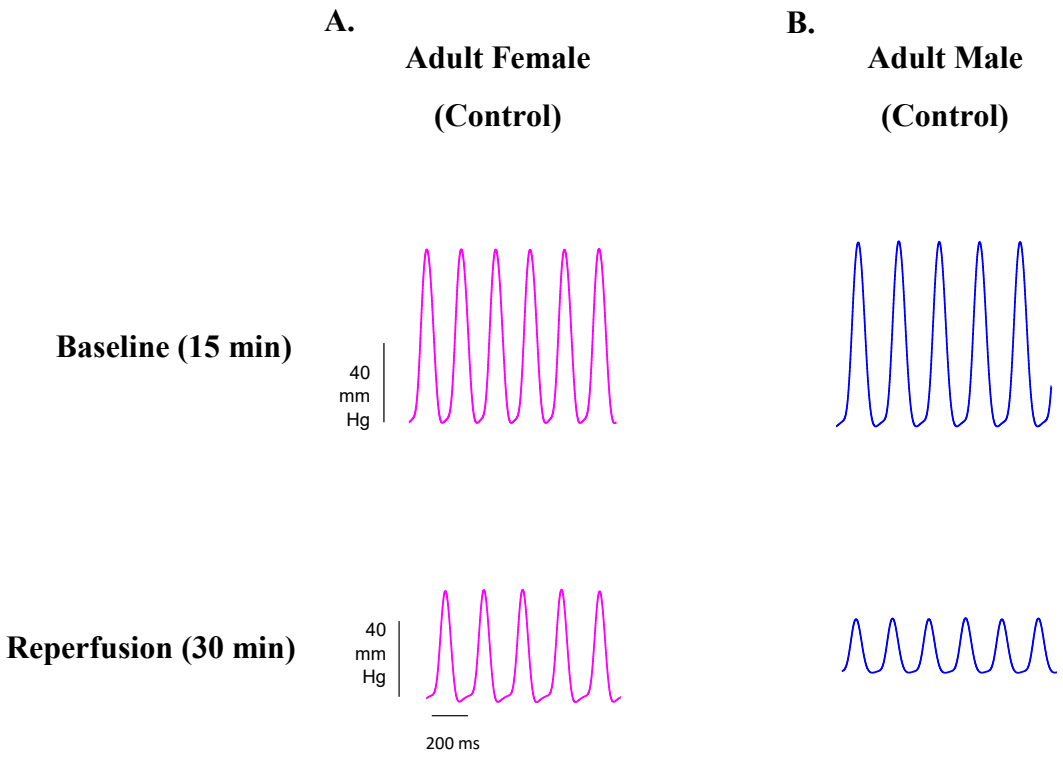
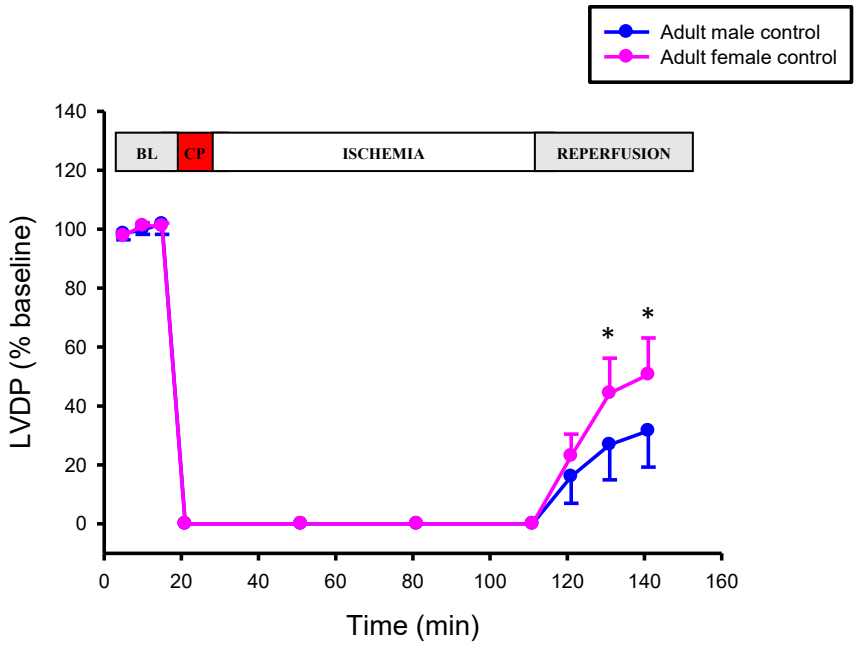


Figure 3.2 Representative experimental recordings from adult female and male mouse hearts. Recovery of contractile function after I/R injury was better in hearts from females **(A)** than in males **(B)** when compared to their respective baseline traces.

A.



B.

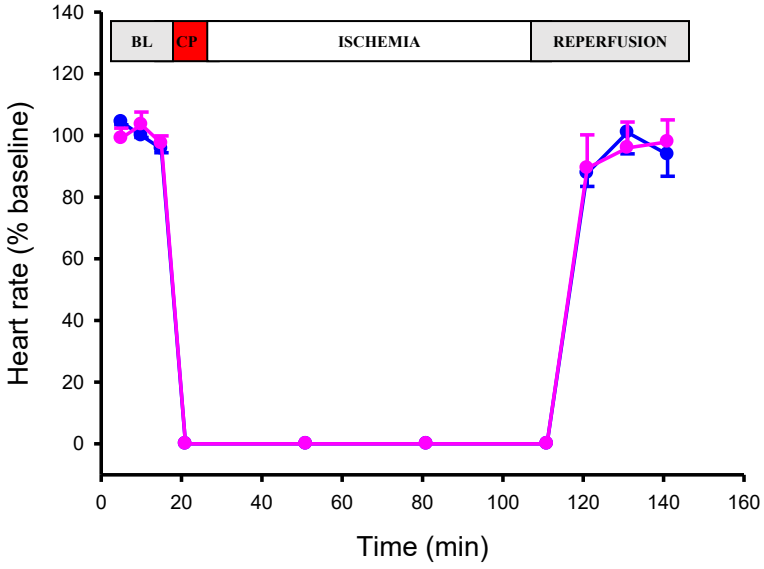
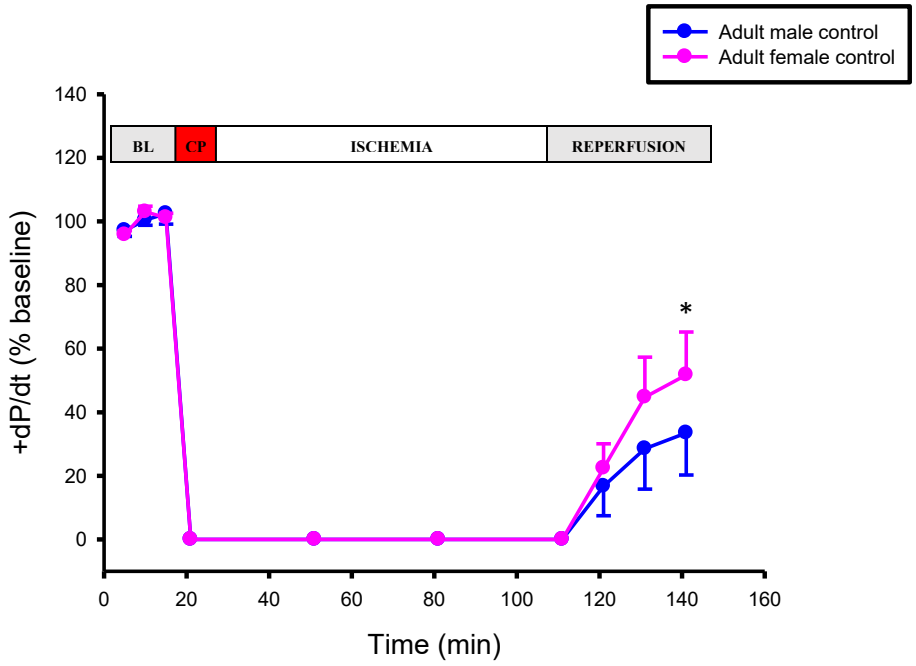


Figure 3.3 Sex differences in response to I/R in Langendorff-perfused hearts: LVDP and HR. (A) Recovery of LVDP was significantly better during reperfusion (at 131 min and 141 min time points) in female compared to male control hearts (cardioplegia + DMSO; * $p < 0.05$; 2-way RM ANOVA). **(B)** By contrast, HR recovered completely during reperfusion regardless of sex. Adult male control n=6; adult female control n=6.

A.



B.

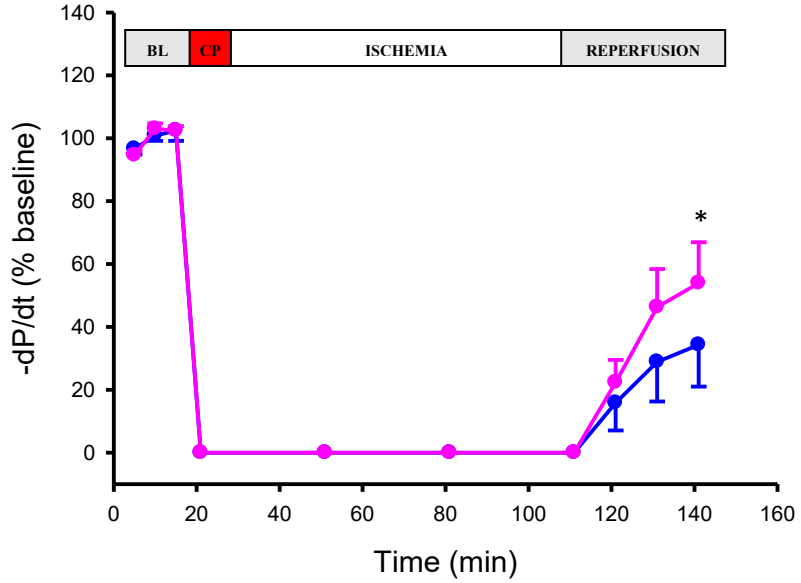


Figure 3.4 Recovery of contractile function after I/R was worse in hearts from males compared to females. (A) Recovery of +dP/dt was significantly better during reperfusion (at 141 min time point) in female compared to male control hearts (* $p < 0.05$; 2-way RM ANOVA). **(B)** Similar results were seen when –dP/dt was compared. Adult male control n=6; adult female control n=6.

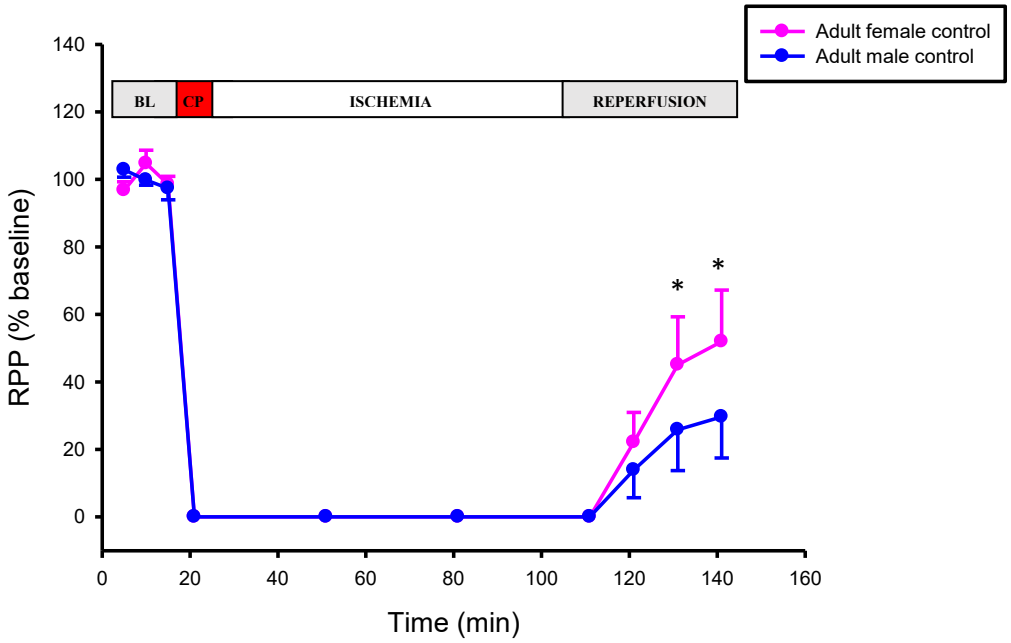


Figure 3.5 Recovery of the RPP was better in female hearts than in male hearts in reperfusion. Adult female mouse hearts recovered significantly better in reperfusion (at 131 and 141 min time points) compared to male mouse hearts (* $p<0.05$). Adult female control n=6; adult male control n=6; 2-way RM ANOVA**.**

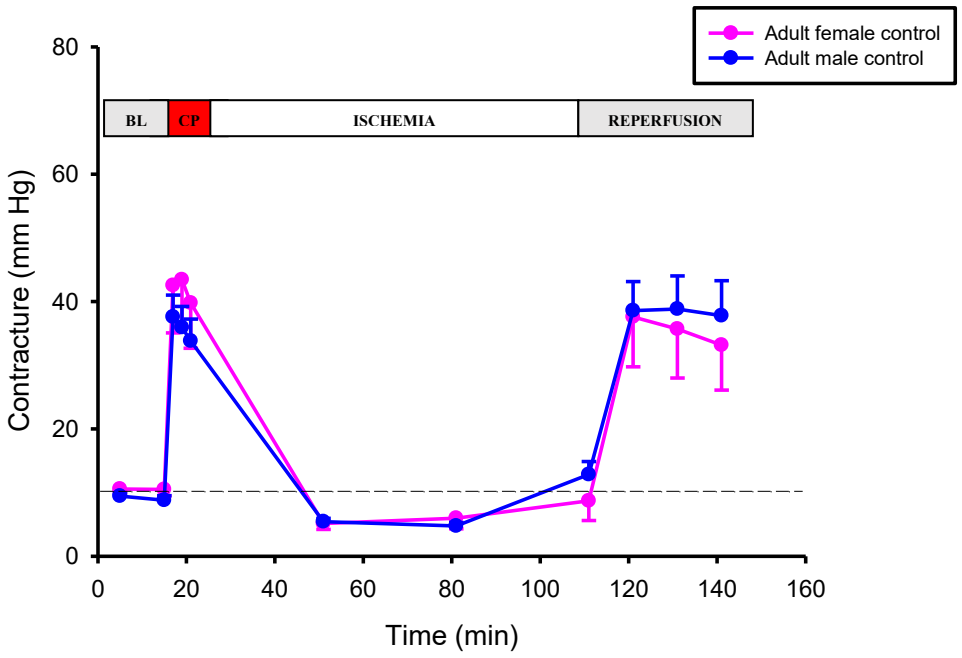


Figure 3.6 The contracture level in reperfusion was similar in adult female and male mouse hearts. Similar contracture amplitudes were observed in adult female and male mouse hearts throughout the experiments. Adult female control n=6; adult male control n=6. 2-way RM ANOVA.

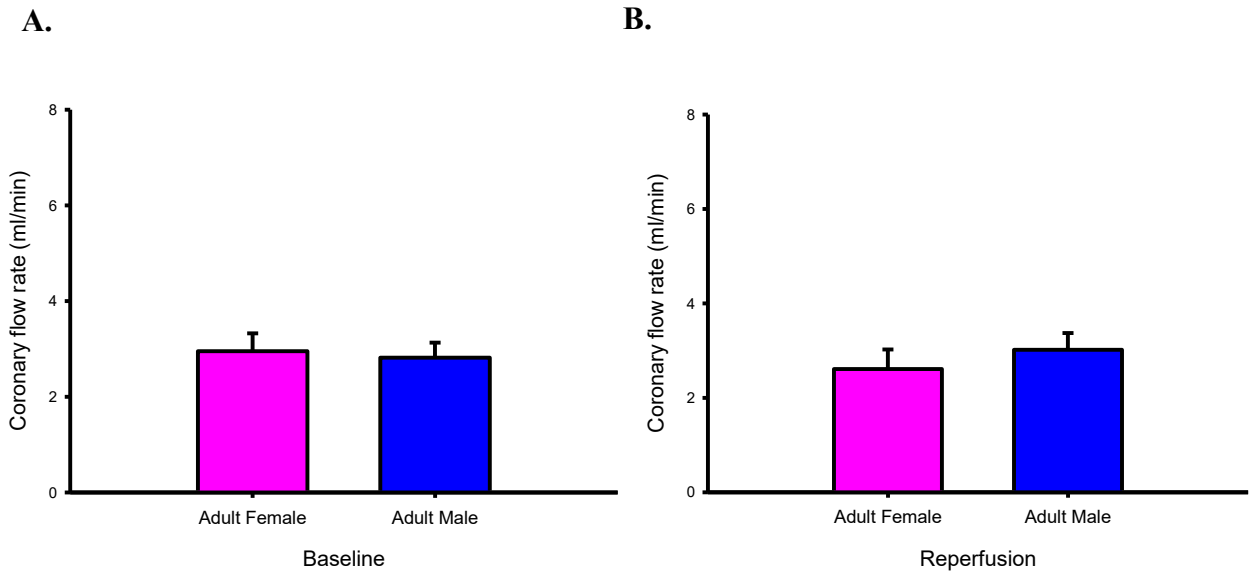
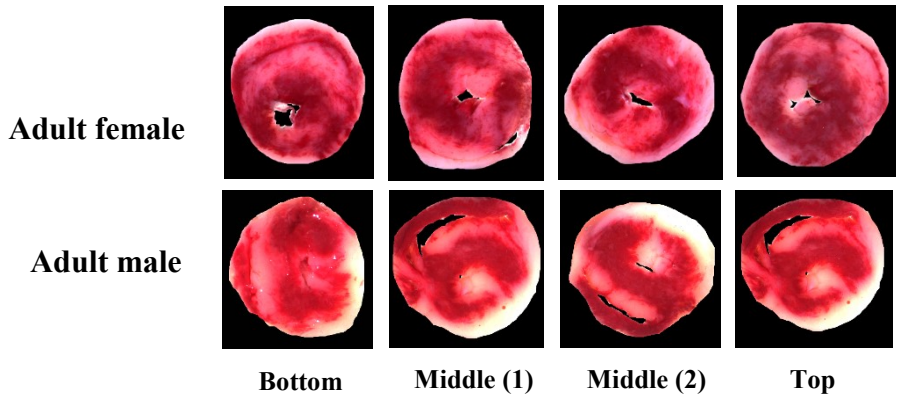


Figure 3.7 No change in coronary flow rate was observed in baseline and reperfusion, between adult female and male mouse hearts. In control hearts from adult female and male mice, there was no difference in coronary flow rate in either baseline **(A)** or reperfusion **(B)** of the hearts. Adult female n=6; adult male n=6. Student's t-test.

A.



B.

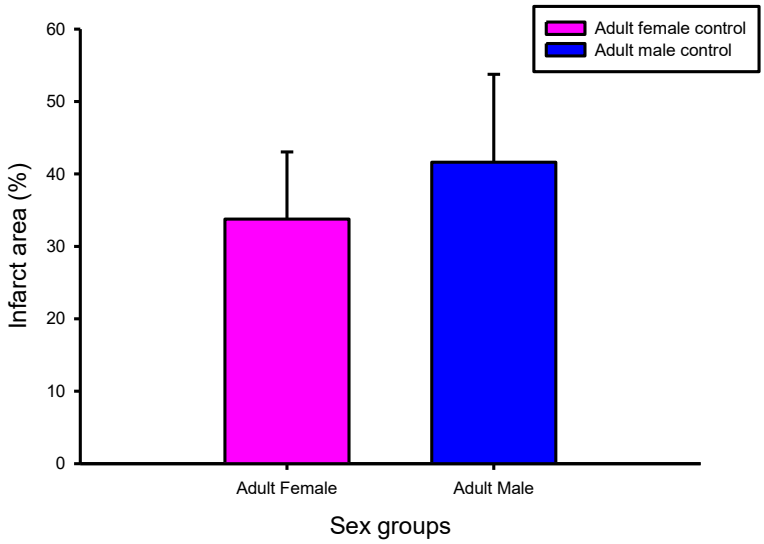


Figure 3.8 There was no sex difference in infarct area. (A) When the hearts were perfused with TTC, the dye stained the viable tissue red and the non-viable tissue remained unstained. The heart slices from bottom (apex), middle and top (base) were taken from adult female and male hearts and their infarct areas were compared. **(B)** No differences were seen in the percentage of infarct area between the sexes. Adult female control n=4; adult male control n=3. Student's t-test.

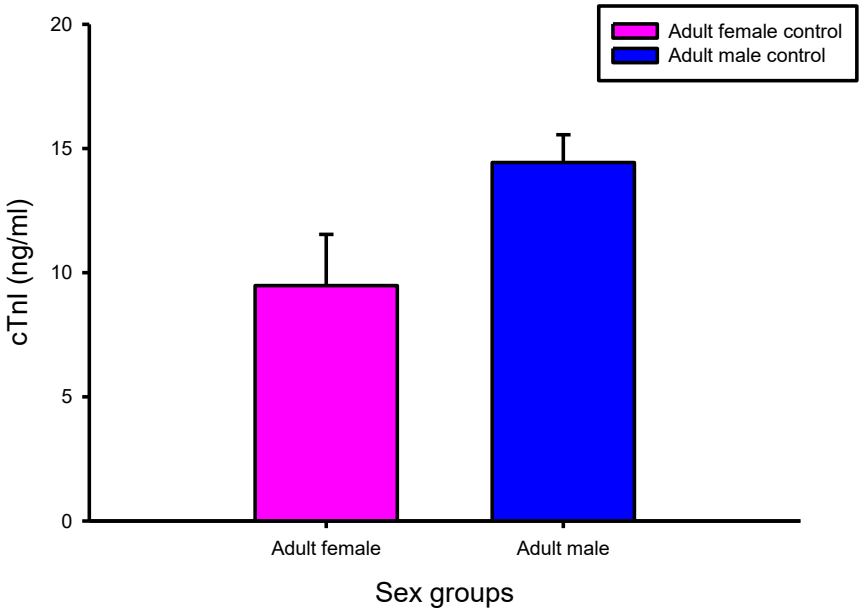


Figure 3.9 The concentration of cTnI released did not differ between adult female and male mouse hearts. There was no significant difference seen in the concentration of cTnI released in samples collected during reperfusion (at 141 min) in adult female and male effluent samples. Adult female control n=6; adult male control n=6. Student's t-test.

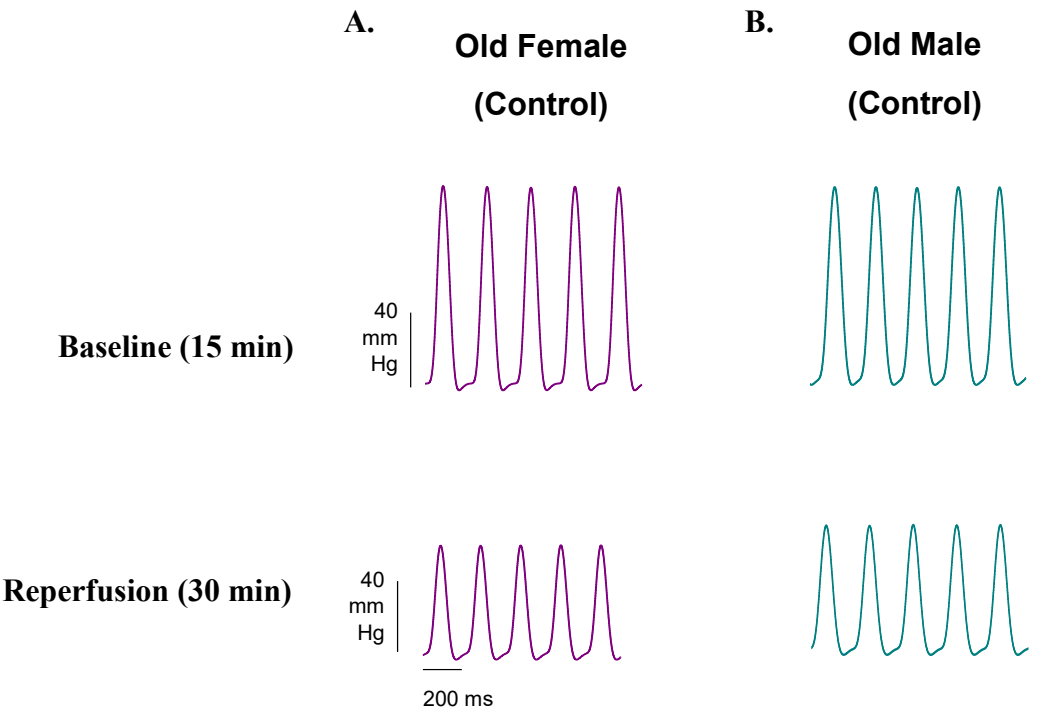


Figure 3.10 Representative experimental recordings from old female and male mouse hearts. Contractile function partially recovered after I/R injury in hearts from both **(A)** old female **(B)** and old male mice.

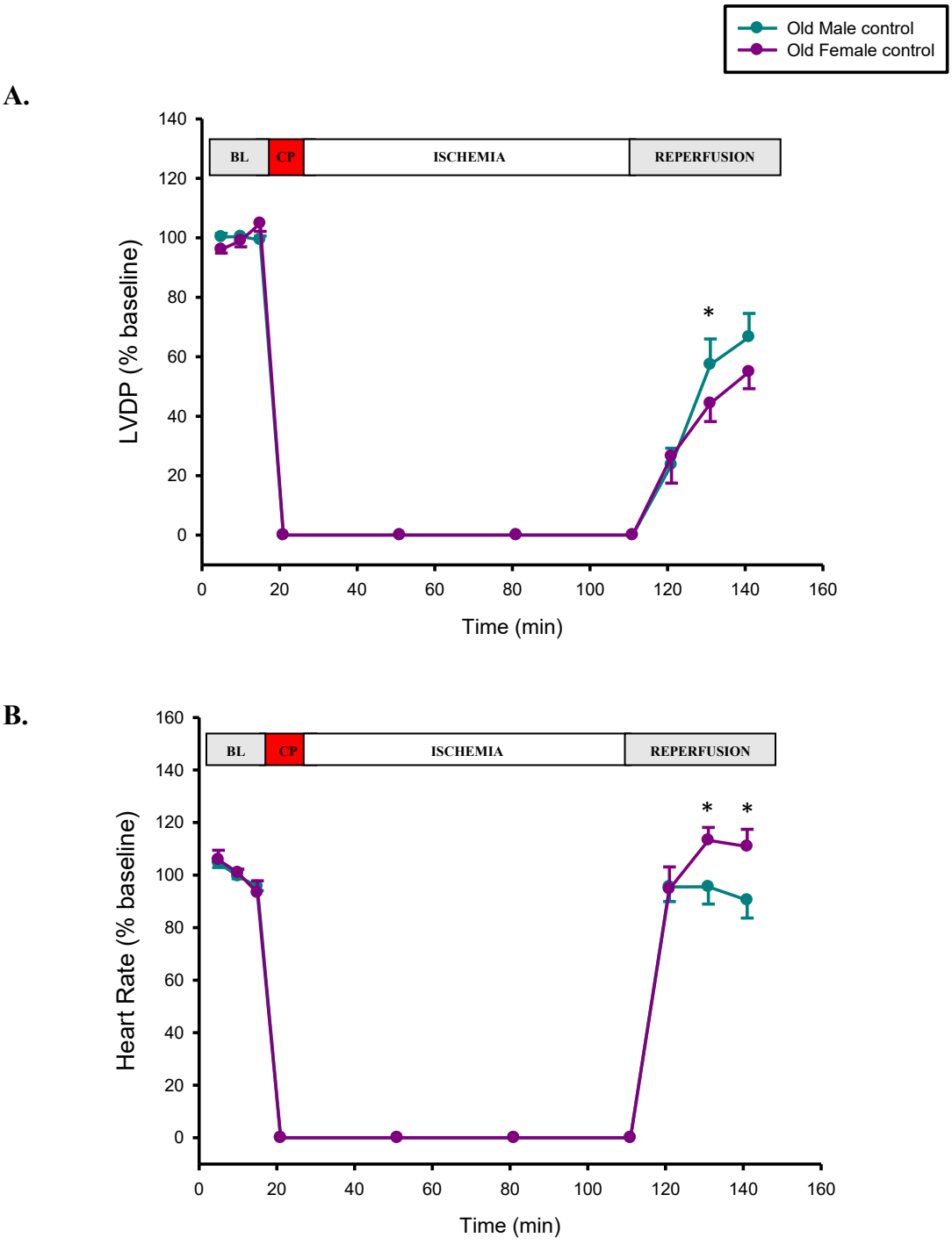
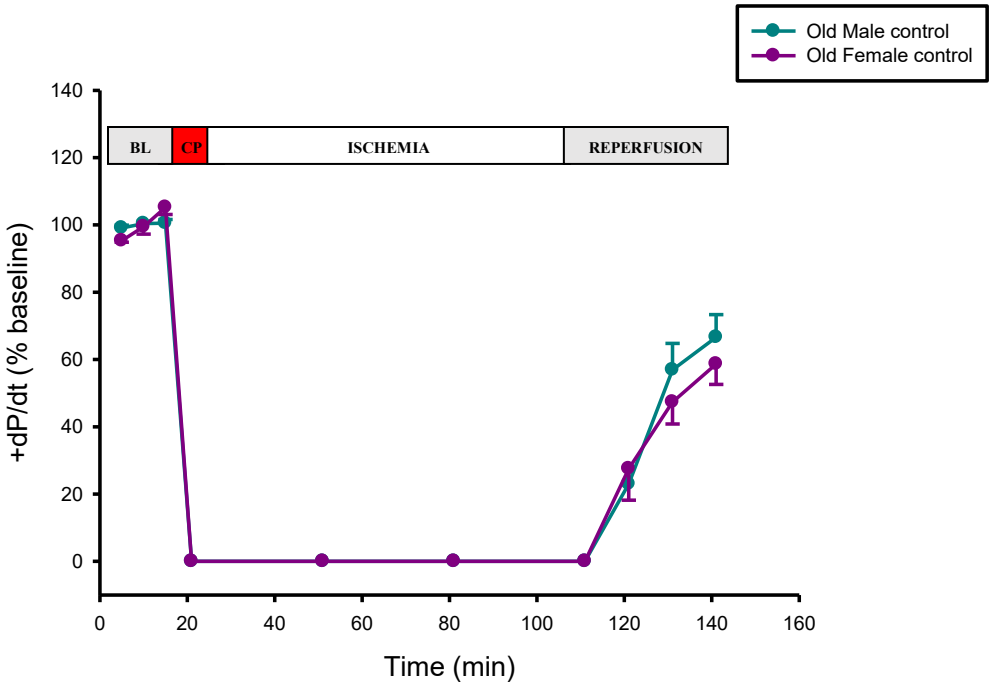


Figure 3.11 Comparison of LVDP and HR between old female and male control groups. (A) Recovery of LVDP was significantly better during reperfusion (at 131 min time point) in old male hearts compared to old female hearts (* $p < 0.05$). **(B)** HR recovered completely during reperfusion, although recovery in old female hearts recovered to a higher level than in old males. Old male control n=6; old female control n=5. 2-way RM ANOVA.

A.



B.

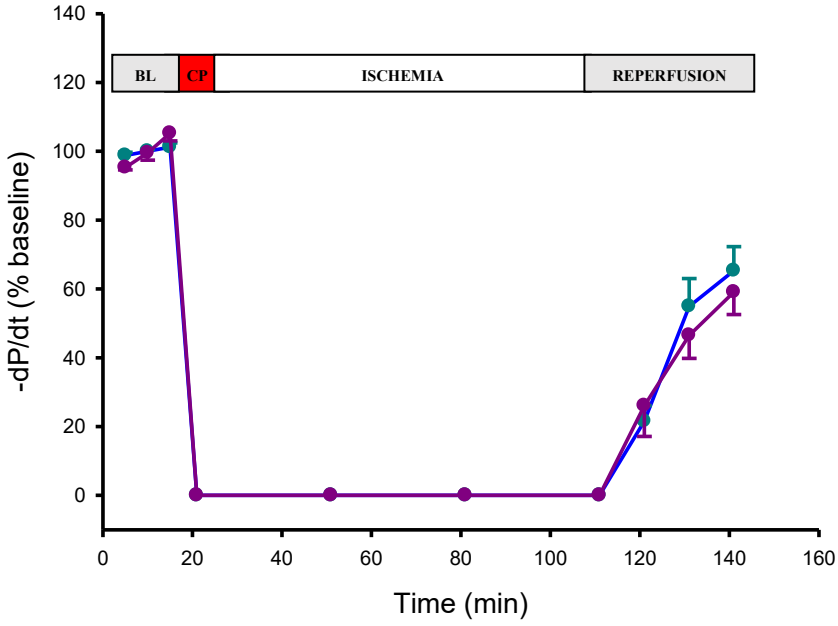


Figure 3.12 +dP/dt and –dP/dt recovered in reperfusion to a similar extent in hearts from old male and old female mice. (A) +dP/dt and **(B)** –dP/dt in old male and female hearts recovered similarly at all three time points in reperfusion. Male hearts tended to recover slightly better than females, although, there was no statistical difference in recovery between the two groups. Old male control n=6; old female control n=5. 2-way RM ANOVA.

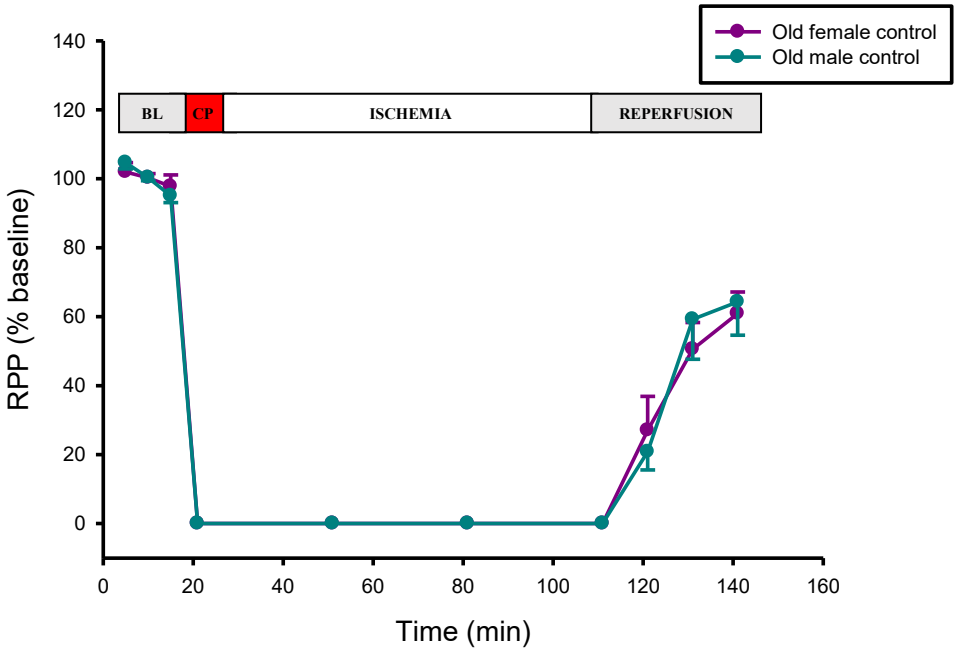


Figure 3.13 Old male and female hearts exhibited similar recovery of RPP in reperfusion. RPP recovered similarly in both old male and female hearts in reperfusion. Old female control n=5; Old male control n=6; 2-way RM ANOVA.

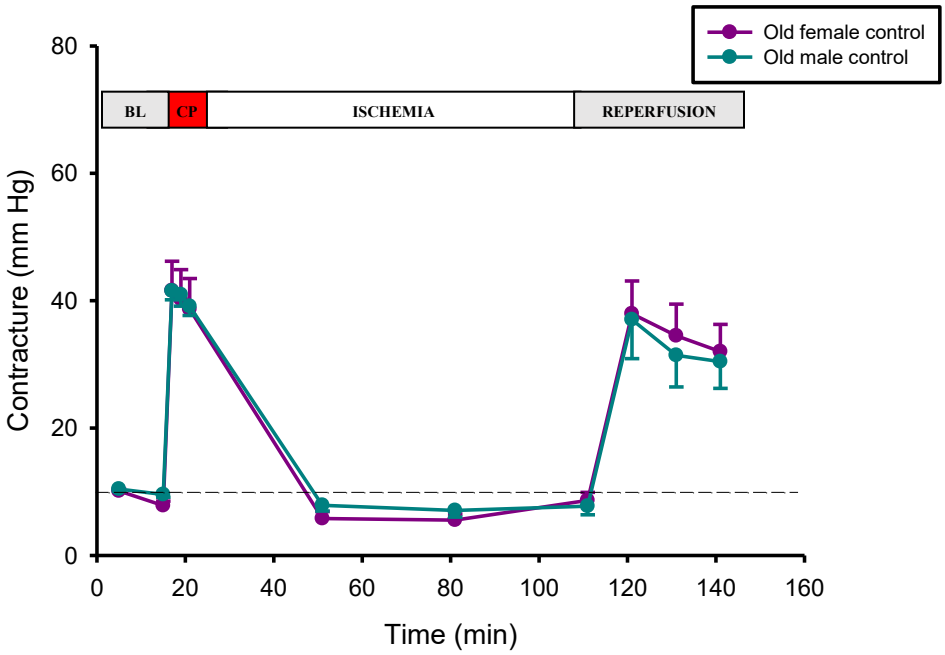


Figure 3.14 The contracture level in reperfusion was similar in old female and male mouse hearts. In reperfusion, similar contracture levels were observed in old female and male mouse hearts. Old female control n=5; old male control n=6. 2-way RM ANOVA.

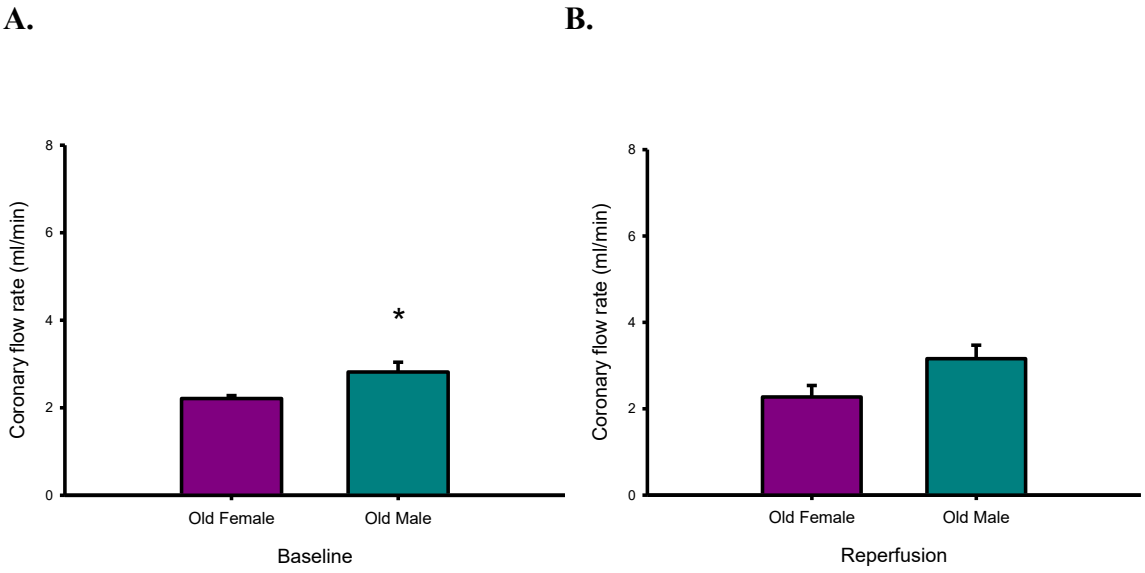


Figure 3.15 The baseline coronary flow rate was better in old male compared to old female mouse hearts; however, no difference in flow rate was seen in reperfusion. **(A)** In control hearts, the baseline coronary flow rate was higher in old male mouse hearts compared to old females (* $p < 0.05$). **(B)** However, in reperfusion, the coronary flow rate was similar in old male and female mouse hearts. Old female n=5; old male n=6. Student's t-test.

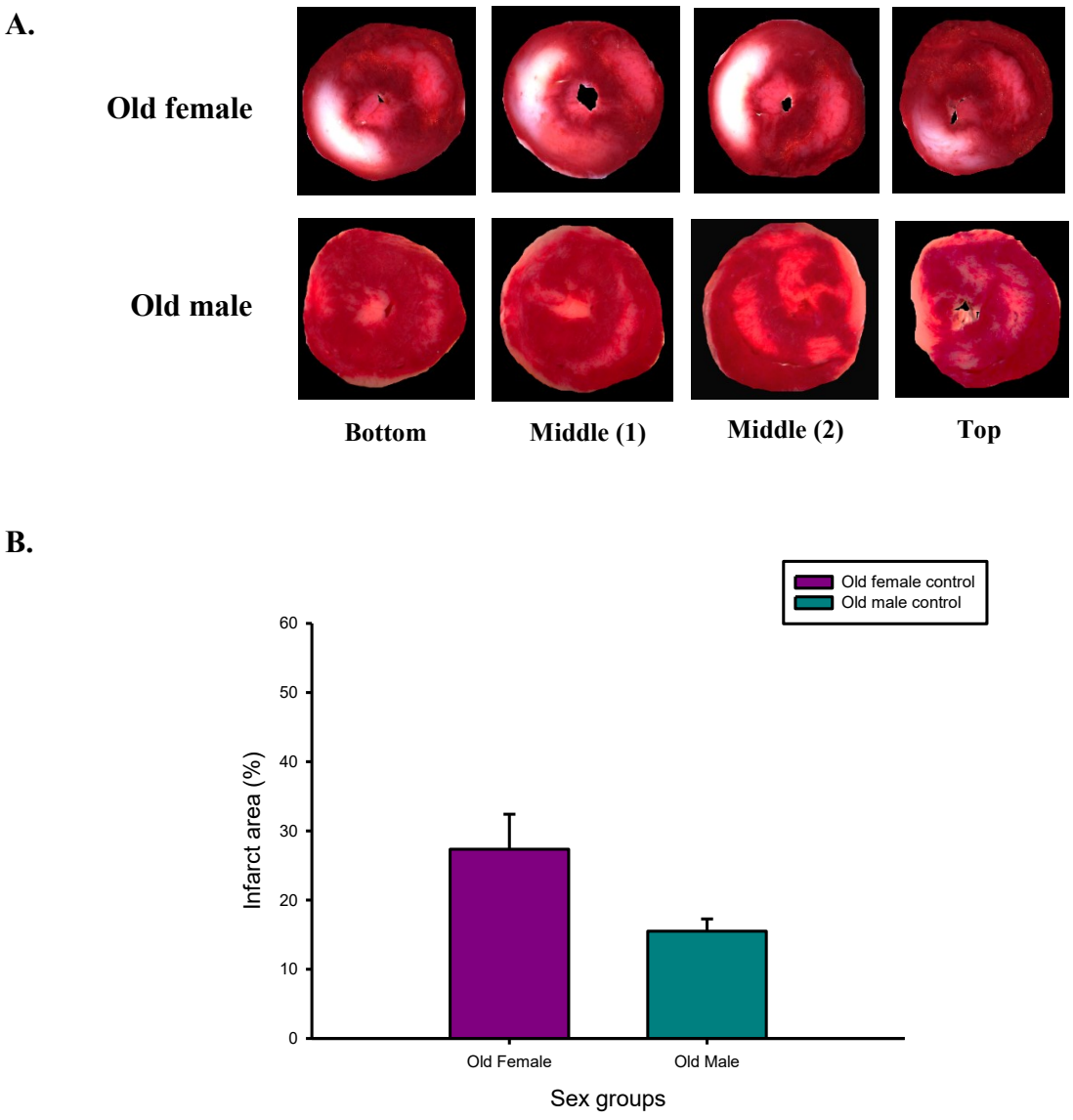


Figure 3.16 The infarct area did not differ between old female and old male mouse hearts. (A) Heart slices from the bottom (apex), middle and top (base) were taken from old female and male hearts and their infarct areas were compared. **(B)** No differences in the percentage of infarct area were observed between old female and male mouse hearts. Old female control n=5; old male control n=6. Student's t-test.

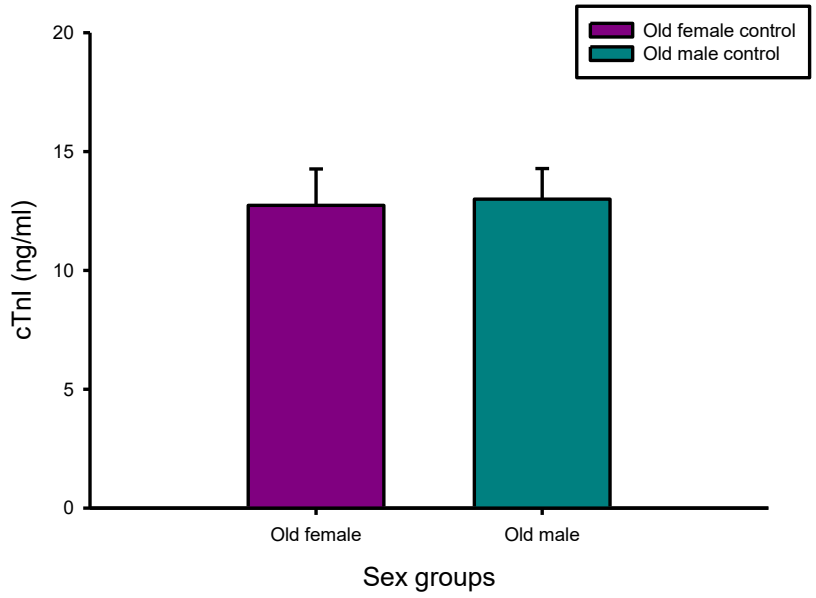


Figure 3.17 The concentration of cTnI released did not differ between the sexes in the older group. There was no significant difference in the concentration of cTnI released in effluents collected during reperfusion (at 141 min). Old female control n=5; adult male control n=6. Student's t-test.

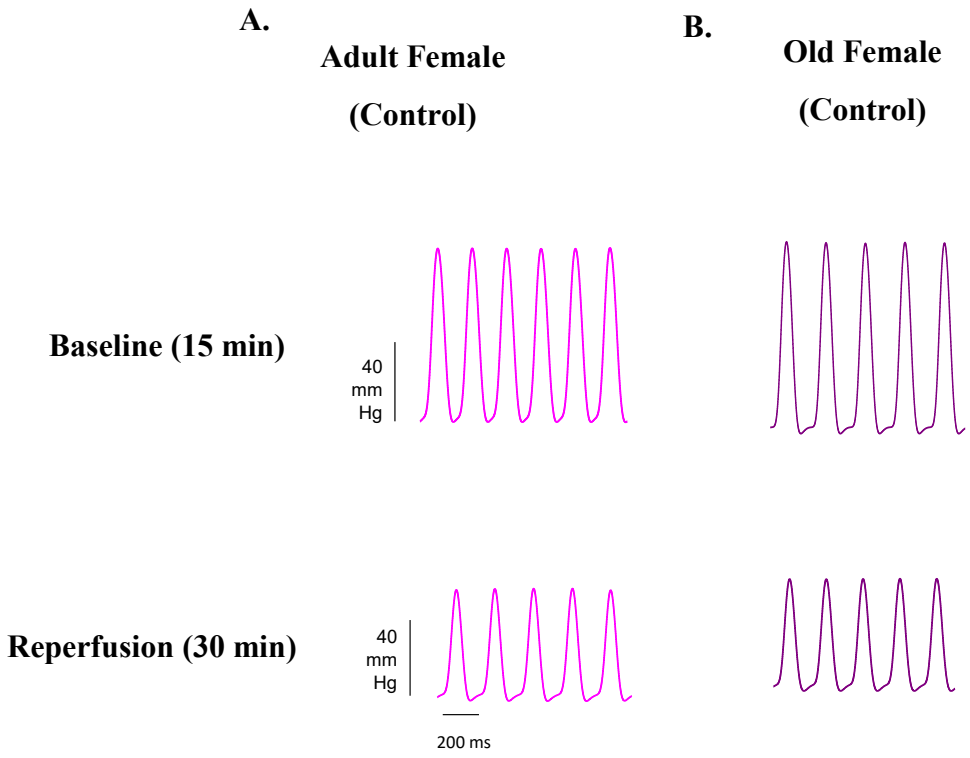
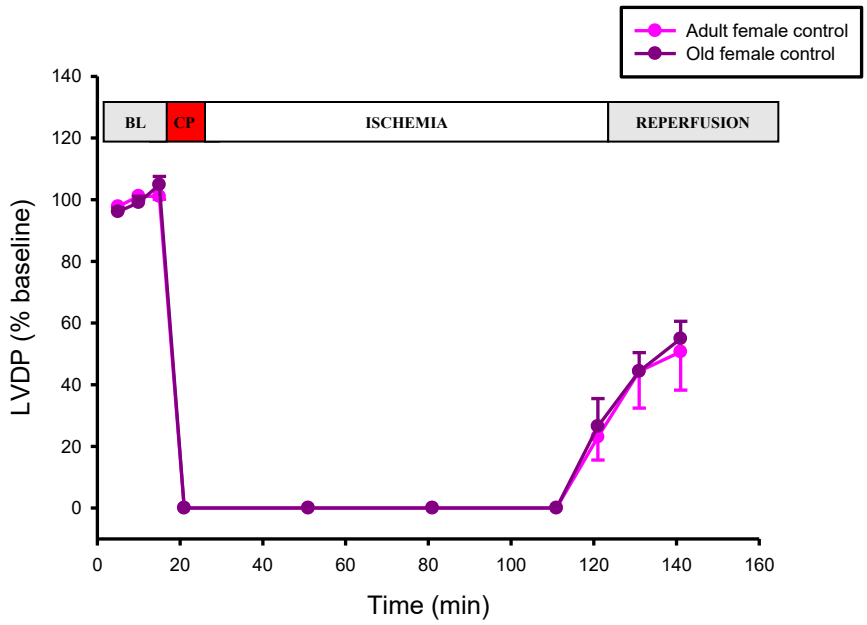


Figure 3.18 Representative traces from adult and old female mouse hearts. Contractile function recovered similarly in adult **(A)** and old **(B)** female mouse hearts in reperfusion.

A.



B.

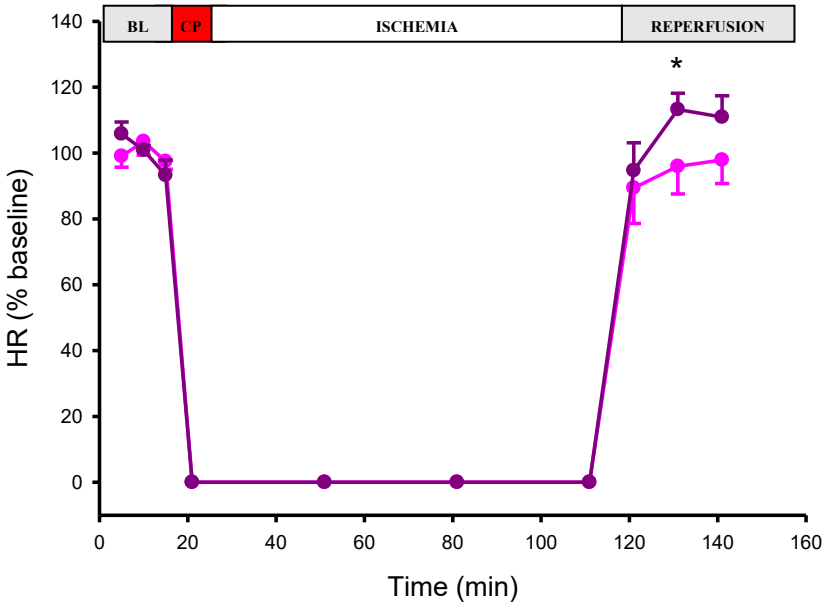
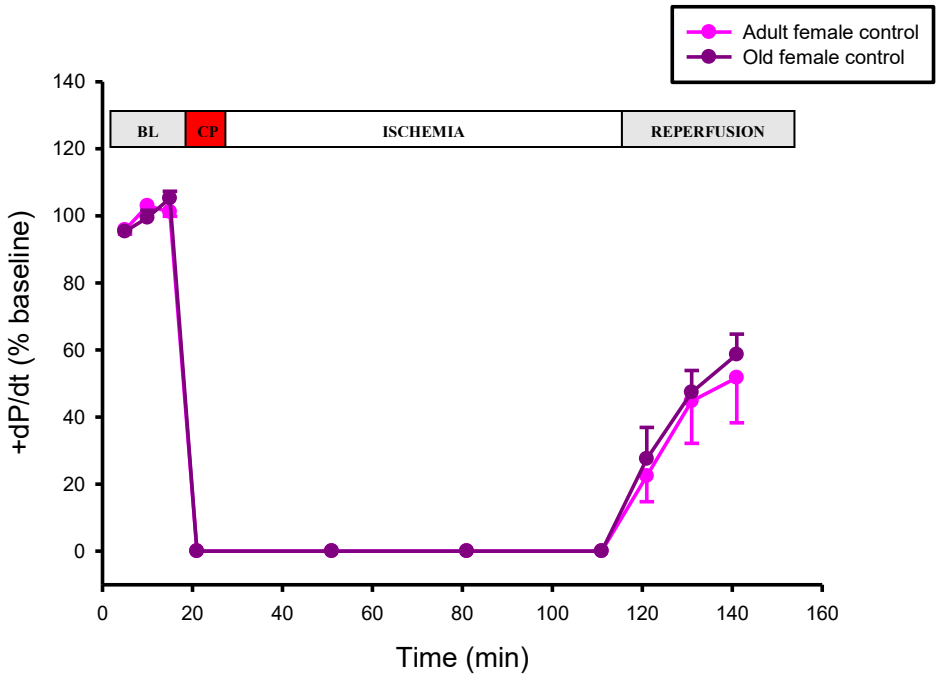


Figure 3.19 Comparison of LVDP and HR between adult and old female mouse hearts. **(A)** Recovery of LVDP was similar in both adult and old female mouse hearts in reperfusion. **(B)** HR recovered completely during reperfusion, although HR in old female hearts was significantly higher than in adult females at 131 min of reperfusion (* $p<0.05$). Adult female control n=6; old female control n=5. 2-way RM ANOVA.

A.



B.

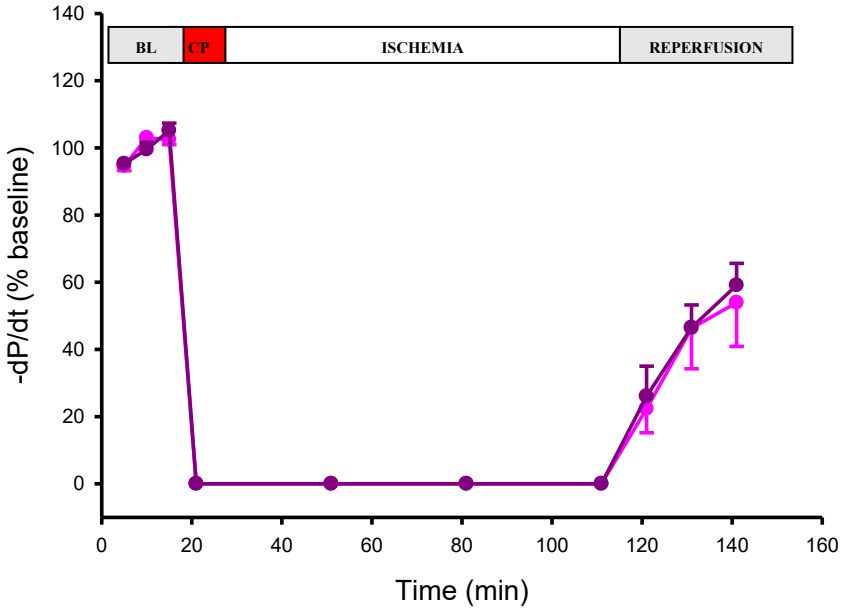


Figure 3.20 +dP/dt and –dP/dt recovered similarly in adult and old female hearts in reperfusion. (A) +dP/dt and **(B)** –dP/dt showed similar extent of recovery at all three time points in reperfusion in hearts from adult and old female mice. Adult female control n=6; old female control n=5. 2-way RM ANOVA.

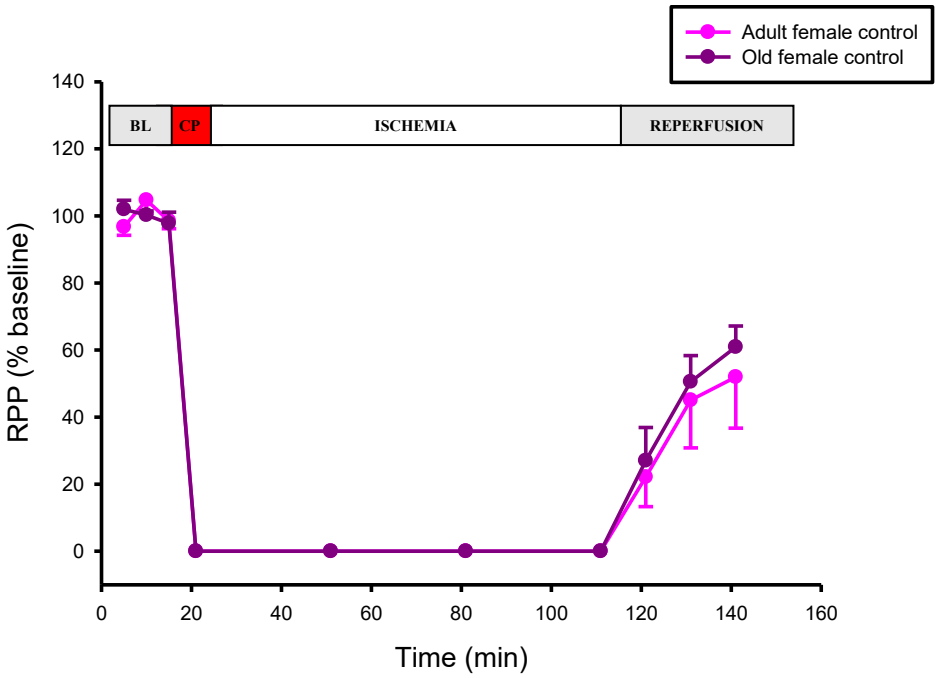


Figure 3.21 The recovery of RPP was similar in adult and old female mouse hearts in reperfusion. Both adult and old female mouse hearts exhibited similar recovery of RPP. Adult female control n=6; old female control n=5. 2-way RM ANOVA.

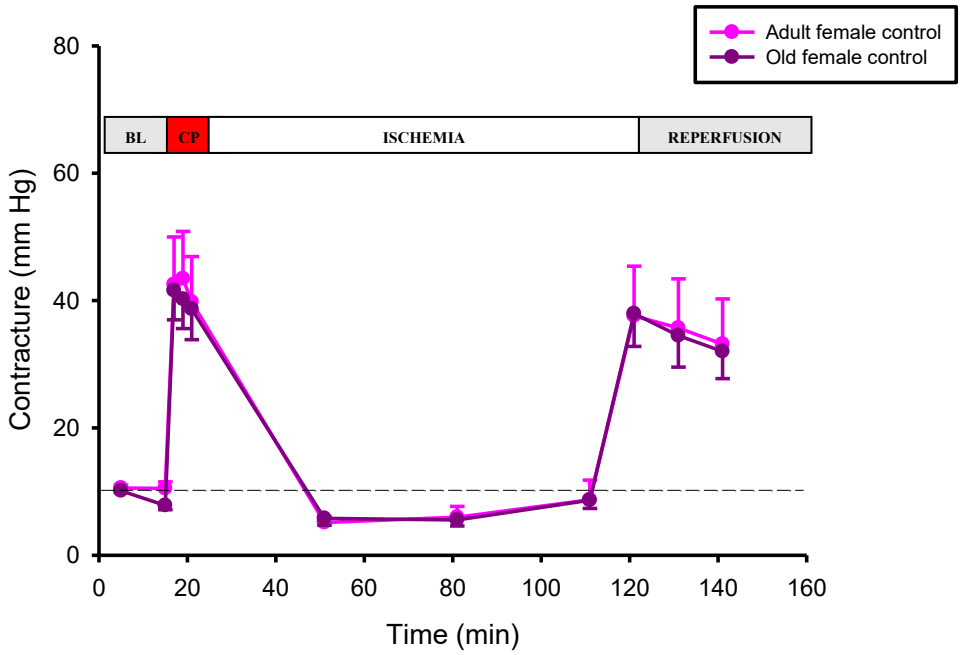


Figure 3.22 The level of contracture in adult and old female mouse hearts was similar in reperfusion. No difference was observed in the level of contracture thoughout the experimental protocol in adult and old female mouse hearts. Adult female control n=6; old female control n=5. 2-way RM ANOVA.

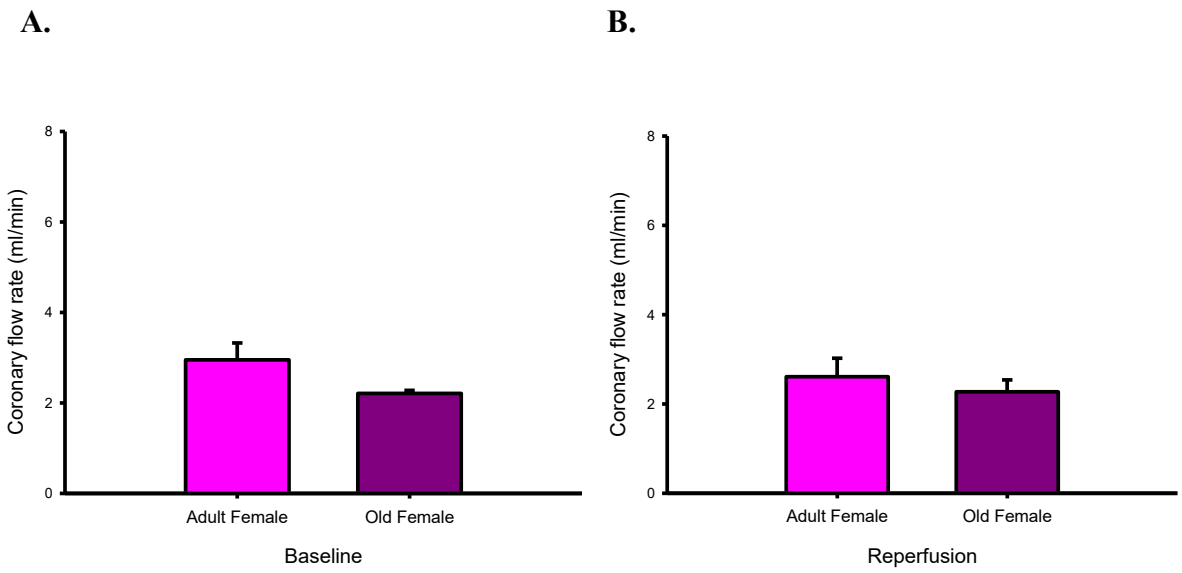
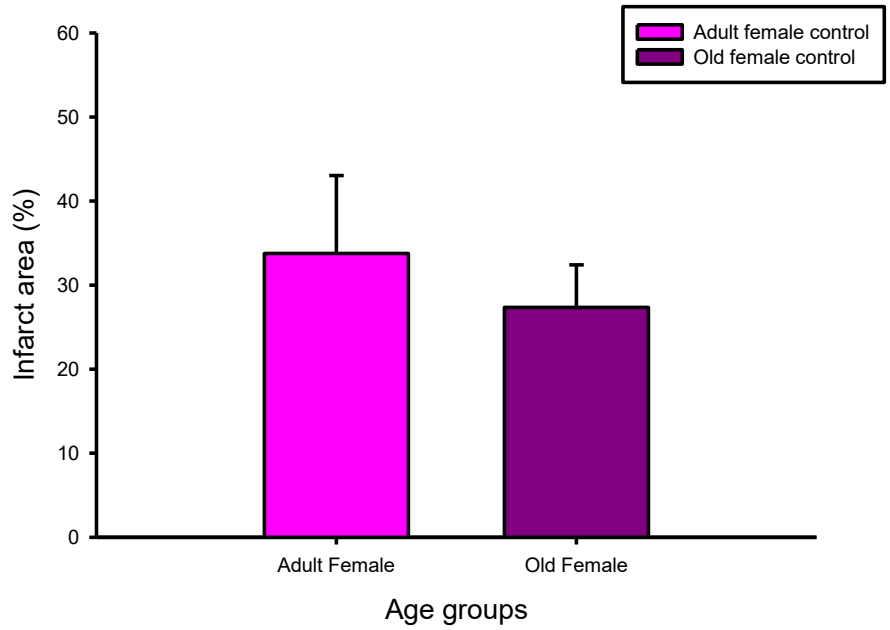


Figure 3.23 No change in coronary flow rate in baseline and reperfusion between adult and old female mouse hearts. There was no difference in the coronary flow rate between adult and old female mouse hearts in baseline **(A)** as well as in reperfusion **(B).** Adult female n=6; old female n=5. Student's t-test.

A.



B.

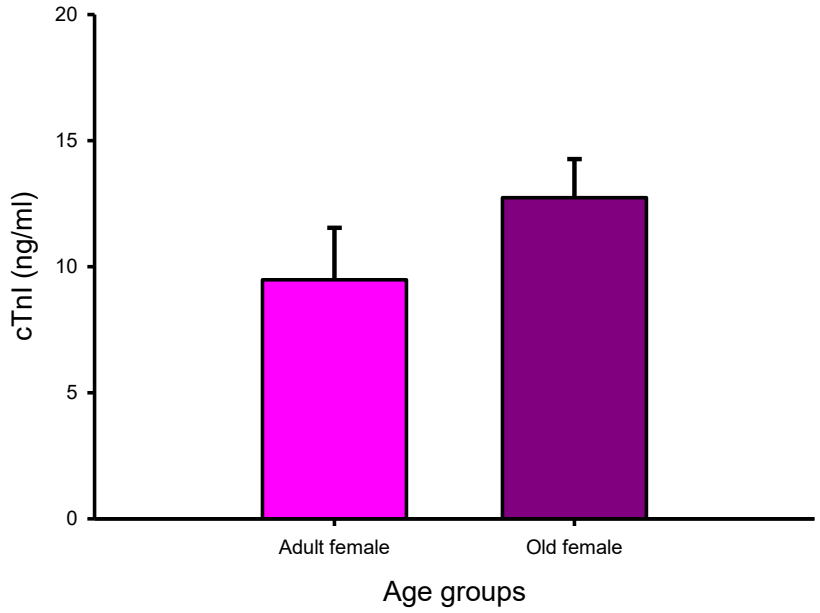


Figure 3.24 The infarct area and concentration of cTnI released did not differ between adult and old female mouse hearts. (A) No significant difference in infarct area was seen when adult female hearts were compared to old female hearts. Adult female control n=4, old female control n=5. **(B)** There was no significant difference in the extent of cTnI release in reperfusion between adult and old female effluent samples. Adult female control n=6; old female control n=5. Student's t-test.

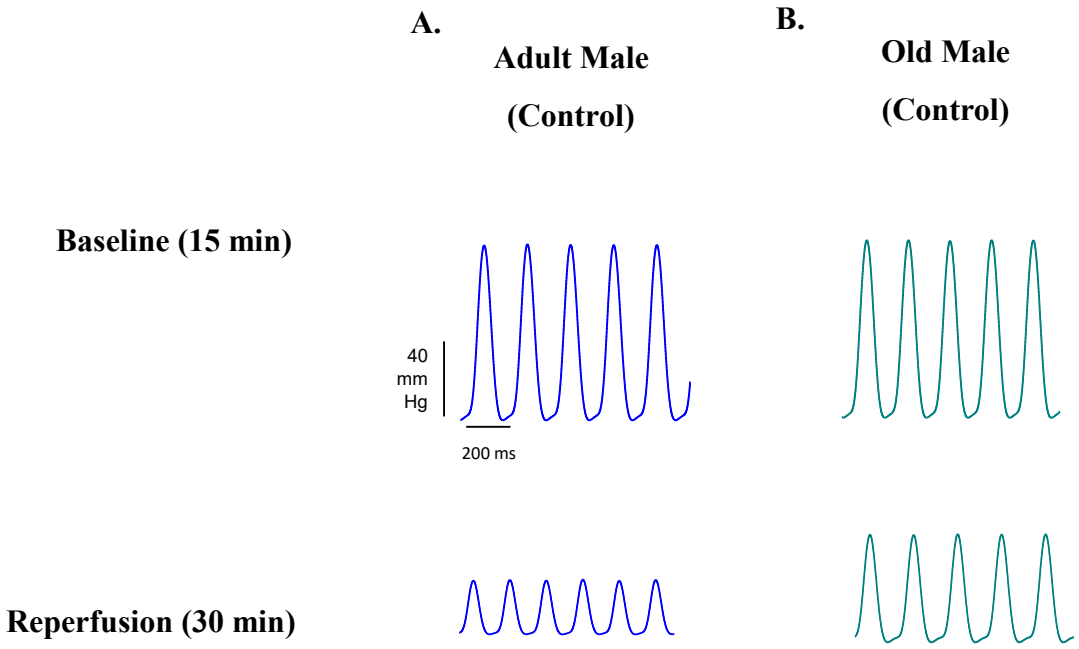
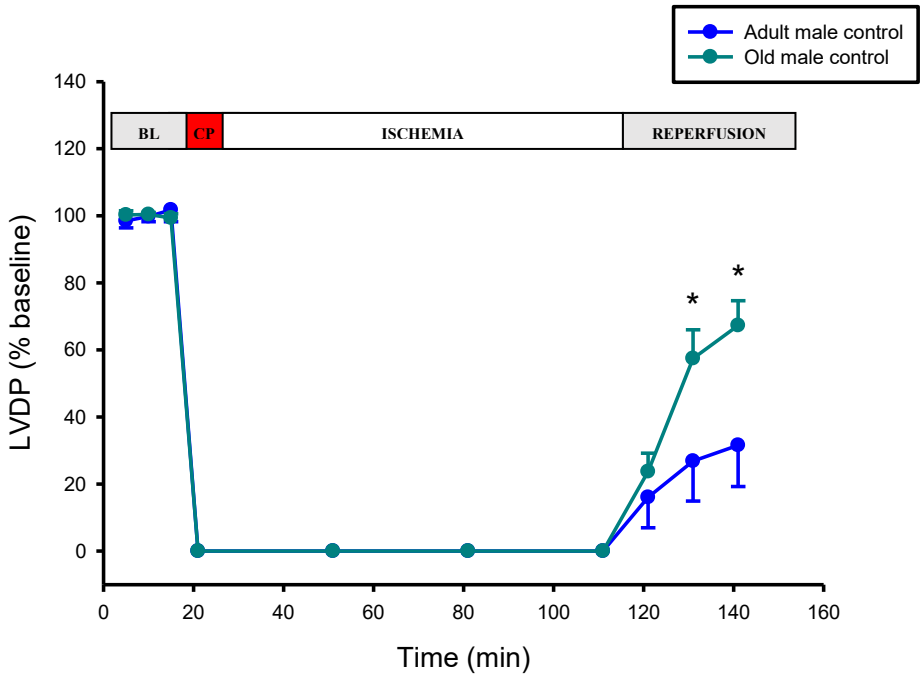


Figure 3.25 Representative experimental recordings from adult and old male mouse hearts. Recovery of contractile function after I/R injury was worse in hearts from adult males **(A)** than in old males **(B)** when compared to their respective baseline traces.

A.



B.

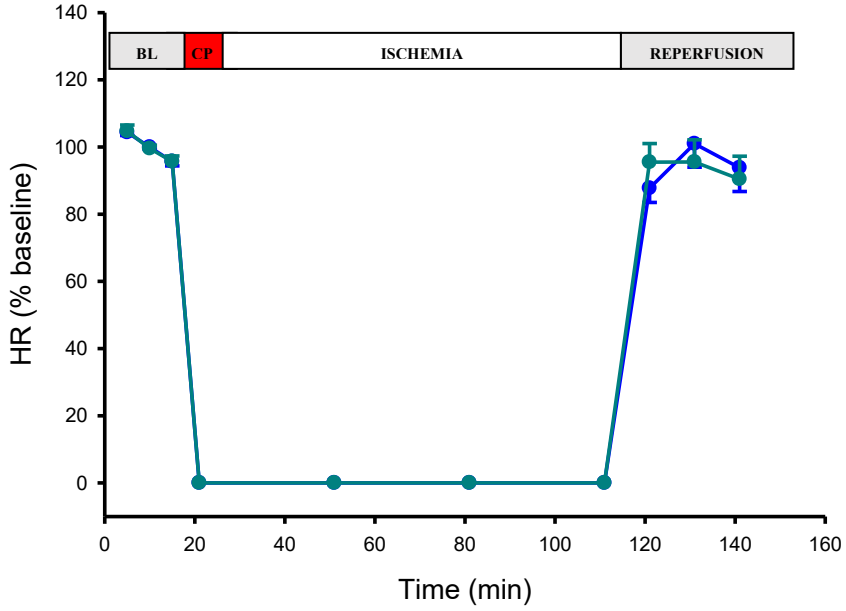


Figure 3.26 Differences in the recovery of LVDP and HR in adult and old male mouse hearts. (A) LVDP recovered significantly better in old male hearts compared to adult male hearts in reperfusion (* $p < 0.05$). **(B)** By contrast, HR recovered completely during reperfusion regardless of age. Adult male control n=6; old male control n=6. 2-way RM ANOVA.

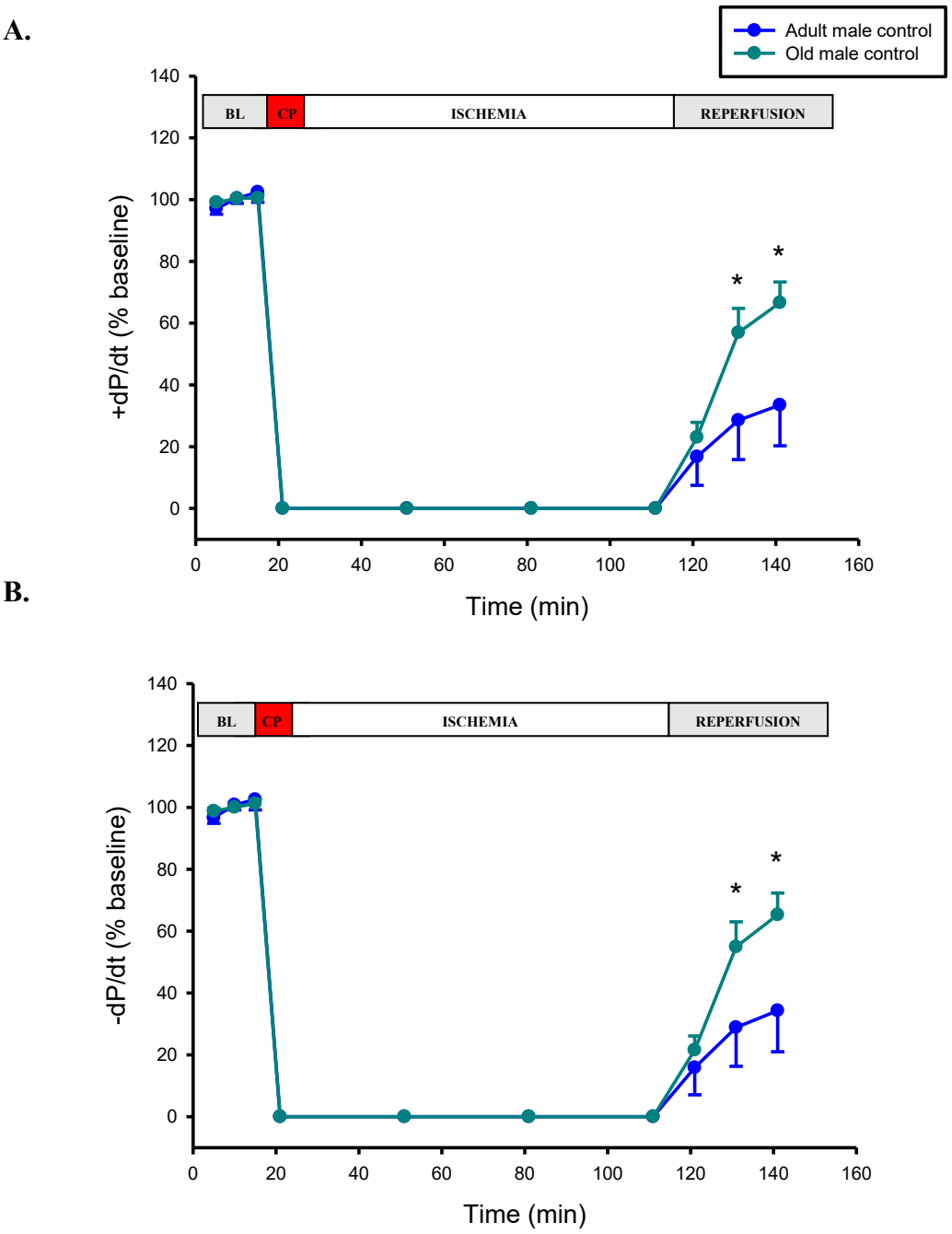


Figure 3.27 Recovery of +dP/dt and –dP/dt in adult and old male mouse hearts in reperfusion. (A) +dP/dt and **(B)** –dP/dt recovered significantly better in old male mouse hearts in reperfusion when compared to the recovery in adult male mouse hearts. Adult male control n=6; old male control n=6. 2-way RM ANOVA.

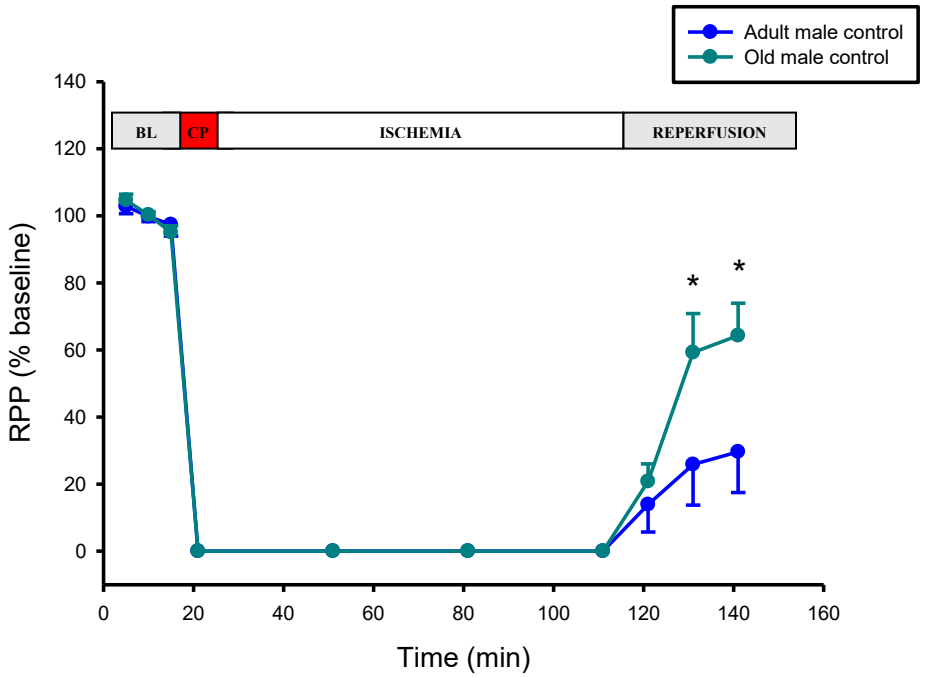


Figure 3.28 Recovery of the RPP was better in old male hearts than in adult male hearts in reperfusion. Old male hearts recovered significantly better in reperfusion compared to adult male hearts when the RPP was calculated (* $p < 0.05$). Adult male control n=6; old male control n=6. 2-way RM ANOVA.

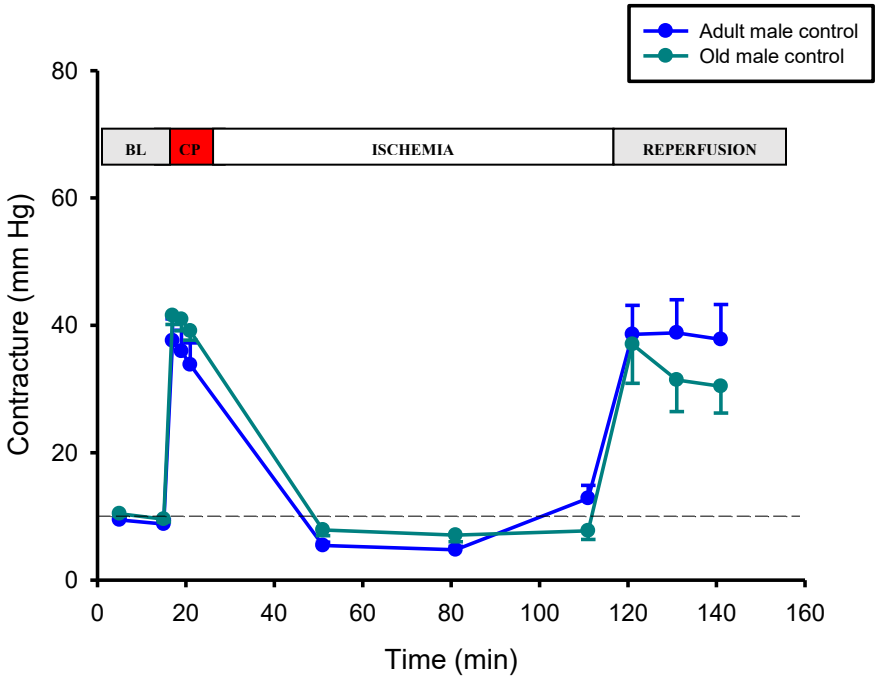


Figure 3.29 The level of contracture did not differ between adult and old male hearts in reperfusion. No significant difference in contracture was seen when adult male hearts were compared to old male hearts at any time point in the experiment. Adult male control n=6; old male control n=6. 2-way RM ANOVA.

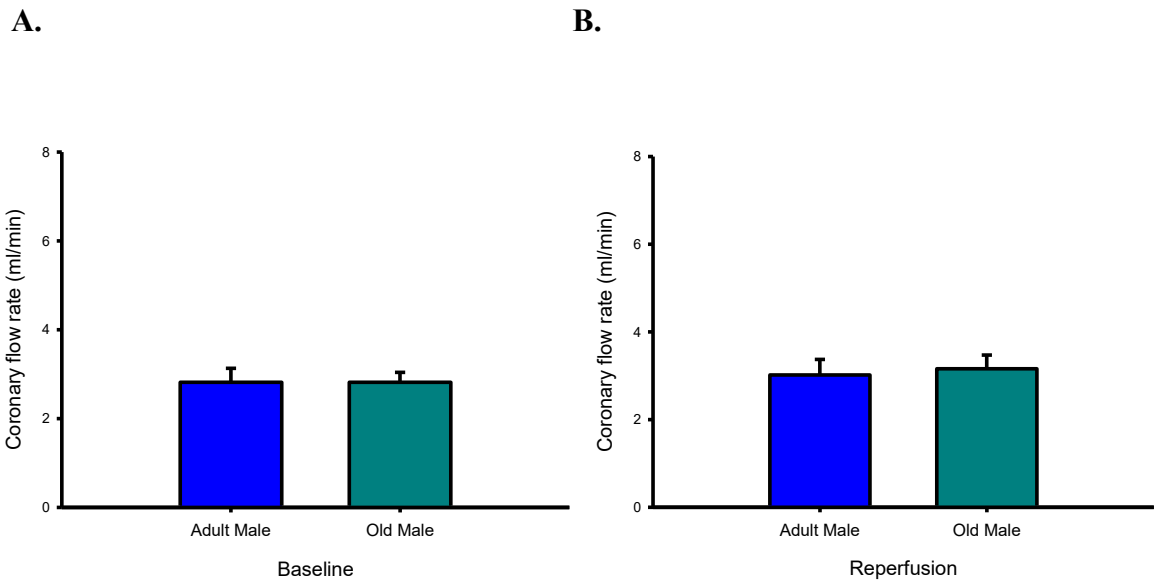


Figure 3.30 The coronary flow rate was similar between adult and old male mouse hearts in baseline and reperfusion. In control hearts from adult and old male mice, there was no difference in coronary flow rate in baseline **(A)** as well as in reperfusion **(B)**. Adult male n=6; old male n=6. Student's t-test.

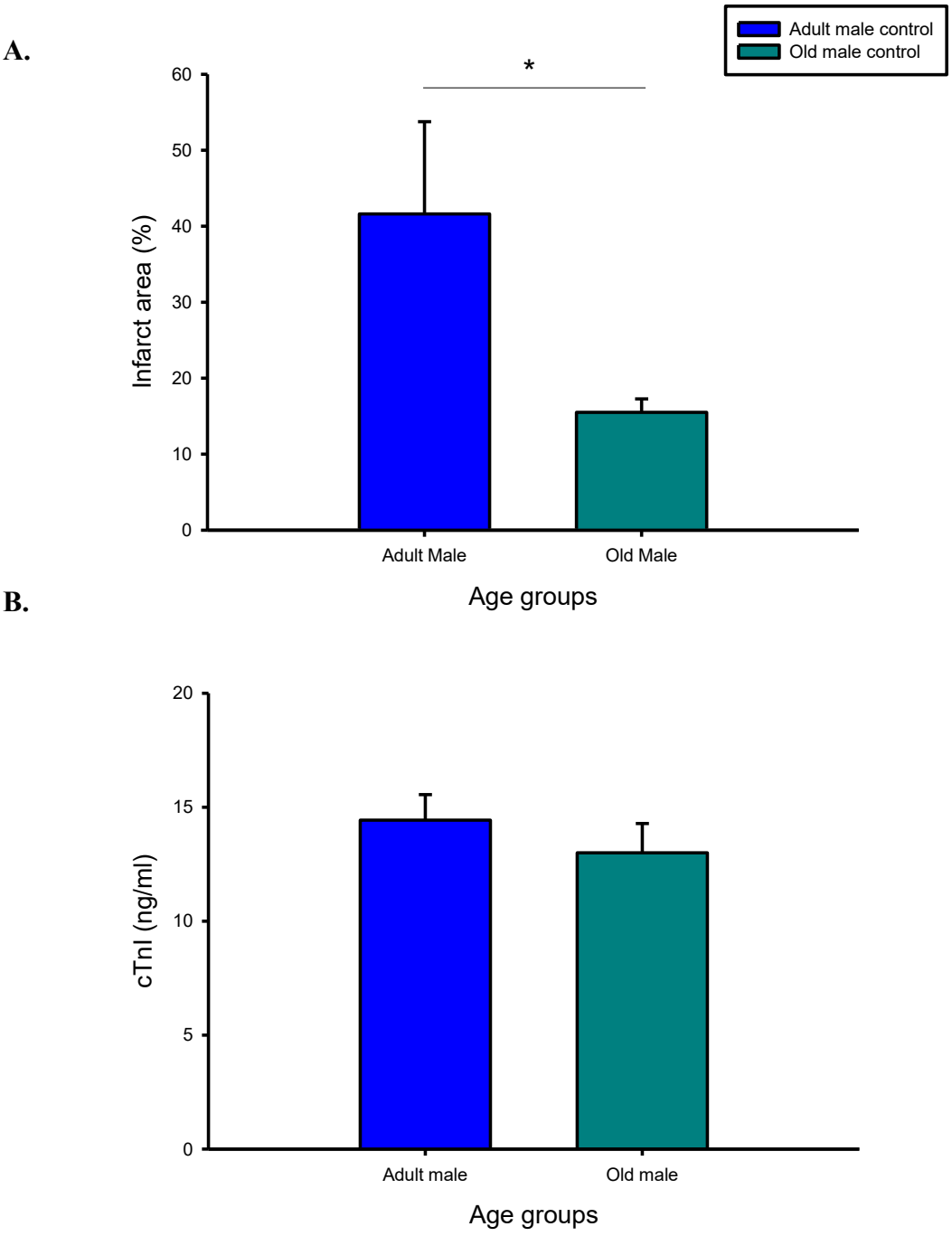


Figure 3.31 Comparison of the infarct area and the concentration of cTnI released in adult and old male mouse hearts. (A) A significant reduction in infarct area was seen in old male mouse hearts compared to adult male hearts (* $p < 0.05$). Adult male control n=3, old male control n=6. **(B)** However, in terms of cTnI release between the two groups, there was no significant difference. Adult male control n=6, old male control n=6. Student's t-test.

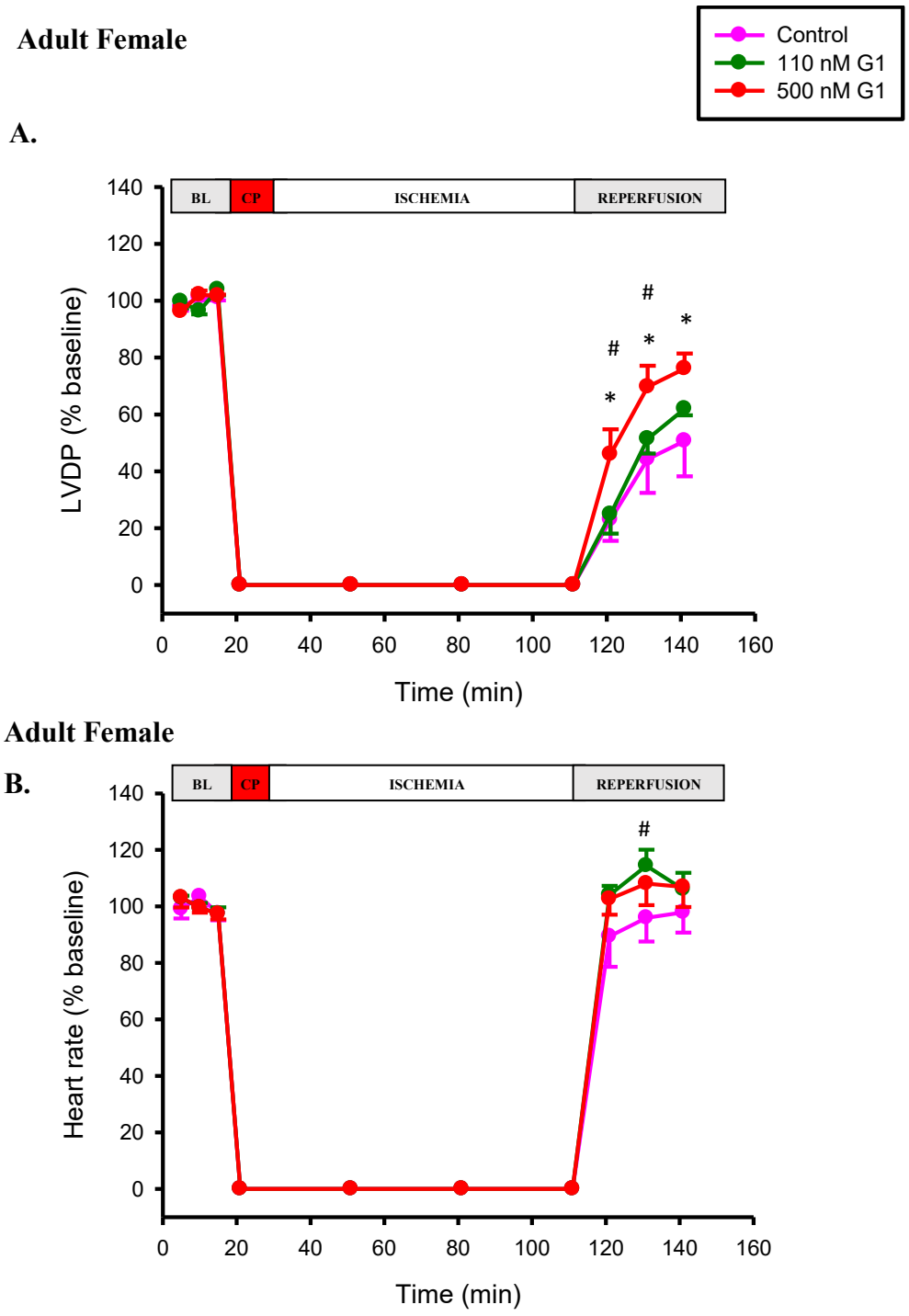
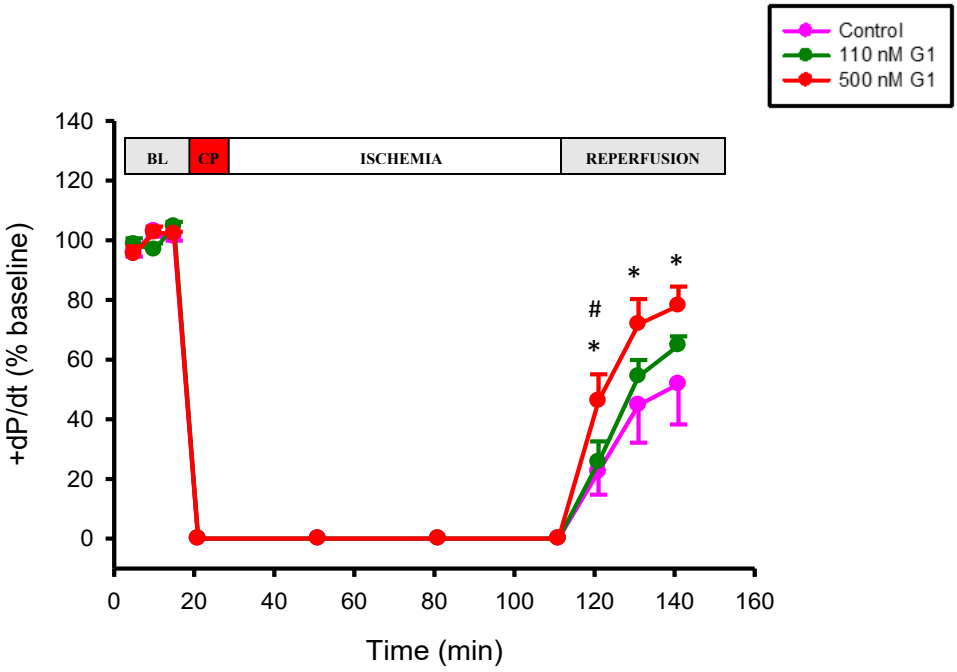


Figure 3.32 Recovery of LVDP and HR in adult female mice when hearts were perfused with cardioplegia with or without G1 (110 nM or 500 nM). (A) Recovery of LVDP was significantly better during reperfusion when the heart was perfused with cardioplegia + 500 nM G1 compared to either vehicle control (* $p < 0.05$) or 110 nM G1 (# $p < 0.05$). **(B)** However, HR recovered completely in reperfusion, except at the 131 min time point with 110 nM G1, where the HR was significantly higher than in baseline (#$p< 0.05$). Control n=6; 500 nM G1 n=6; 110 nM G1 n=4. 2-way RM ANOVA.

Adult Female

A.



Adult Female

B.

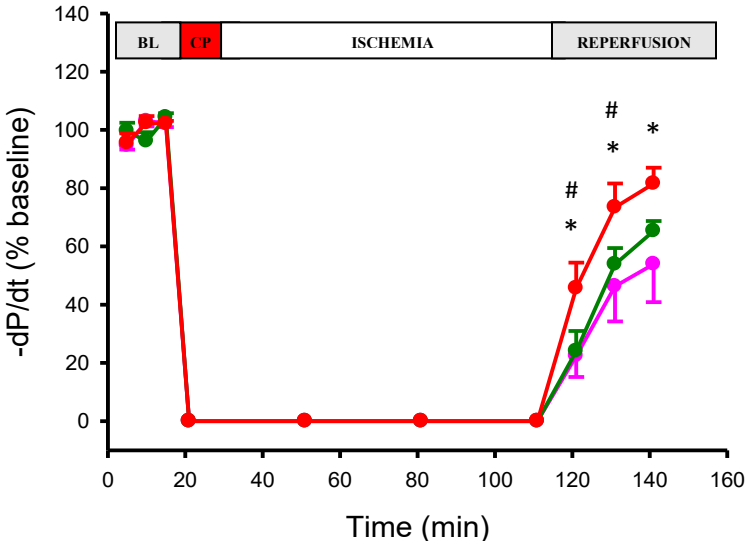
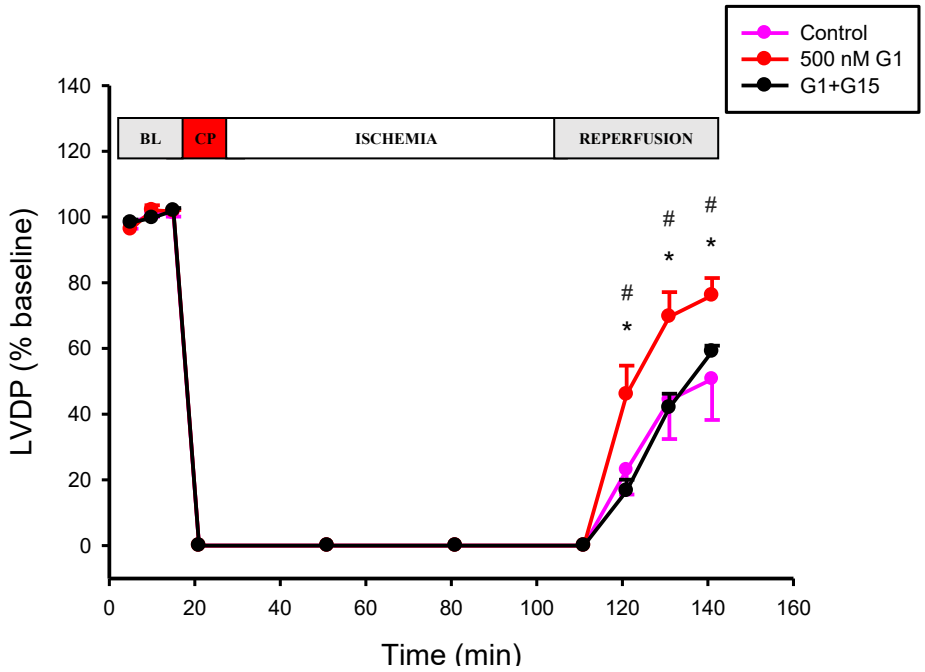


Figure 3.33 Recovery of +dP/dt and –dP/dt in adult female mice when hearts were perfused with cardioplegia with or without G1 (110 nM and 500 nM). Recovery of +dP/dt **(A)** and –dP/dt **(B)** was significantly better during reperfusion when the heart was perfused with cardioplegia + 500 nM G1, when compared to vehicle control (* $p < 0.05$) or 110 nM G1 (# $p < 0.05$). Control n=6; 500 nM G1 n=6; 110 nM G1 n=4. 2-way RM ANOVA.

Adult Female

A.



Adult Female

B.

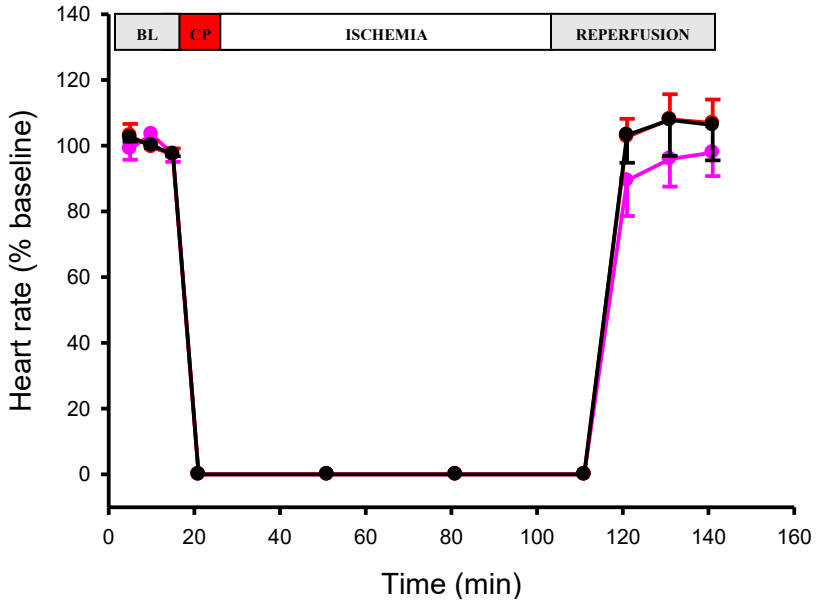
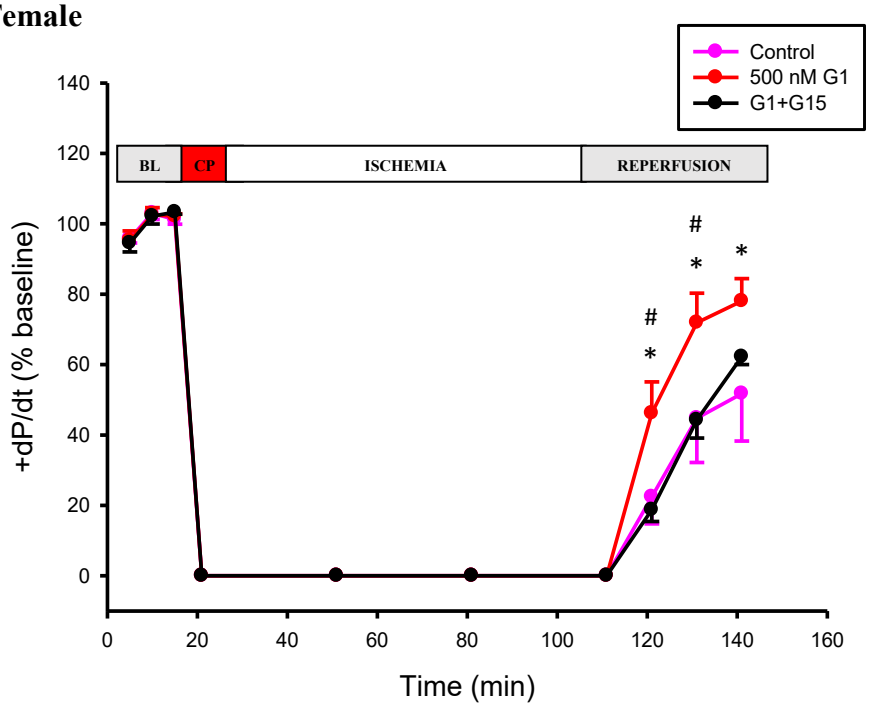


Figure 3.34 G15 blocked the beneficial effects of G1 on LVDP during reperfusion but G15 had no effect on HR in adult female mouse hearts. (A) Recovery of LVDP was significantly better during reperfusion when the heart was perfused with cardioplegia + G1 compared to vehicle control (* $p < 0.05$); this effect was blocked by G15 (1 µM; # $p < 0.05$). **(B)** In contrast, HR recovered completely in reperfusion regardless of drug. Control n=6; 500 nM G1 n=6; G1+G15 n=5. 2-way RM ANOVA.

Adult Female

A.



Adult Female

B.

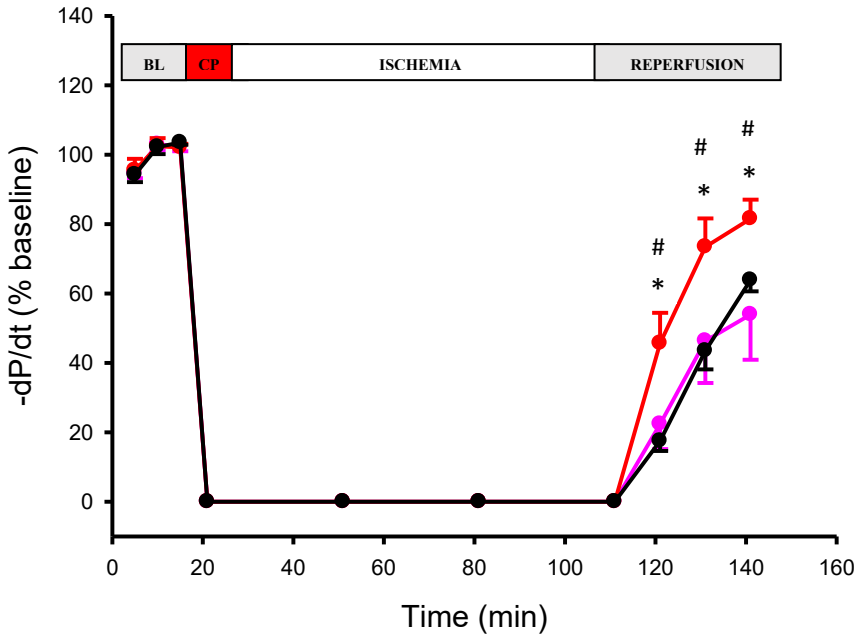


Figure 3.35 G15 blocked the beneficial effects of G1 on +dP/dt and –dP/dt during reperfusion in hearts from adult females. (A) Recovery of +dP/dt was significantly better during reperfusion when hearts were perfused with cardioplegia + G1 compared to vehicle control (* $p < 0.05$); this effect was blocked by G15 (1 µM; # $p < 0.05$). **(B)** Similar results were seen when –dP/dt was measured. Control n=6; 500 nM G1 n=6; G1+G15 n=5. 2-way RM ANOVA.

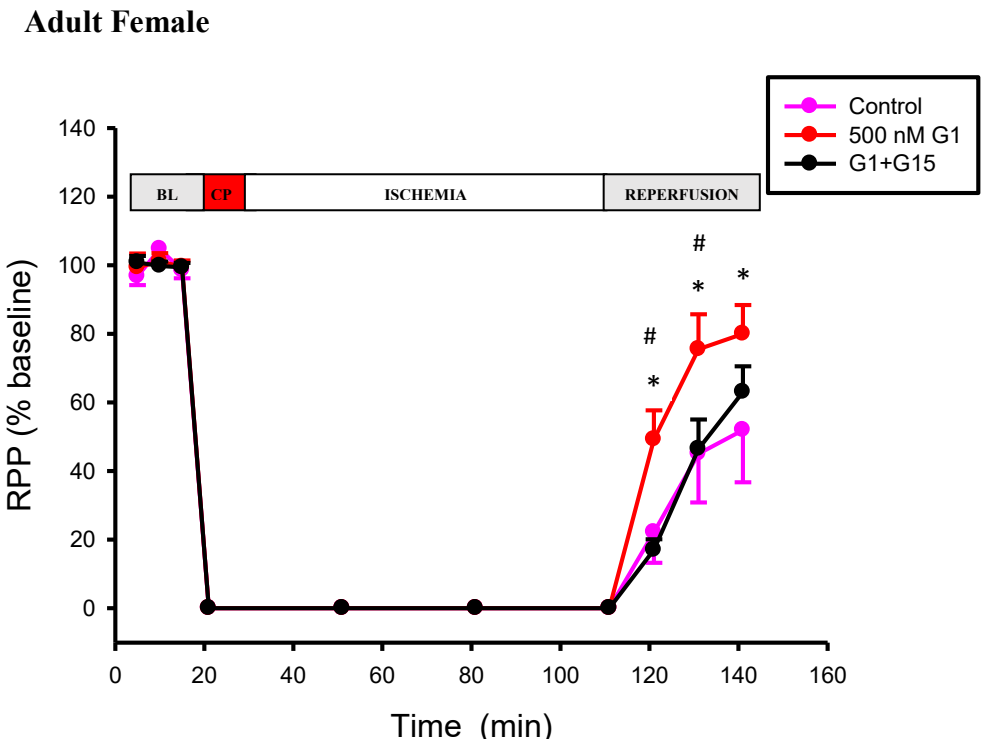


Figure 3.36 Recovery of RPP was better in hearts treated with G1 compared to either control or G1+G15 group in hearts from female mice. RPP recovered significantly better with cardioplegia + G1 in reperfusion compared to the control group (* $p<0.05$); this effect was blocked by G15 (1 µM; # $p < 0.05$) at the 121 and 131 min time points in reperfusion. Control n=6; 500 nM G1 n=6; G1+G15 n=5. 2-way RM ANOVA.

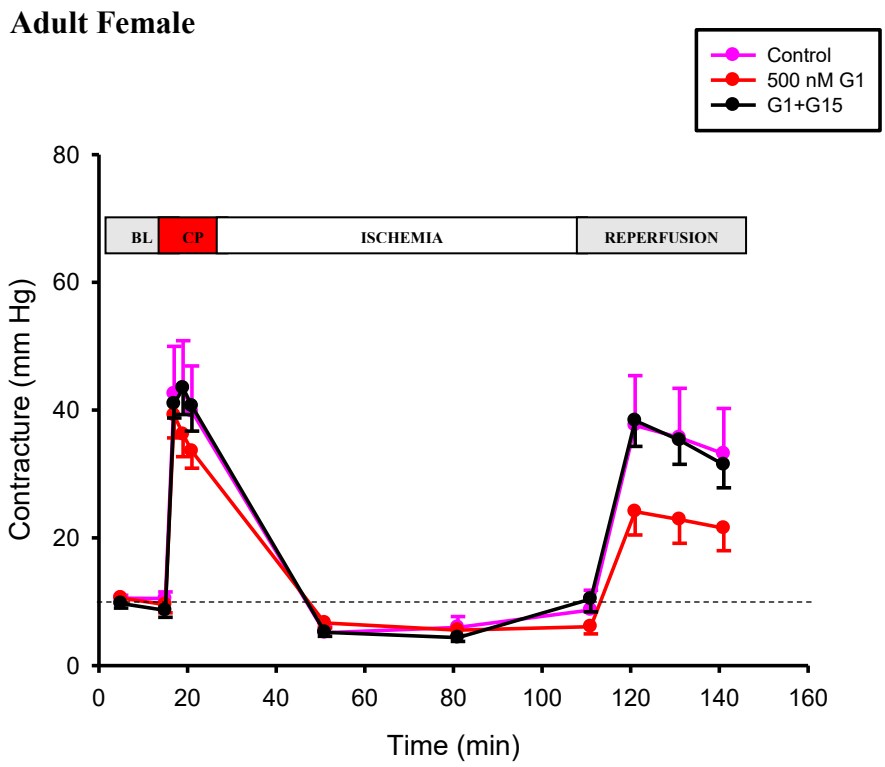


Figure 3.37 G1 did not affect the contracture level compared to either the vehicle control or hearts perfused with G1+G15 in female mouse hearts. The minimum baseline LV pressure was maintained at 10 ± 2 mm Hg; a rapid cooling contracture was seen during hypothermic cardioplegia perfusion. The heart was then subjected to 90 min of ischemia where it relaxed. This was followed by 30 min reperfusion, which was accompanied by contracture. There was no significant reduction in contracture in the G1 group compared to control and G1+G15 groups. Control n=6; G1 n=6; G1+G15 n=5. 2-way RM ANOVA.

Adult Female

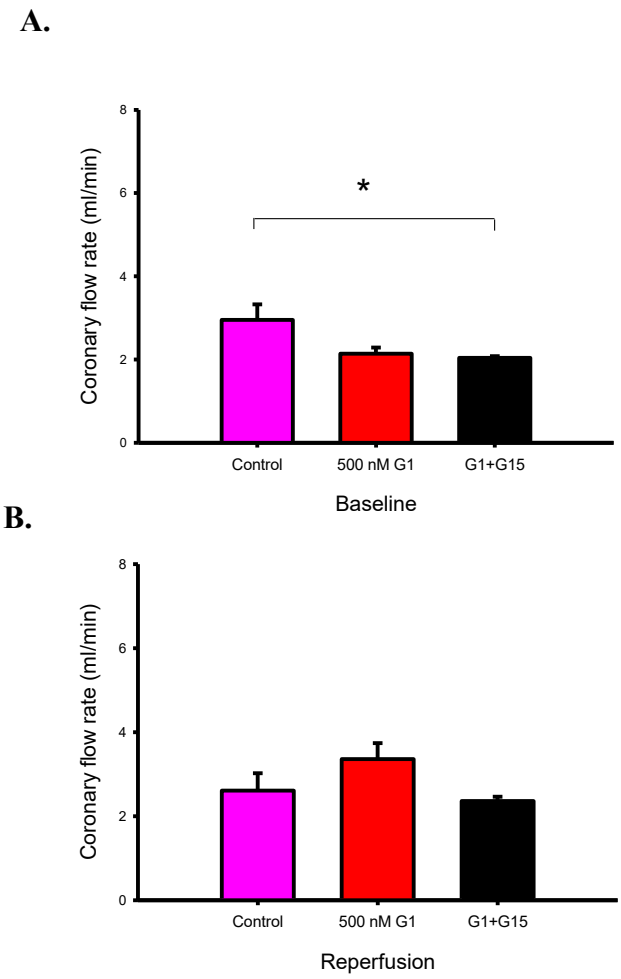
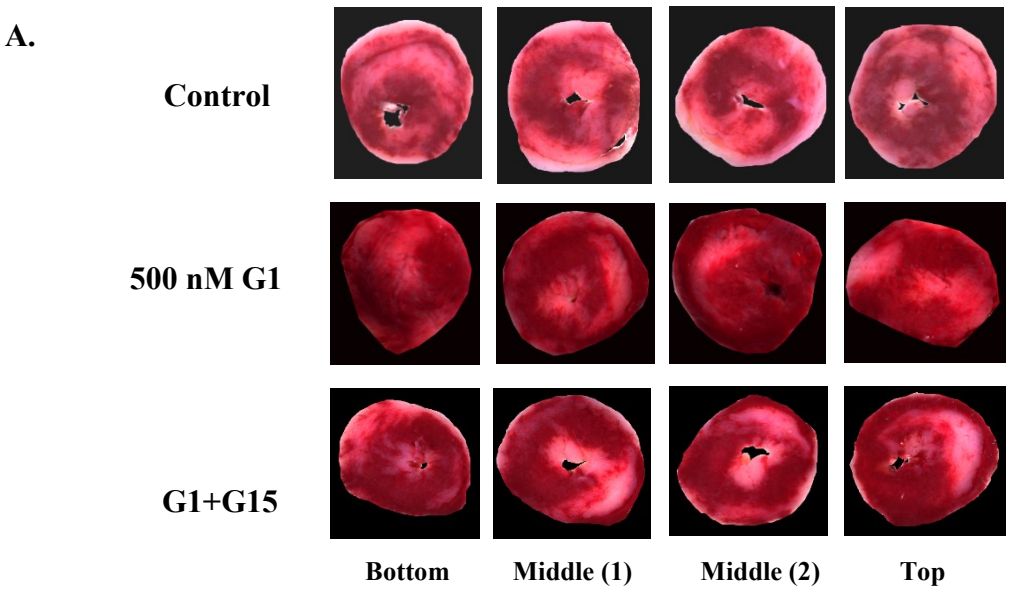


Figure 3.38 Coronary flow rate was not affected by G1 in reperfusion compared to control or G1+G15 treated hearts. (A) No significant change in flow rate was observed in baseline between control and G1-treated hearts, however, hearts in G1+G15 treated group showed significantly lower flow rate compared to control hearts. **(B)** In reperfusion, G1-treated hearts had higher coronary flow rate compared to control and G1+G15 treated hearts, however this was not statistically significant. Control n=6; 500 nM G1 n=6; G1+G15 n=5. One way ANOVA.

Adult Female

A.



Adult Female

B.

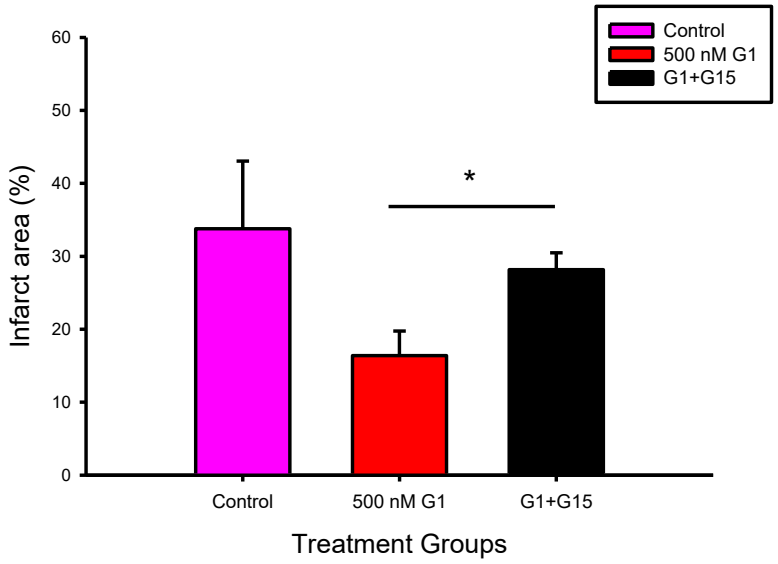


Figure 3.39 G1 did not affect infarct area in adult female hearts. (A) Slices (bottom, middle and top) of hearts treated with vehicle (control), G1 (500 nM) and G1 (500 nM) + G15 (1 μM) were compared for their infarct areas. **(B)** No significant reduction of infarct area was seen when hearts treated with G1 were compared to control hearts; however, infarct area was significantly reduced by G1 when compared to the hearts treated with G1+G15. Control n=4; G1 n=5; G1+G15 n=5. Student's t-test.

Adult Female

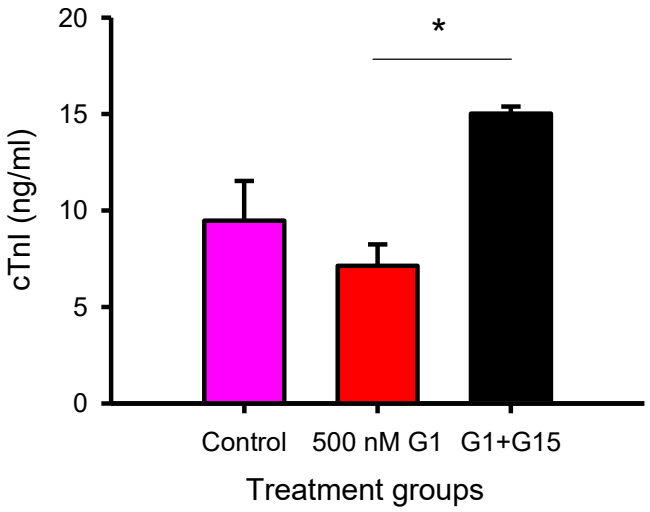
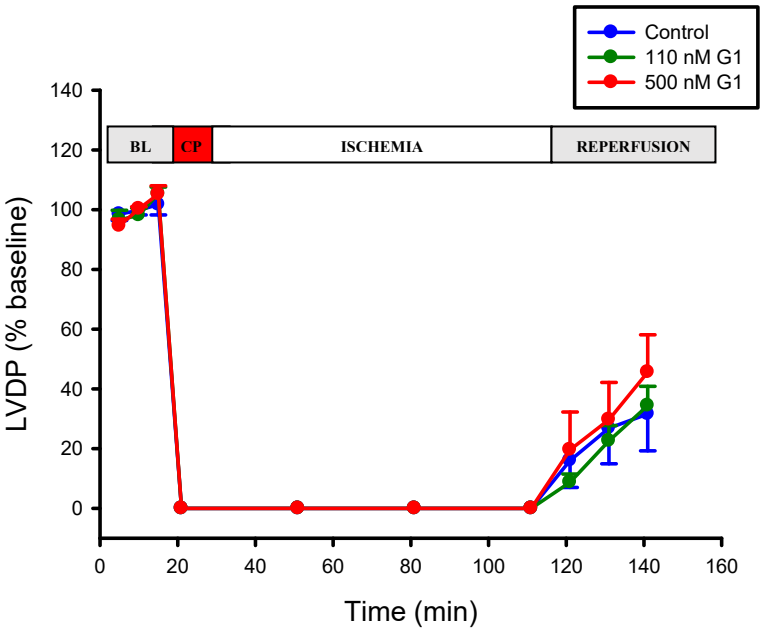


Figure 3.40 Concentration of cTnI released in reperfusion (141 min time point) did not differ between vehicle and G1 but was higher in G1+G15 treated adult female hearts. There was no significant difference in the concentration of cTnI released between control and G1 treated hearts. However, G1+G15 treated hearts had significantly more cTnI released compared to G1 treated hearts. Control n=6; 500 nM G1 n=6; G1+G15 n=4. One way ANOVA.

Adult male

A.



Adult male

B.

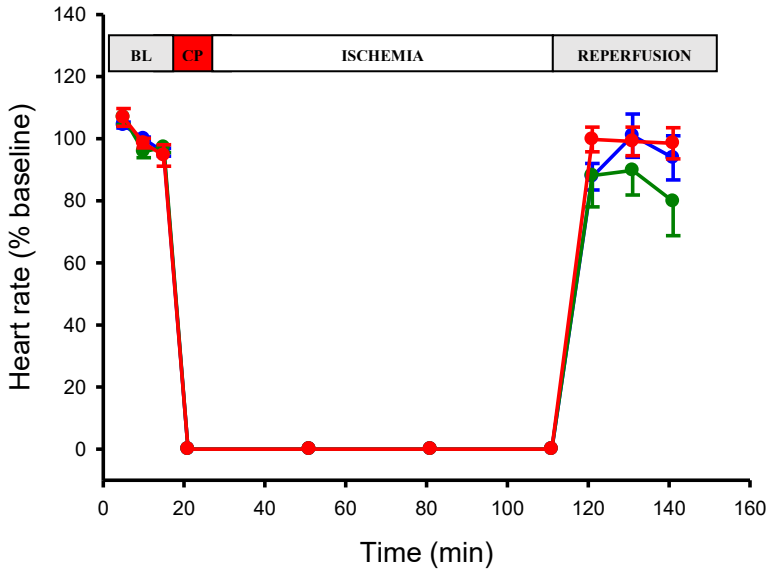
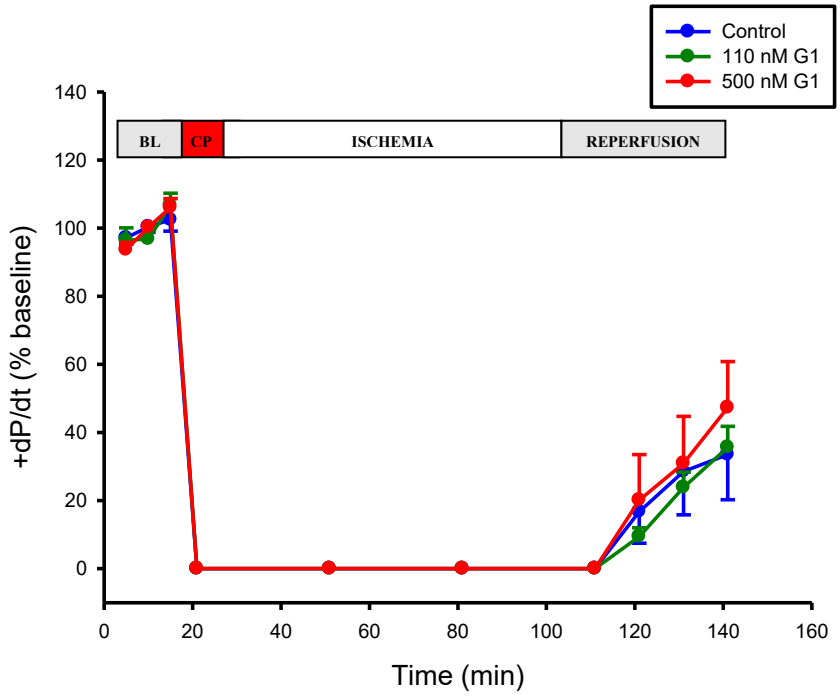


Figure 3.41 G1 had no effect on the recovery of LVDP and HR in adult male hearts. **(A)** Recovery of LVDP in reperfusion was not affected by G1 (110 nM or 500 nM) compared to the control hearts. **(B)** Also, HR did not change, regardless of drug treatment. Control n=6; 110 nM G1 n=3; 500 nM G1 n=5. 2-way RM ANOVA.

Adult male

A.



Adult male

B.

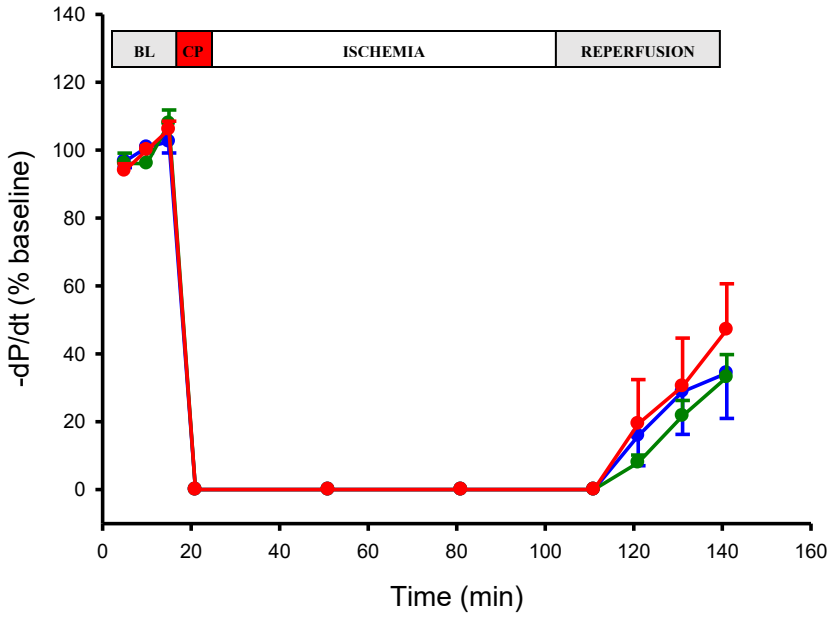


Figure 3.42 G1 had no beneficial effect on recovery of +dP/dt and -dP/dt in adult male hearts when compared to control. (A) Recovery of +dP/dt and **(B)** –dP/dt in reperfusion was not affected by any concentration of G1 (110 nM or 500 nM) when compared to vehicle control. Control n=6; 110 nM G1 n=3; 500 nM G1 n=5. 2-way RM ANOVA.

Adult male

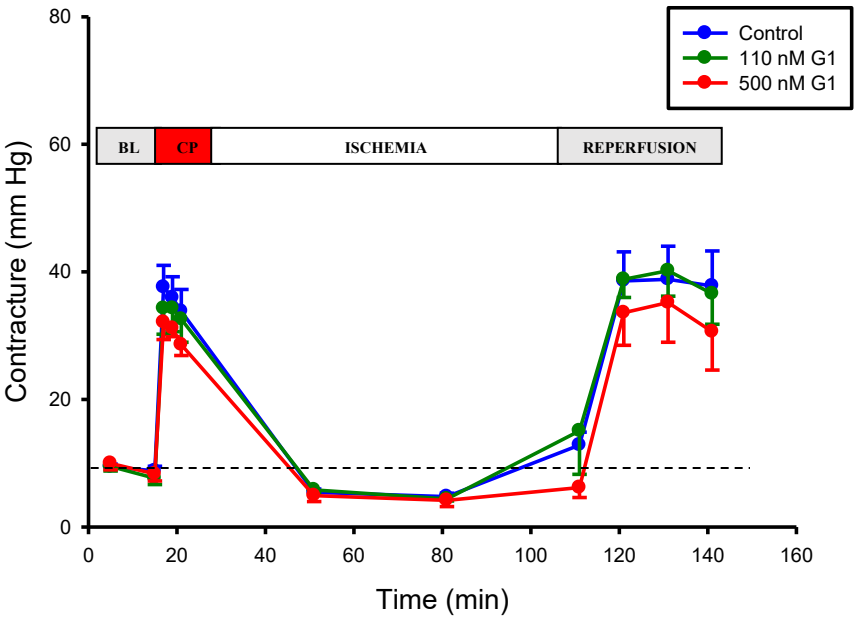


Figure 3. 43 G1 did not affect contracture levels when compared to vehicle control in male mouse hearts. G1 (110 nM or 500 nM) did not affect contracture amplitude. Control n=6; 110 nM G1 n=3; 500 nM G1 n=5. 2-way RM ANOVA.

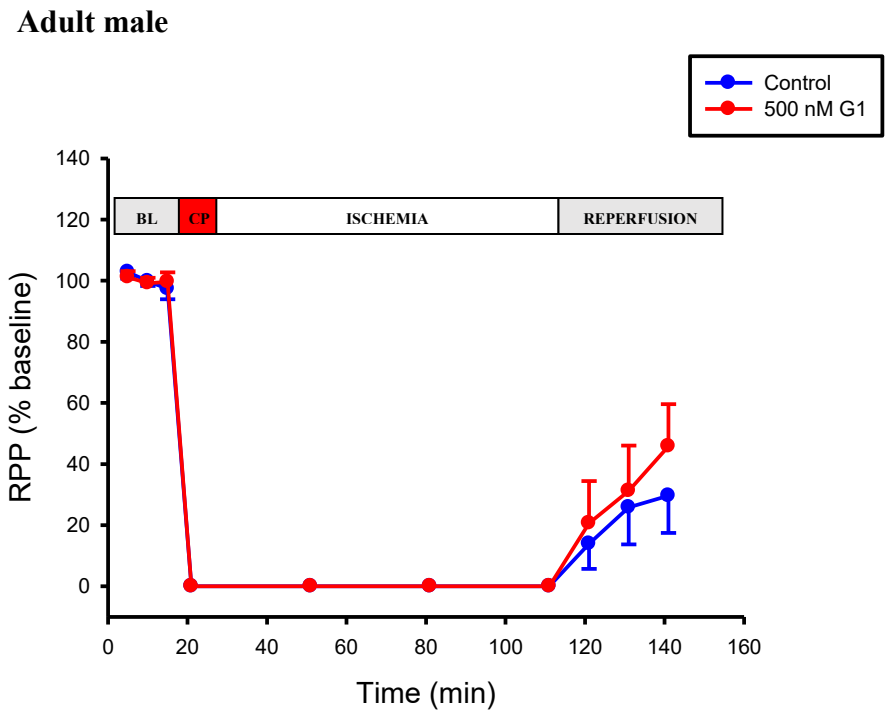


Figure 3.44 G1 had no effect on the recovery of RPP compared to the vehicle control in adult male hearts. The recovery of RPP in reperfusion was similar in control and G1-treated hearts from adult males. Control n=6; 500 nM G1 n=5. 2-way RM ANOVA.

Adult male

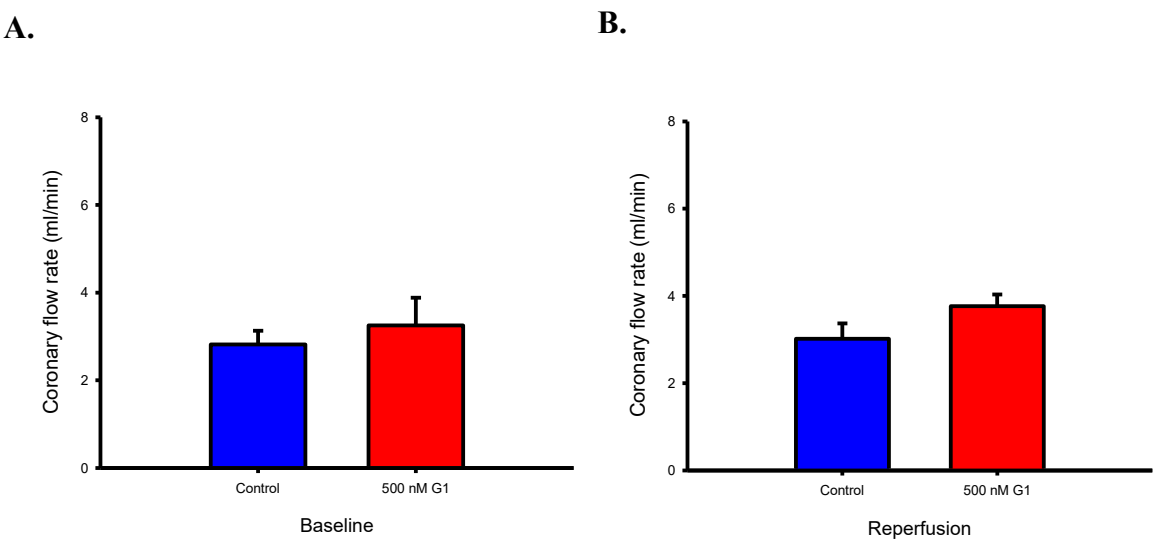
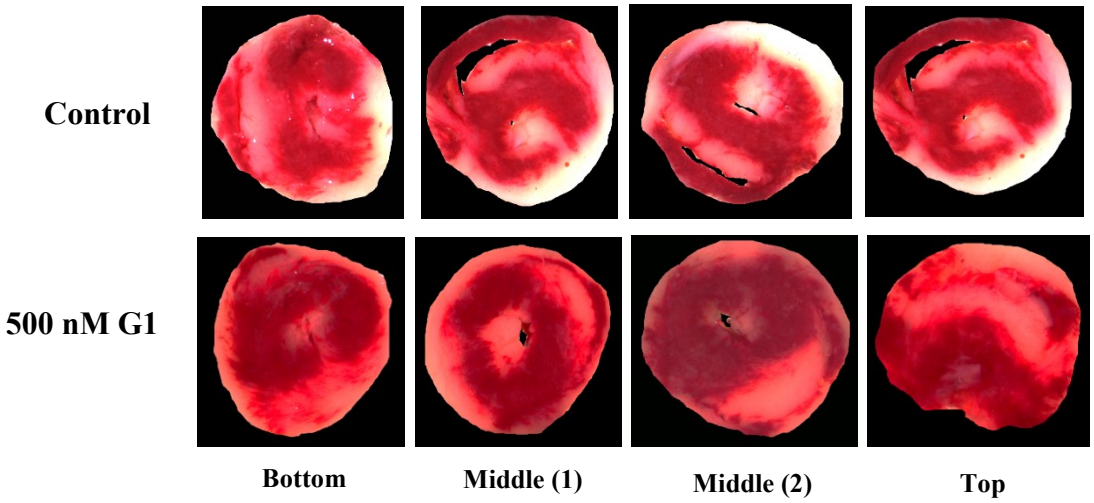


Figure 3.45 G1 had no effect on coronary flow rate in reperfusion compared to control adult male hearts. (A) No significant change in coronary flow rate was observed in baseline between control and G1-treated hearts. **(B)** Similar results were obtained in reperfusion, where there was no difference in flow rate between control or G1-treated hearts. Control n=6; 500 nM G1 n=5. Student's t-test.

Adult male

A.



Adult male

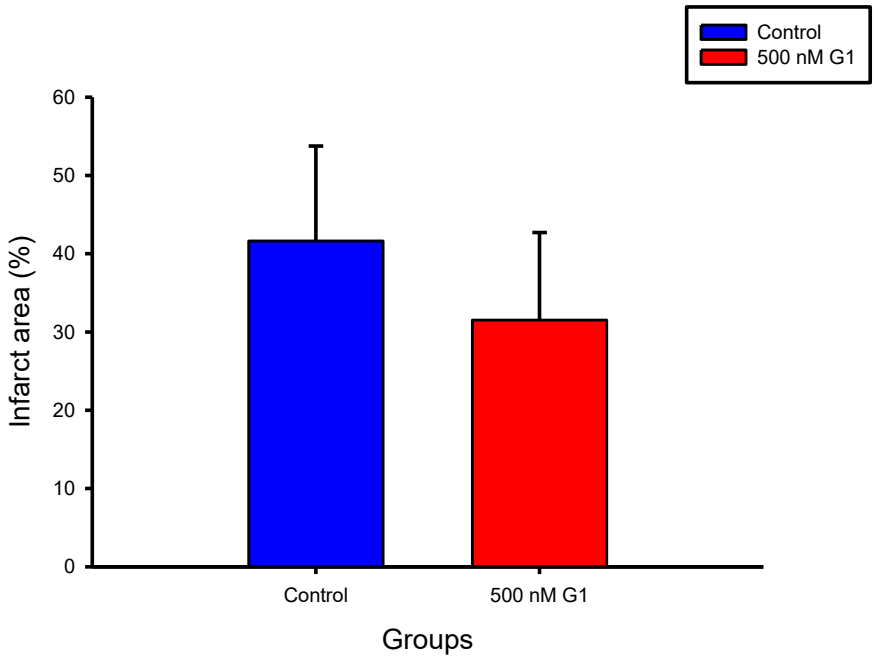


Figure 3.46 G1 had no effect on infarct area in adult male hearts. (A) Heart slices from the bottom (apex), middle and top (base) were taken from vehicle and G1 (500 nM) treated groups and compared for their infarct areas. **(B)** No significant reduction of infarct area was seen when hearts treated with G1 were compared to control hearts. Control n=3; G1 n=4; Student's t-test.

Adult male

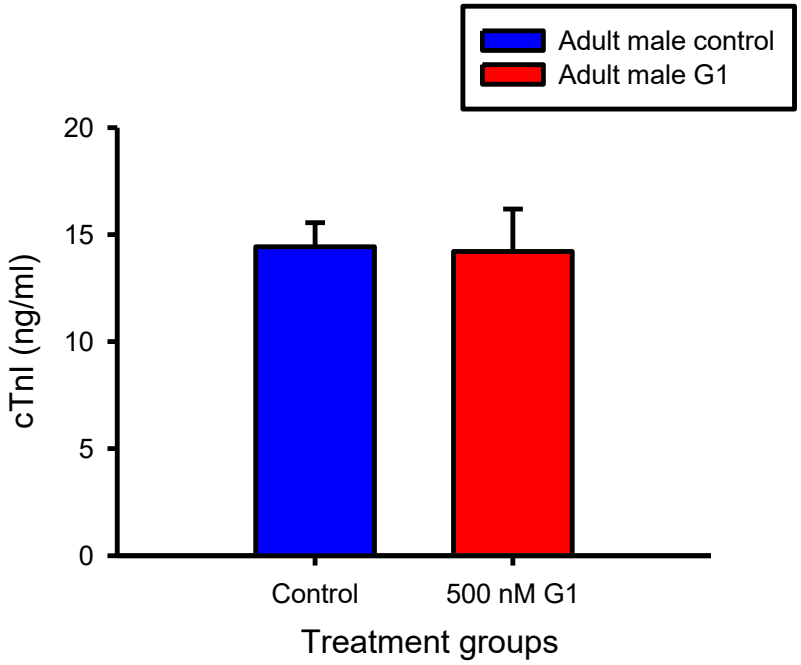
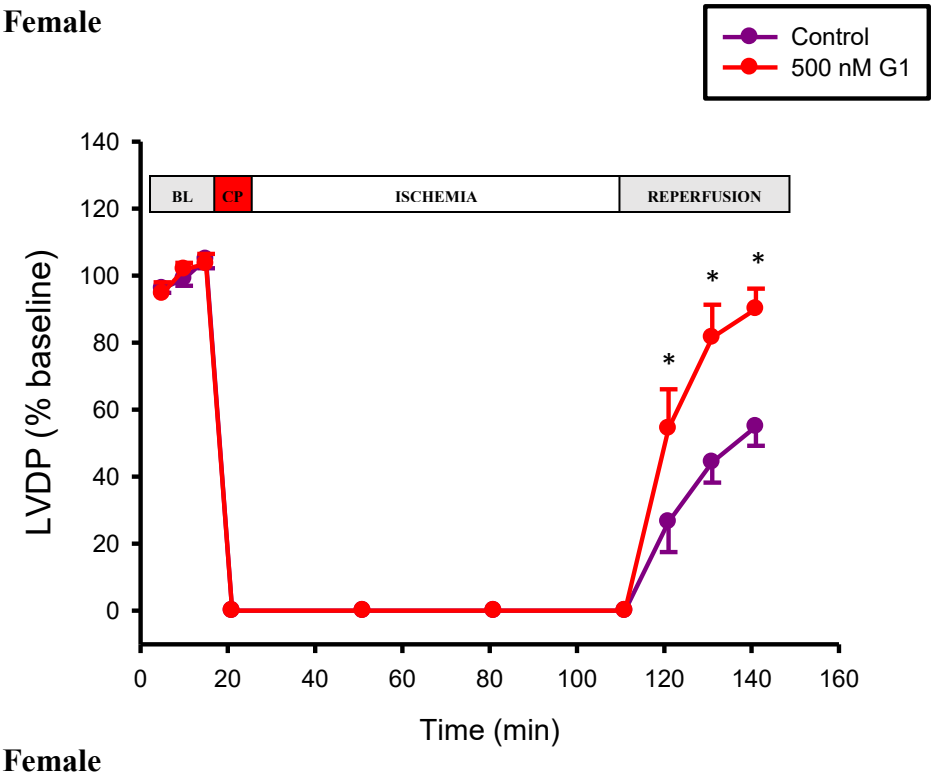


Figure 3.47 Concentrations of cTnI released in reperfusion (141 min time point) did not differ between vehicle and G1-treated adult male hearts. The concentration of cTnI released in reperfusion from adult male hearts was not different from the concentration in control samples. Adult male control n=6; adult male G1 n=6. Student's t-test.

Old Female

A.



Old Female

B.

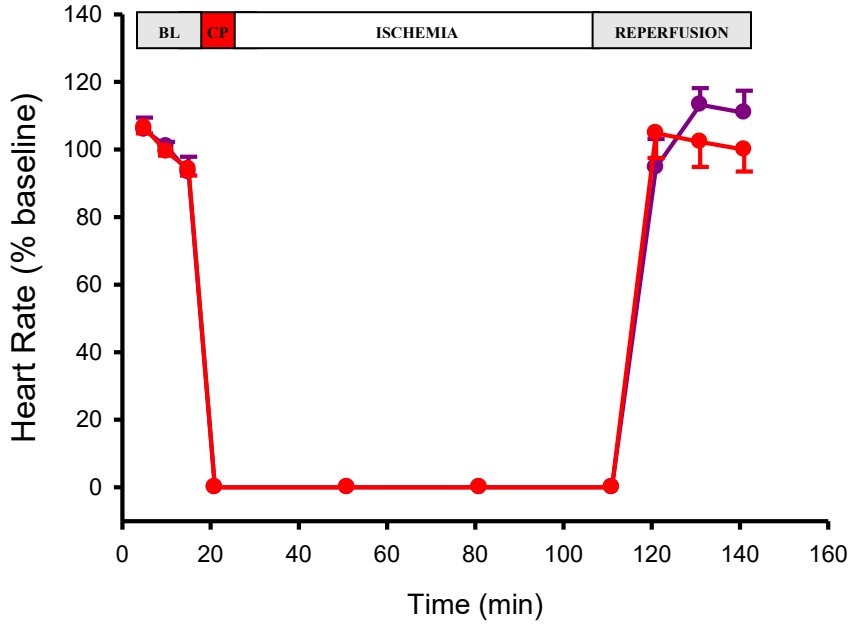
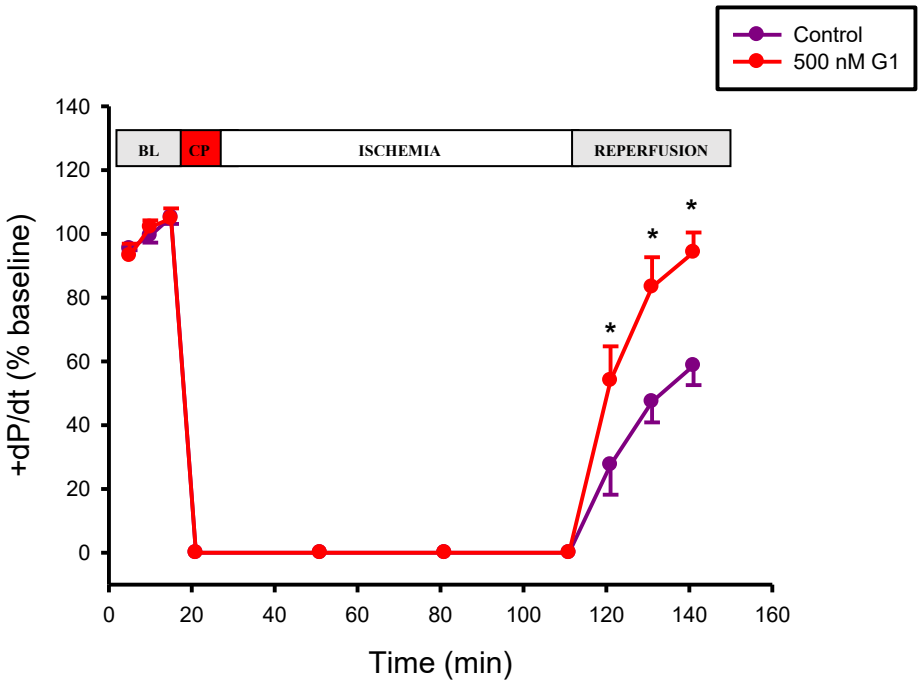


Figure 3.48 Recovery of LVDP and HR in hearts from old female mice when hearts were perfused with cardioplegia in the absence or presence of 500 nM G1. (A) Recovery of LVDP was significantly better during reperfusion when hearts were perfused with cardioplegia + 500 nM G1 compared to vehicle control (* $p < 0.05$). **(B)** HR recovered completely in reperfusion regardless of drug. Control n=5; 500 nM G1 n=5. 2-way RM ANOVA.

Old Female

A.



Old Female

B.

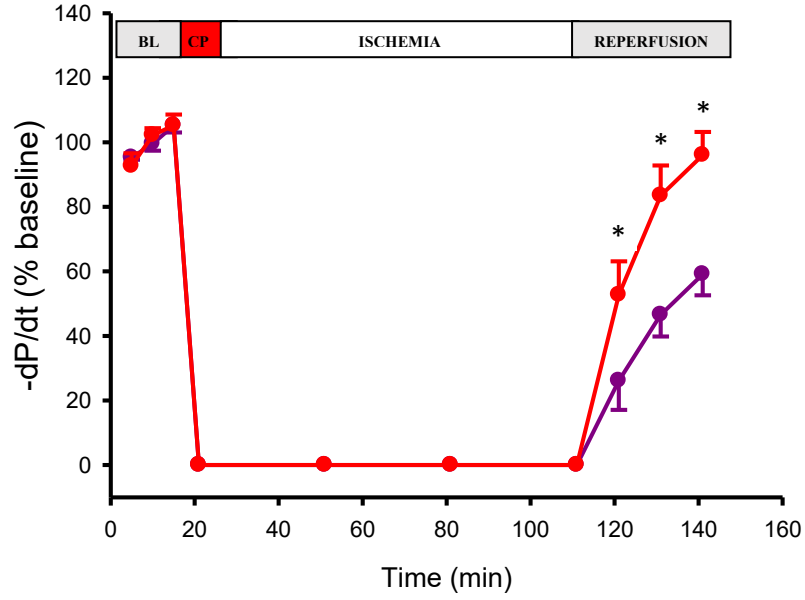


Figure 3.49 G1 (500 nM) in cardioplegia improved recovery of +dP/dt and –dP/dt in old female mice. Recovery of +dP/dt **(A)** and –dP/dt **(B)** was significantly better during reperfusion when the heart was perfused with cardioplegia + 500 nM G1 compared to vehicle control (* $p < 0.05$). Control n=5; 500 nM G1 n=5. 2-way RM ANOVA.

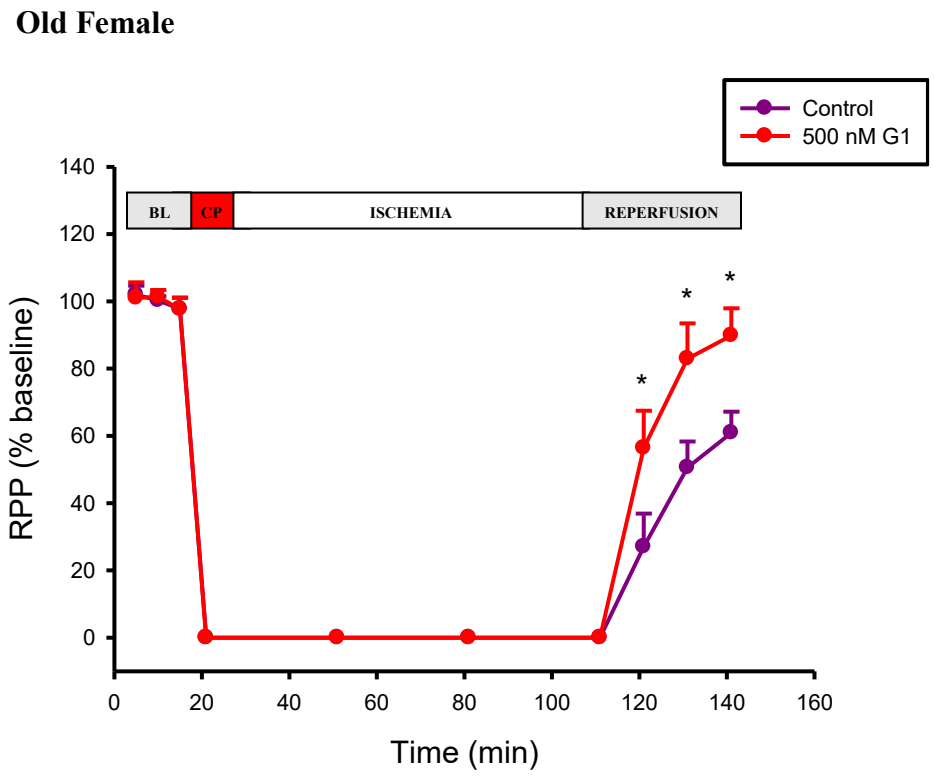


Figure 3.50 Recovery of RPP was significantly better in hearts treated with G1 compared to vehicle control in old female mouse hearts. RPP recovered significantly better with cardioplegia + G1 in reperfusion compared to the control group (* $p<0.05$). Control n=5; 500 nM G1 n=5. 2-way RM ANOVA.

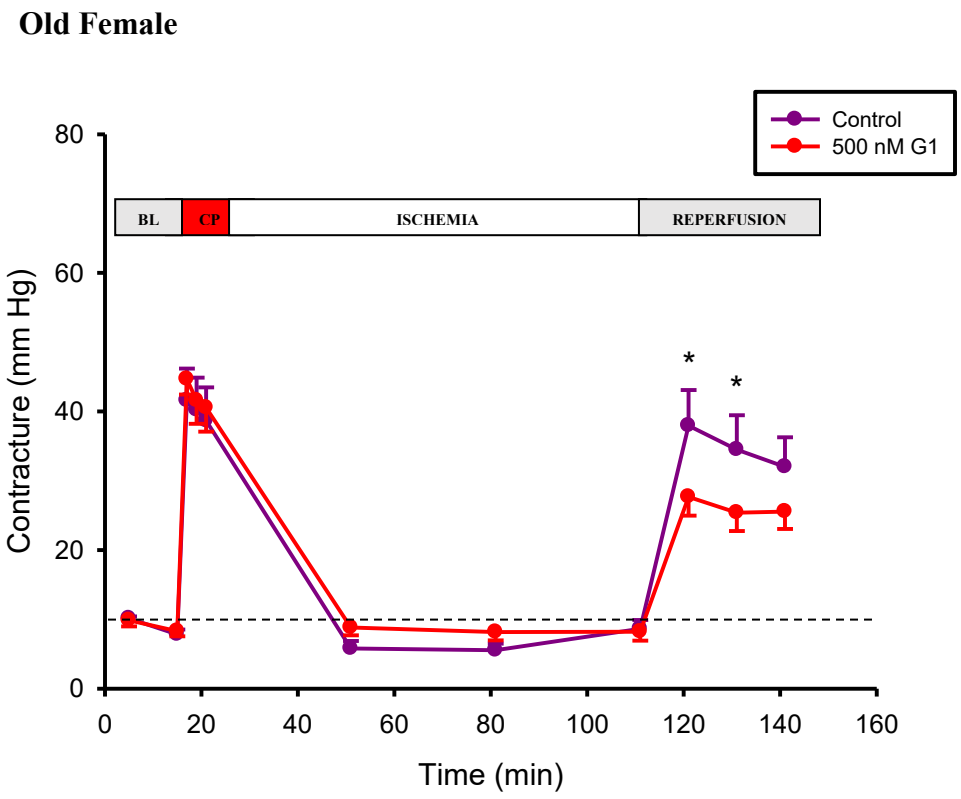


Figure 3.51 G1 reduced the contracture level in reperfusion compared to vehicle control in old female mouse hearts. G1 (500 nM) significantly reduced the contracture level at 121 and 131 min in reperfusion compared to the control group. Control n=4; 500 nM G1 n=4. 2-way RM ANOVA.

Old Female

A. **B.**

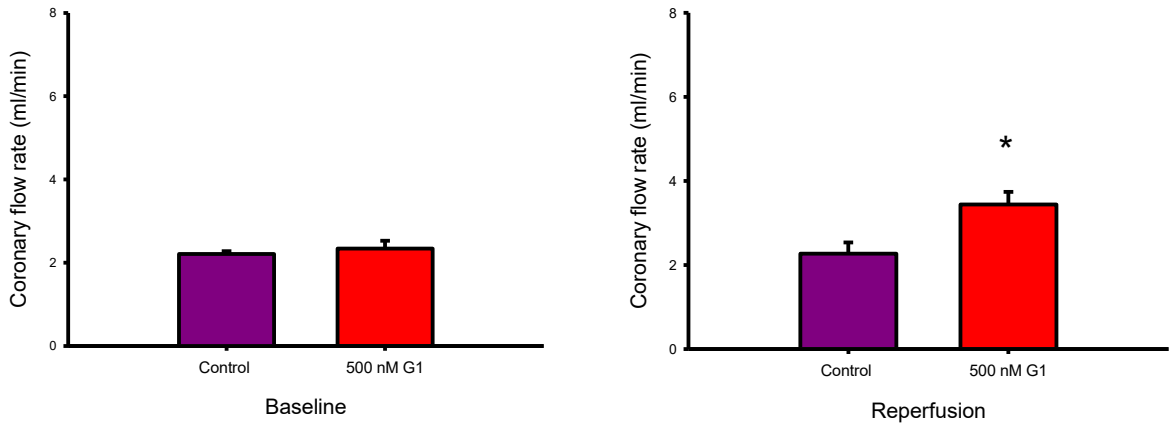


Figure 3.52 G1 improved coronary flow rate in reperfusion in old female hearts when compared control hearts. (A) No significant change in the flow rate was observed in baseline between control and G1-treated hearts. **(B)** However, coronary flow rate in reperfusion was significantly higher in G1-treated hearts compared to control hearts. Control n=5; 500 nM G1 n=5. Student's t-test.

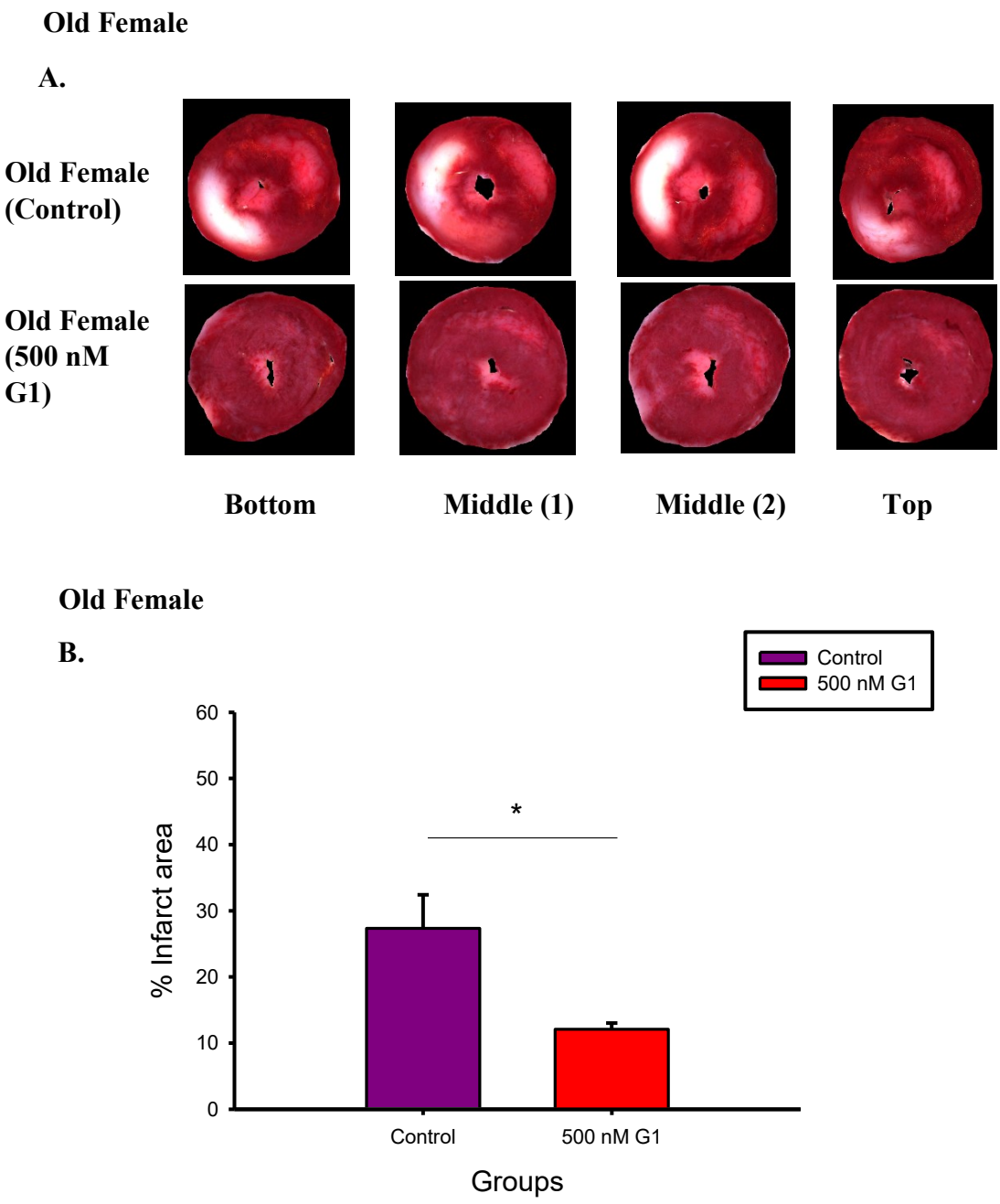


Figure 3.53 G1 (500 nM) significantly reduced infarct area in old female hearts. (A) TTC stained the viable tissue red, and the non-viable tissue remained unstained. The heart slices (bottom (apex), middle and top (base)) from vehicle (control) and G1 (500 nM) treated groups were compared for their infarct areas. **(B)** A significant reduction of infarct area was seen when hearts treated with G1 were compared to control hearts. Control n=5; 500 nM G1 n=4. Student's t-test.

Old Female

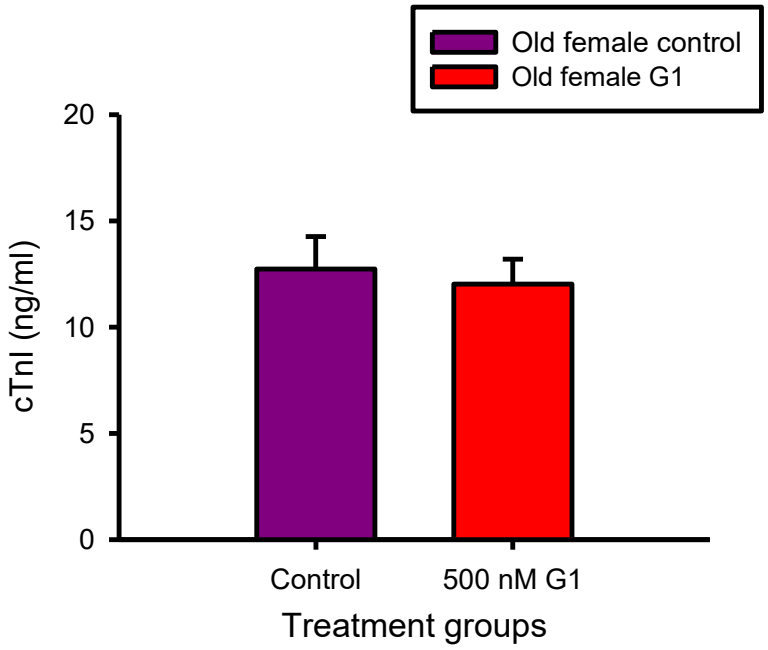
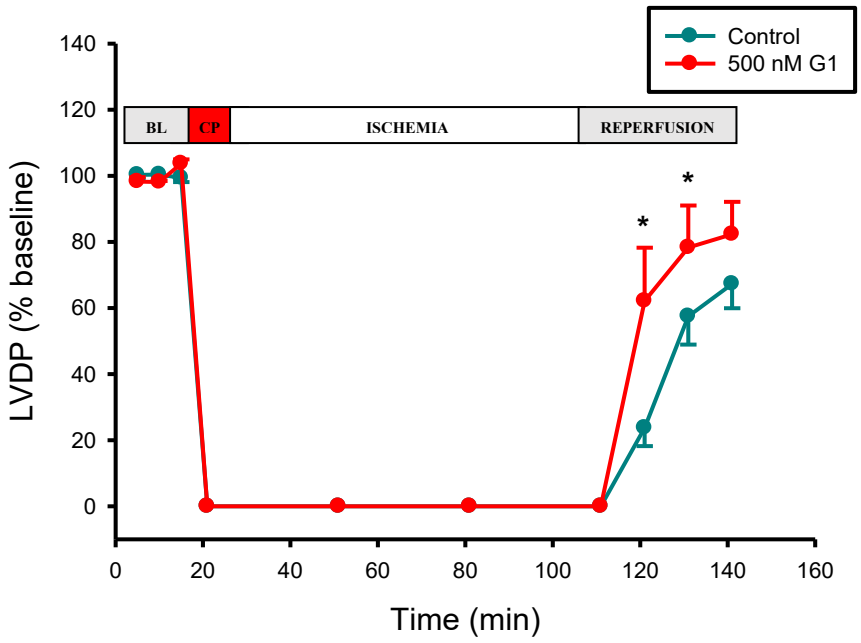


Figure 3.54 cTnI released in reperfusion (141 min time point) did not differ between vehicle and G1-treated old female hearts. The concentration of cTnI released from old female hearts in reperfusion was not different from control. Old female control n=5; old female G1 n=5. Student's t-test.

Old male

A.



Old male

B.

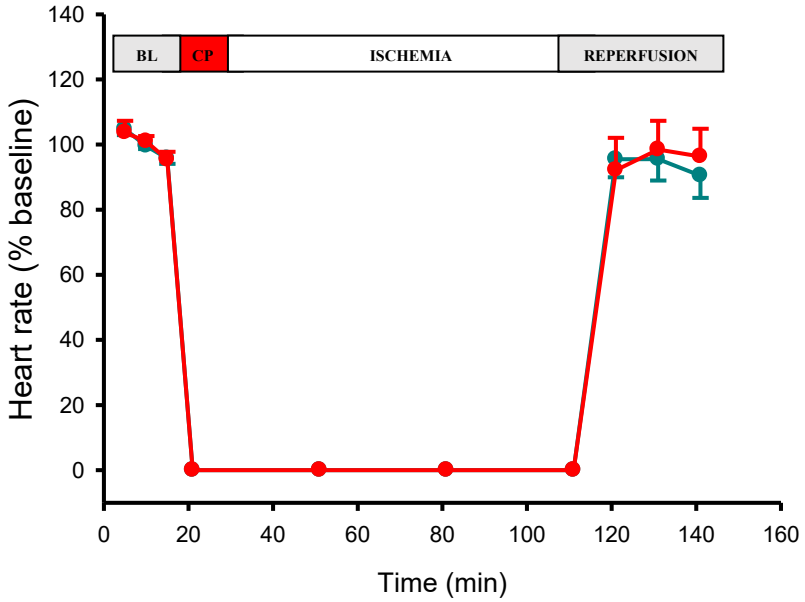
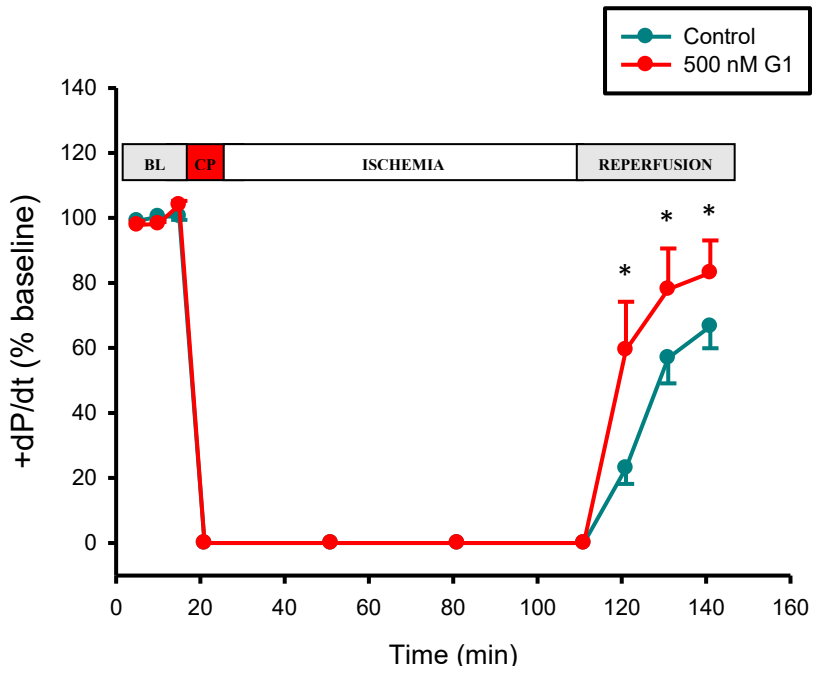


Figure 3.55 LVDP and HR in hearts from old male mice in the absence and presence of 500 nM G1 in cardioplegia. (A) Recovery of LVDP was significantly better in reperfusion when hearts were perfused with cardioplegia + 500 nM G1 compared to vehicle control (* $p < 0.05$). **(B)** HR recovered completely in reperfusion regardless of drug. Control n=6; 500 nM G1 n=6. 2-way RM ANOVA.

Old male

A.



Old male

B.

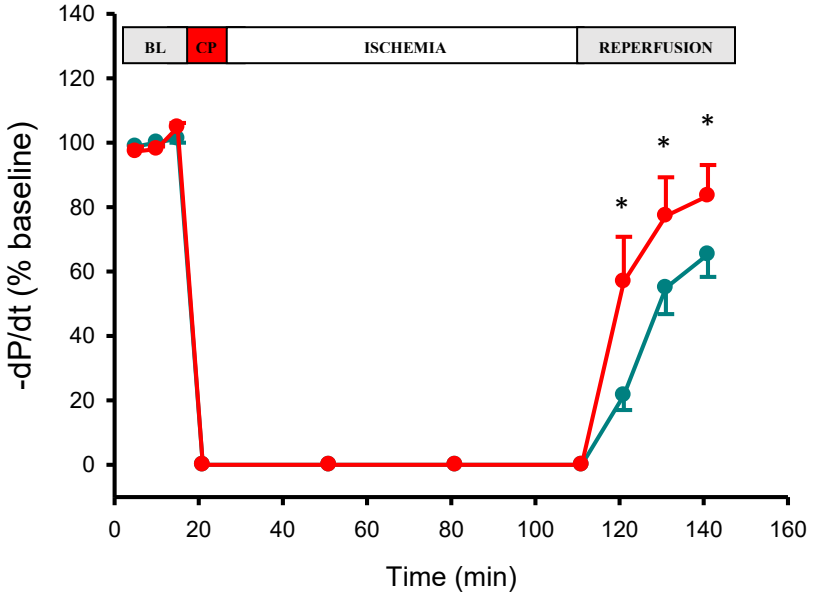


Figure 3.56 G1 caused a marked improvement in the recovery of +dP/dt and –dP/dt in old male mice when hearts were perfused with cardioplegia plus 500 nM G1. Recovery of +dP/dt **(A)** and –dP/dt **(B)** was significantly better during reperfusion when hearts were perfused with cardioplegia + 500 nM G1 compared to vehicle control (* $p < 0.05$). Control n=6; 500 nM G1 n=6. 2-way RM ANOVA.

Old male

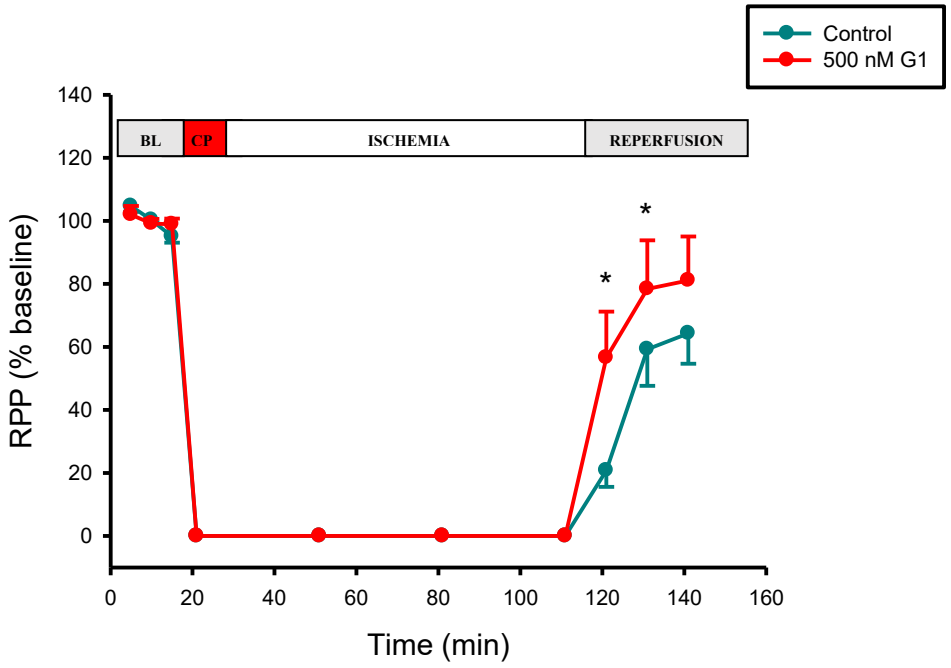


Figure 3.57 G1 improved the recovery of RPP in reperfusion in old male hearts compared to vehicle control. RPP recovered significantly better (at 121 and 131 min) in reperfusion with cardioplegia + G1 compared to the control group (* $p<0.05$). Control n=6; 500 nM G1 n=6. 2-way RM ANOVA.

Old male

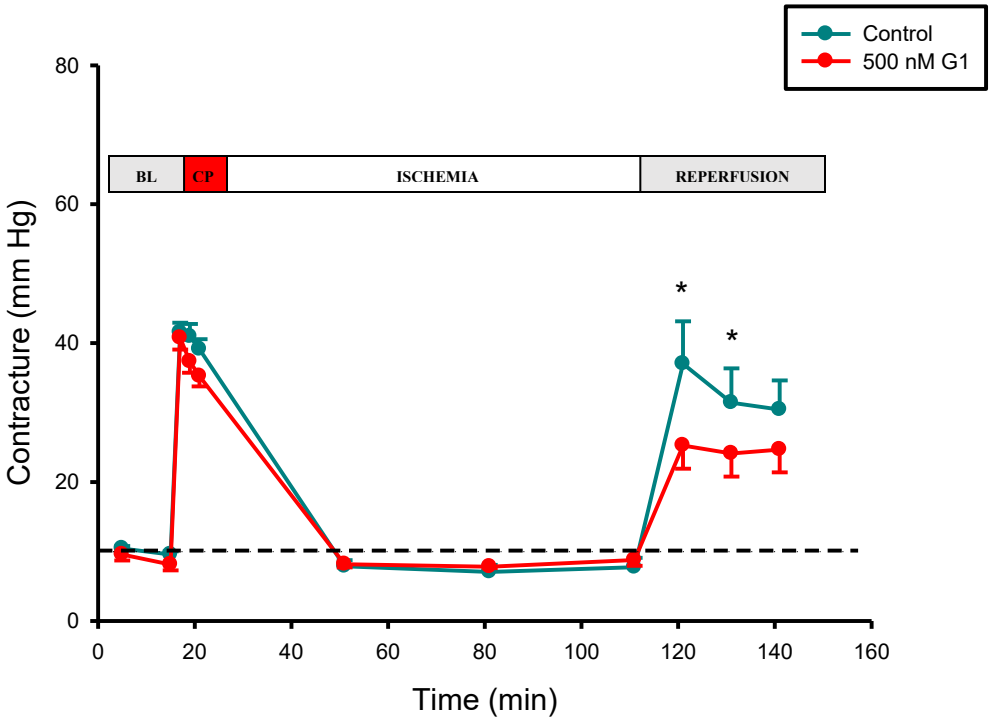


Figure 3.58 G1 reduced the contracture level in reperfusion compared to the vehicle control in old male mouse hearts. G1 (500 nM) significantly reduced contracture level at two time points (121 and 131 min) in reperfusion compared to the control group. Control n=6; 500 nM G1 n=6. 2-way RM ANOVA.

Old male

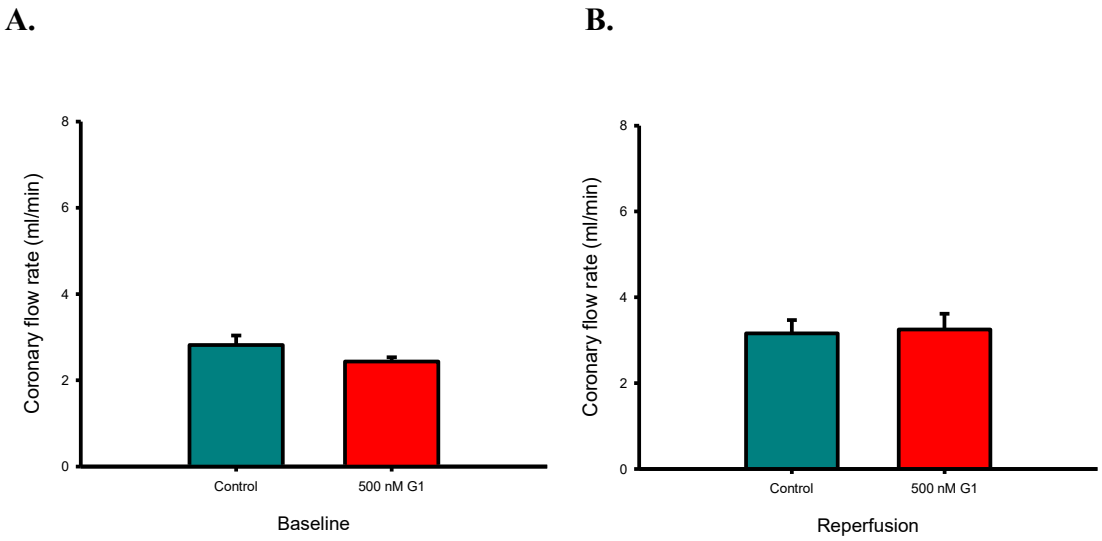


Figure 3.59 G1 did not affect the coronary flow rate in reperfusion compared to control hearts. The coronary flow rate did not differ between control and G1-treated hearts either in baseline **(A)** or in reperfusion **(B)**. Control n=6; 500 nM G1 n=6. Student's t-test.

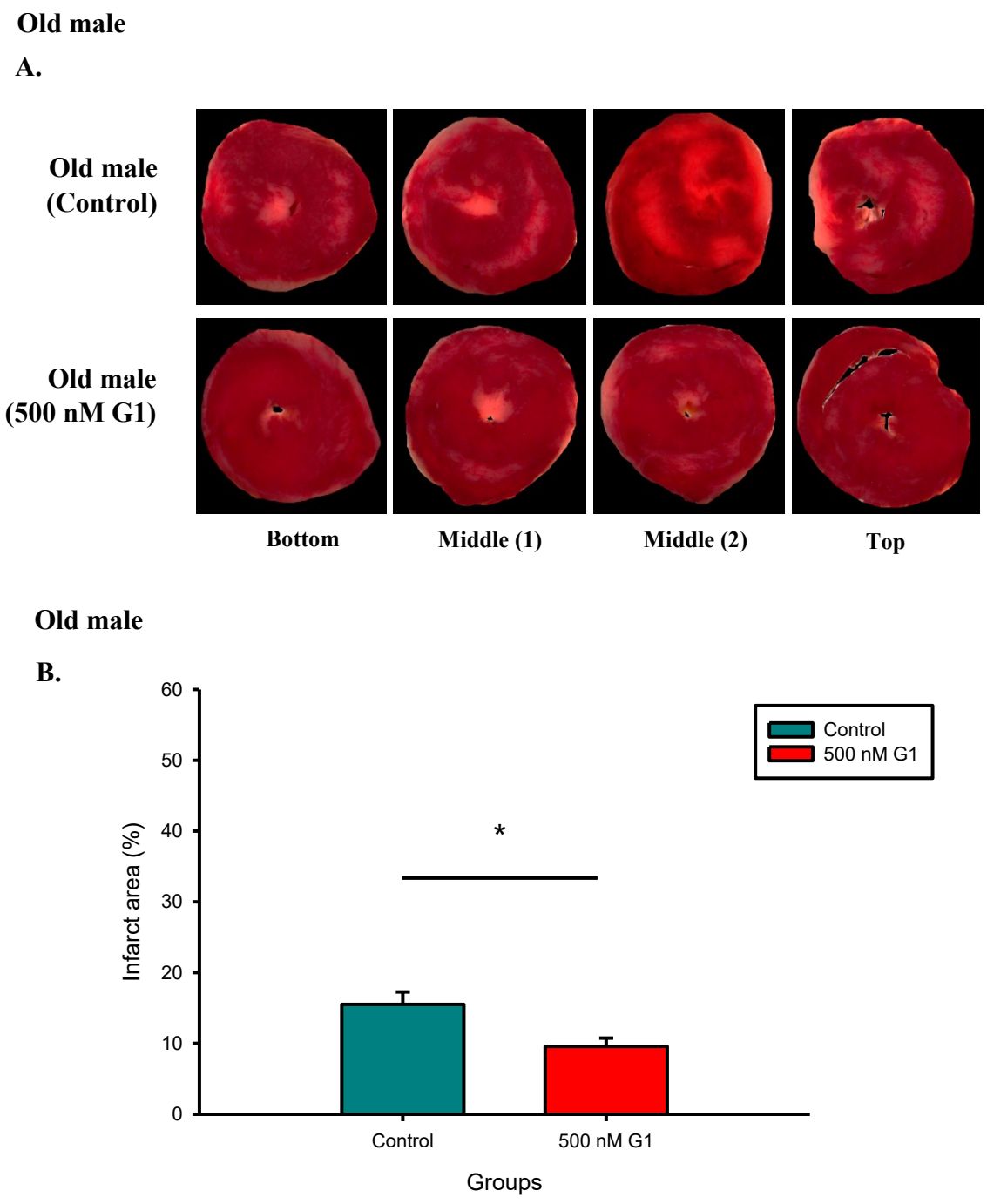


Figure 3.60 G1 reduced infarct area in old male hearts compared to vehicle control hearts. (A) Hearts slices (bottom (apex), middle and top (base)) from different groups (G1 vs. control) were compared for their infarct areas. **(B)** A significant reduction of infarct area was seen when hearts treated with G1 were compared to control hearts. Control n=6; 500 nM G1 n=5. Student's t-test.

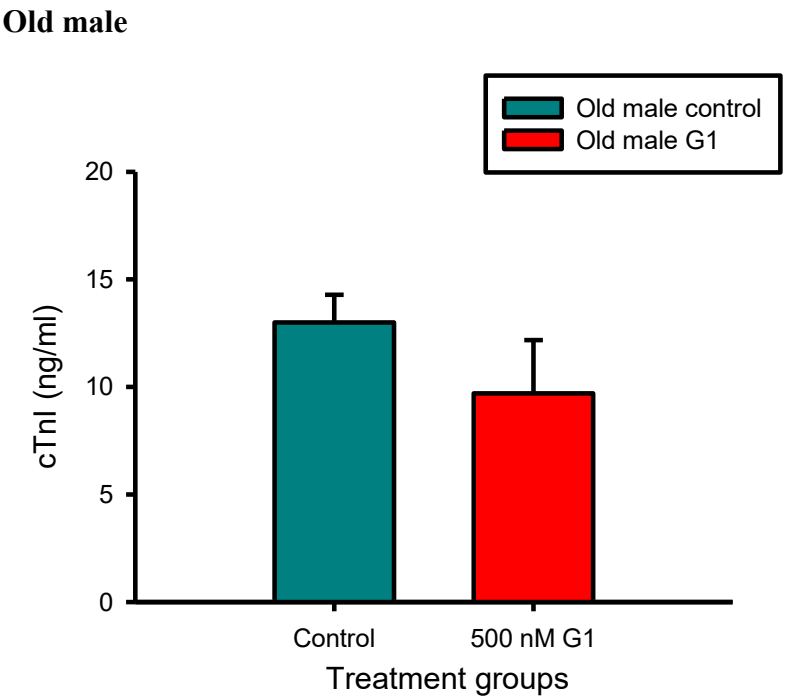


Figure 3.61 G1 had no effect on cTnI release in reperfusion when compared to vehicle treated old male hearts. The concentration of cTnI released in reperfusion from old male hearts did not differ from control. Old male control n=6; old male G1 n=6. Student's t-test.

CHAPTER 4: DISCUSSION

4.1 Overview of key findings

The overall hypothesis of this study was to determine whether incorporation of G1 in STH2 cardioplegia enhanced its cardioprotective properties in adult and aged hearts from both sexes. Before exploring the effects of G1, it was important to investigate the responses of adult and old hearts from both sexes to STH2 cardioplegia alone. Therefore, hearts from adult and old mice of both sexes were perfused with STH2 cardioplegia and then exposed to I/R injury. When sex-specific responses to I/R injury were analyzed, adult female mice showed significantly better recovery of the majority of cardiac parameters compared to adult male mice in reperfusion. By contrast, hearts from aged males showed either similar or even better recovery of cardiac function compared to aged females. When age-specific responses to I/R injury were analyzed, adult and old female mouse hearts recovered to the same extent in reperfusion. Interestingly, old males recovered significantly better than adult males in reperfusion. Taken together, these data suggested that there was variation in the degree of recovery of cardiac function between the sexes and in the different age groups. Even so, the extent of recovery was still suboptimal.

To improve the beneficial properties of STH2, the GPER agonist G1 was incorporated in STH2 cardioplegia and sex- and age-specific responses to the drug were examined. Results showed that incorporating G1 in cardioplegia was beneficial in adult female hearts, but not in adult male hearts. This effect of G1 was also blocked by the GPER antagonist, G15, in adult female mouse hearts. Interestingly, G1 significantly improved recovery of cardiac parameters in reperfusion in old hearts from both sexes.

These results demonstrate that G1 was advantageous in adult female mouse hearts and in old mouse hearts from both sexes. By contrast, G1 had no beneficial effects in younger males. Together these findings suggest that incorporation of the GPER agonist G1 in standard cardioplegic solution could be a useful strategy to pursue in older individuals who are undergoing cardiac surgery.

4.2 Sex-specific responses to I/R in hearts perfused with STH2 cardioplegia

As discussed in an earlier section (**1.3.2)**, there are many sex-specific changes in the heart that may adversely affect performance after I/R insult. Still, many studies use male animals only, potentially to avoid the influence of fluctuating hormonal activity in their experimental outcomes. Nonetheless, in a meta-analysis of 293 studies, Prendergast *et al.* (2014) looked at different traits in male and female mice and found no significant difference in variability between the sexes. Also, MacDonald *et al.* (2014) have shown that young adult female mice housed in groups did not exhibit regular estrous cycles unless they were exposed to male pheromones. Therefore, inclusion of both sexes in pre-clinical studies is justifiable and desirable. Despite that, few studies have examined sex-specific responses to I/R injury in hearts subjected to cardioplegic arrest followed by reperfusion. The following sections will discuss what is known about differences in the recovery of cardiac function in adult and old mice of both sexes.

4.2.1 Recovery of cardiac parameters was better in adult female than in adult male mouse hearts

Poor outcomes following cardiac surgery are mainly due to I/R injury. To attenuate myocardial injury, STH2 cardioplegia was used in a mouse heart perfusion model. The results of this study demonstrated variable levels of protection of contractile function (LVDP, +dP/dt, -dP/dt and RPP), which were better in adult females than in adult males. Previous studies of sex differences in responses to global I/R show increased resistance to I/R injury in adult female hearts and myocytes when compared to male hearts and cells (Ross and Howlett, 2012; Murphy and Steenbergen, 2007). Bell *et al.* (2008) reported that young adult female rat hearts exposed to global ischemia had better recovery of contractile function with fewer arrhythmias in reperfusion, compared to age-matched male rat hearts. Adult female mouse hearts subjected to global ischemia are also protected from the deleterious effects of adrenergic stimulation and Ca^{2+} loading in I/R via a nitric oxide synthase (NOS)-mediated mechanism while males are not (Cross *et al.,* 2002). Cross *et al.* (2002) suggested that these cardioprotective mechanisms were estrogen-mediated in adult female mice by activation of genomic ERs, either ERα or ERβ. They noted that NOS activity is higher in adult females than in males, which could provide cardioprotection by decreasing SR Ca^{2+} release, SR Ca^{2+} cycling or Ca^{2+} influx across sarcolemma in ischemia (Cross *et al.,* 2002). A study done by Sun and colleagues showed less I/R injury in adult females when compared to adult male mouse hearts and suggested that the protection was due to S-nitrosylation of the L-type-Ca^{2+} channel resulting in reduced Ca^{2+} entry in females in ischemia (Sun *et al.,* 2006). This effect is also possibly caused by a NOS-mediated mechanism (Sun *et al.,* 2006). However, when ventricular myocytes from adult female and male rat hearts are subjected to I/R injury, no difference in intracellular Ca^{2+} loading is observed in reperfusion (Ross and Howlett,

2012). This suggests that the underlying cardioprotective mechanism in females may involve an increase in myofilament Ca^{2+} sensitivity in reperfusion (Ross and Howlett, 2012). Also, less inflammatory cytokine production is associated with improved functional recovery in females and this may help protect female hearts from I/R damage (Willems *et al.,* 2005; Lagranha *et al.,* 2010).

The results presented in this thesis extend what is known about sex differences in response to myocardial ischemia to a clinically relevant setting. This work shows, for the first time, that there are sex-specific differences in the levels of protection in mouse hearts perfused with cardioplegic solution prior to I/R insult. As with the outcomes seen in global I/R injury models, the results of this study suggest that adult female mouse hearts are more resistant to I/R injury and better protected by cardioplegia compared to adult males. Along with the improved recovery of contractile function, adult female hearts had reduced infarct area and less cTnI release compared to adult male hearts, although this was not statistically significant. Together these results suggest that STH2-induced cardioplegic arrest had better recovery in adult female hearts, possibly by maintaining Ca^{2+} homeostasis in myocytes (Sun *et al.,* 2006; Ross and Howlett, 2012). This, in part, might be mediated by higher levels of estrogen in adult females than in adult males, as estrogen exerts cardioprotection via activation of ERα and ERβ through a NOS mediated mechanism and/or by modulating Ca^{2+} loading in cardiomyocytes as discussed above (Cross *et al*., 2002; Willems *et al.,* 2005).

4.2.2 Recovery of cardiac parameters was either similar in both sexes or better in old male than in old female mice

Pre-menopausal women are less likely to experience ischemic heart disease when compared to age-matched men (Lloyd-Jones *et al.,* 1999). However, after menopause women become equally susceptible to ischemic insult (Jousilahti *et al.,* 1999). Also, in an I/R study in rat cardiomyocytes, high Ca^{2+} levels in ischemia promote contractile dysfunction and cell death in aged and in ovariectomized female rats (Ross and Howlett, 2012). This suggests that low estrogen levels, as seen in postmenopausal women, alter myocyte Ca^{2+} homeostasis and promote I/R injury. Hence, with age the estrogen mediated cardioprotection is lost in females and they become as susceptible as males to I/R injury. A study done in an isolated mouse heart model reported impaired recovery of ventricular contractility after global I/R injury in aged male and female mice compared to their younger counterparts (Willems *et al.,* 2005). Also, the extent of recovery of developed pressure, rate of pressure development and diastolic pressure is similar in aged male and female mice (Willems *et al.,* 2005). In a study using perfused rabbit hearts, older females are at higher risk of global I/R injury than older males (McCully *et al.,* 2006). Even in a model of cardioplegic arrest, post-ischemic functional recovery is worse and infarcts are larger in old female than in old male rabbit hearts (McCully *et al.,* 2006; Black *et al.,* 2012). These investigators also added diazoxide (K_{ATP} channel opener) in cardioplegia to improve cardioprotection. Surprisingly, addition of diazoxide further worsens recovery in aged female hearts, but is beneficial in aged male hearts (McCully *et al.,* 2006; Black *et al.,* 2012). They showed that opening the mitochondrial K_{ATP} channel is less effective in limiting infarct size in aged female rabbit hearts compared to aged males (McCully *et al.,* 2006). Though mechanisms contributing to age- and sex-related

differences in cardioprotection are yet to be elucidated, these results suggest that estrogen might play an important role in modulating I/R injury in females (McCully *et al.,* 2006).

In summary, the results presented in this thesis, in agreement with previous studies, showed either similar recovery of cardiac function in reperfusion in both sexes or better recovery in old male hearts compared to old female hearts. This increase in susceptibility to I/R injury in females may be due to the loss of estrogen-mediated cardioprotection with age in females.

4.3 Age-specific differences in responses to I/R with STH2 cardioplegia

There are differences in the recovery of cardiac function after I/R insult that vary at different ages (McCully *et al.,* 2006; Willems *et al.,* 2005). As discussed in section **(1.3.1),** there are many age-related changes that occur in male and female hearts. These changes in the heart may influence it's recovery after surgery. The following sections will discuss the differences in the recovery of hearts from adult and old mice of both sexes subjected to cardioplegic arrest.

4.3.1 Recovery of cardiac parameters was similar in adult and old female mice

Animal studies show better recovery of cardiac parameters in adult females than in aged females following I/R injury, with or without cardioplegic arrest (McCully *et al.,* 2006, Willems *et al.,* 2005). However, in this thesis cardiac function recovered similarly in adult and old female mice. Ischemic preconditioning of the heart may help explain the enhanced recovery of old female hearts in reperfusion (Casos *et al.,* 2017). Ischemic preconditioning is an experimental technique in which hearts are subjected to repeated

short ischemic episodes prior to a more prolonged ischemic insult (Iliodromitis *et al.,* 2007). Adenosine released during myocardial ischemia plays an important role in mediating cardioprotective effects of ischemic preconditioning (Burns *et al.,* 1996; Liang and Jacobson*,* 1999). Adenosine stimulates A_1 and A_3 receptors in myocytes which activates protein kinase C, and opens mitochondrial K_{ATP} channels, which may be involved in cardioprotection (Iliodromitis *et al.,* 2007; Burns *et al.,* 1996; Liang *et al.,* 1999). In the work presented in this thesis, ischemic preconditioning may occur during the heart cannulation, when the aorta is temporarily cut off and the heart is not perfused for a brief duration (Herr *et al.,* 2015; Motayagheni, 2017). Also, while inserting balloons and adjusting the initial myocardial temperature to ~ 37^{o}C there is a chance that cardiac preconditioning will occur (Herr *et al.,* 2015; Motayagheni, 2017).

The clinical equivalent of ischemic preconditioning is preinfarction angina. Elderly patients who have angina during the week before myocardial infarction exhibit better recovery and smaller infarcts following thrombolytic therapy compared to those without preinfarction angina (Andreotti *et al.,* 1996). Therefore, it is likely that ischemic preconditioning can improve outcomes in people. Two studies of ischemic preconditioning in animals show improved post-ischemic recovery of cardiac function in young adult animals but not in hearts from aged animals (Abete *et al.,* 1996; Fenton *et al.,* 2000). By contrast, a study done in sheep demonstrates protection of the myocardium with ischemic preconditioning even in senescent sheep hearts (Burns *et al.,* 1996). Nevertheless, these studies were either limited to males or did not specify the sex used, so whether sex differences were present is unclear. Some studies have analyzed age- and sex-specific responses to ischemic preconditioning. However, these studies considered

only very young (12-wks) or adult (18 wks) rats or mice (Turcato *et al.,* 2006; Ledvenyiova *et al.,* 2013), so they were not true studies of aging.

The experimental model used here employed hypothermic STH2 cardioplegia before subjecting hearts to I/R injury. Our results suggest that hypothermic STH2 cardioplegia may be equally effective in both adult and old female mouse hearts. Moreover, the old mice used in this study were heavier than the adult mice, which suggest possible higher fat/adipose tissue accumulation in old mice. The level of the enzyme aromatase increase in animals with higher deposition of adipose tissue (Blakemore and Naftolin, 2016; Bernasochi *et al.,* 2017). Aromatase is a key enzyme in the biosynthesis of estrogen (Bernasochi *et al.,* 2017; Ostadal and Ostadal, 2013). Hence, higher levels of aromatase in aging female mice could counteract the natural loss of estrogen with age (Jousilahti *et al.,* 1999) and promote estrogen-mediated cardioprotection. Additional studies of the mechanisms involved in cardioprotection would be of interest.

4.3.2 Recovery of cardiac parameters was better in old male than in adult male mice

While aging is thought to be associated with increased myocardial susceptibility to I/R injury this is not, in fact, observed in all studies. A study done by Boucher *et al.* (1998), showed that the severity of myocardial damage following I/R insult increased progressively in male rats between 4 and 16 mos of age, but then declined up to 24 mos. Boucher and colleagues found similar recovery of LVDP in 4 mos (young) and 24 mos (senescent) old rats in reperfusion (Boucher *et al.,* 1998). In addition, myocardial catalase activity and glutathione peroxidase were similar in young and senescent rats (Boucher *et al.,* 1998). These cardiac enzymes are involved in reactive oxygen species (ROS)

elimination; ROS are suspected to be involved in reperfusion induced arrhythmias (Boucher *et al.,* 1998; Euler, 1995) as well as promote tissue injury (Sugamura and Keaney, 2011). Hence, they concluded that the decrease in susceptibility of the myocardium to I/R injury at 24 mos of age compared to 16 mos of age could be due to a reduced ability of the hearts to eliminate hydrogen peroxide in 16 mos old male rats (Boucher *et al.,* 1998). This could lead to the accumulation of hydrogen peroxide within the heart and cause the production of O_2 free radicals, which could damage myocytes and impair contractile function (Sugamura and Keaney, 2011). A similar mechanism could also help explain the resistance of old female hearts to injury following cardioplegic arrest, although there is no evidence for this yet.

Other protective mechanisms in old male mice could be estrogen-mediated protection and ischemic preconditioning as discussed previously in section (**4.3.1)**. Estrogen-mediated protection in males could arise via estrogen production by aromatase in adipose tissue, as old males were substantially heavier than adult males. In addition, O'Brien *et al.* (2008) showed that ischemic preconditioning enhanced the recovery of contractile function in reperfusion in ventricular myocytes from young adult and aged male rats. Larger Ca^{2+} transients and reduced diastolic Ca^{2+} accumulation in ischemia in aged hearts promoted better recovery of cardiac parameters (O'Brien *et al.* 2008). By contrast, other studies suggest that aging reduces the cardioprotective effects of ischemic preconditioning in male hearts (Fenton *et al.,* 2000; Abete *et al.,* 1996) so, additional work in this area is warranted.

In summary, results from the present study showed that recovery of cardiac function is substantially better in old male mouse hearts compared to adult males. The

age- and sex-specific effects of STH2 cardioplegia on hearts subjected to I/R injury, showed that STH2 alone did not produce superior outcomes after I/R insult. Therefore, further experiments investigated a novel way to enhance the properties of STH2 cardioplegia.

4.4 Effects of G1 on enhancing properties of STH2 cardioplegia for better recovery of cardiac function

A number of studies of cardioprotection have targeted GPER in the heart. G1 is a highly specific GPER agonist, which is used in experimental models to study GPER-activated cardiac responses (Deschamps and Murphy, 2009; Alencar *et al.,* 2017; Patel *et al.,* 2010; Bopassa *et al.,* 2010). Most studies of G1 used animal heart models and subjected hearts to brief periods of global ischemia (Deschamps and Murphy, 2009; Bopassa *et al.,* 2010). Though beneficial effects of G1 can protect hearts against I/R injury, the incorporation of G1 in cardioplegia has not previously been investigated. Therefore, this thesis examined the effects of adding G1 to STH2 cardioplegia in hearts exposed to prolonged ischemic periods (90 min), imitating a clinically relevant setting. The following sections will discuss the effects of G1 on the recovery of adult and aging mouse hearts of both sexes.

4.4.1 G1 incorporation was beneficial in adult female mouse hearts

This thesis showed that incorporation of G1 in STH2 cardioplegia was beneficial for the recovery of adult female mouse hearts in reperfusion. Several studies of G1 have identified different mechanisms of cardioprotection in animal heart models. Deschamps

and Murphy (2009) reported that acute G1treatment induced cardioprotection was mediated via the PI3K/Akt pathway, as blocking PI3K activation (using PI3K inhibitor, Wortmannin + G1) resulted in reduced phosphorylation of Akt and compromised recovery when compared to hearts treated with G1 alone. Bopassa *et al.,* (2010) concluded that the acute cardioprotection of mouse hearts with G1 was mediated by the ERK pathway, leading to the inhibition of mPTP opening. Another putative protective mechanism for G1 is through modulation of intracellular Ca^{2+} levels. Ullrich *et al.,* (2008) found that estrogen inhibited L-type Ca^{2+} current in ventricular myocytes from mouse hearts. They also reported that inhibition of Ca^{2+} current by estrogen was independent of ERα or ERβ activation (Ullrich *et al.,* 2008). This suggests that G1 may exhibit similar effects as those of estrogen in ventricular myocytes. By inhibiting Ca^{2+} current it may suppress Ca^{2+} -induced Ca^{2+} release from the SR and limit Ca^{2+} overload. This may account for the enhanced recovery of cardiac parameters (LVDP, +dP/dt, -dP/dt, RPP) with G1 in adult female hearts. Also, results presented in this thesis showed that the coronary flow rate was comparatively better in reperfusion following G1 treatment in females. Haas *et al.* (2009) concluded that G1 has vasodilatory effects on murine arteries. Thus, G1 may also improve ventricular function by enhancing coronary flow.

To confirm G1-mediated protection via GPER activation, the GPER antagonist G15 was used and incorporated in STH2 cardioplegia. In 2010, Dennis and colleagues identified this antagonist for GPER and called it G15 (Dennis *et al.,* 2011). G15 effectively blocks GPER-dependent PI3K activation and has no effect on ERα and ERβ at concentrations as high as 10 μM (Dennis *et al.,* 2011). In this thesis, G15 was found to

effectively block the activation of GPER by G1 in adult female mouse hearts. This suggests that activation of the PI3K/Akt pathway by G1 contributes to the beneficial effects of this drug in cardioplegia.

4.4.2 No beneficial effects of G1 were observed in adult male mouse hearts

Studies have shown that GPER is present in similar amounts in adult male and female rat cardiomyocytes (Deschamps and Murphy, 2009). Also, G1 was found to be equally protective in young adult male and female rat hearts subjected to 20 min of global ischemia (Deschamps and Murphy, 2009). However, in this thesis when hearts were perfused with G1+cardioplegia and subjected to 90 min of global ischemia, different levels of protection were observed in male and female hearts. Adult male hearts did not benefit from G1 incorporation in STH2 cardioplegia. In a cardiomyocyte-specific GPER knockout mouse model, male GPER knockout mice exhibit a profound increase in LV end diastolic and systolic dimensions compared to their female counterparts (Wang *et al.,* 2017). This study concluded that these different effects of GPER deletion in males and females could be due to effects of endogenous estrogens in female mice via activation of ERα and ERβ (Wang *et al.,* 2017). They also report that mitochondrial genes are enriched in female GPER knockout mice compared to their male counterparts (Wang *et al.,* 2017). This suggests that male hearts are more vulnerable to damage than female hearts. Another study looked at effects of GPER activation in the setting of ischemic stroke in young adult male and female mice (Broughton *et al.,* 2014). They found that G1 aggravated functional outcomes and increased infarct volume poststroke in males but not in females (Broughton *et al.,* 2014). These authors suggested that pretreatment of mice with G1 increased the expression of the apoptotic protein, cleaved caspase-3, in male brains

poststroke (Broughton *et al.,* 2014). This thesis showed that acute application of G1 was not effective in improving recovery of function after cardioplegic arrest in adult male hearts. The higher endogenous estrogen levels in adult females might have added protection in female hearts via activation of nuclear estrogen receptors. Therefore, further studies are required to understand the underlying mechanisms responsible for these differences in recovery between the sexes.

4.4.3 G1 incorporation was beneficial in both old female and male mouse hearts

Interestingly, the present study showed that the incorporation of G1 in STH2 cardioplegia was beneficial in all aged hearts, regardless of sex. Advancing age is associated with many adverse cardiac structural and functional changes including a decrease in myocardial relaxation, increase in filling pressures and systolic and diastolic dysfunction (Alencar *et al.,* 2017). However, as discussed in an earlier section **(4.4.1)**, G1 mediates different pathways to enhance cardioprotection. Interesting results were observed in this study where G1 had no effect on adult male mouse hearts but was advantageous in old male hearts. One possible reason for the protection could be due to excess adipose tissue accumulation in old animals compared to adult mice. As discussed earlier, old mice used in this experiment weighed more than the adult mice, which might be associated with higher adipose tissue deposition causing higher release of aromatase enzyme. The aromatase enzyme is capable of converting androgens to estrogens and hence promotes estrogen-mediated cardioprotection in aging mice (Bernasochi *et al.,* 2017; Ostadal and Ostadal, 2013). Therefore, even in control mouse hearts, the results presented in this thesis showed similar or better recovery of cardiac function in old hearts compared to adult hearts. Haas *et al.* (2009) also showed vasodilatory effects of G1,

which could also help to explain the increased coronary flow in reperfusion in old hearts of both sexes observed in this thesis. Hence, improved coronary flow might also have added the cardioprotective effects of G1 in old hearts.

4.5 Potential clinical significance

The results of this study provide new techniques to enhance cardioplegia for better outcomes after cardiac surgery. Ascione and colleagues showed that, though cold cardioplegia was associated with less ischemic stress and myocardial injury post-surgery compared to warm cardioplegia in human hearts, the overall recovery of the heart post-surgery is still suboptimal (Ascione *et al.,* 2002). Since then, many strategies have been applied to enhance cardioprotection after cardiac surgery. Haas *et al.,* (2009), have shown positive effects of G1 in human arteries in reducing blood pressure via vasodilatory effects. Also, many studies in animals show cardioprotective effects of G1 (Deschamps and Murphy, 2009; Bopassa *et al.,* 2010). However, this study showed, for the first time, that G1 incorporation in STH2 cardioplegia enhanced its protective properties and was beneficial in adult female mouse hearts. Importantly, G1 was also advantageous in old male and female mouse hearts. This is critical because old individuals are much more likely to undergo cardiac surgery.

While developing the experimental protocol used in this thesis, care was taken to follow clinical practice as closely as possible. As done in human heart surgery, a single dose of STH2 cardioplegia was used to arrest the heart before ischemic insult (Sorabella *et al.,* 2014). Also, the myocardial temperature dropped to 6.5 ± 0.3ºC by the end of

cardioplegic perfusion, which is similar to clinical practice (Sorabella *et al.,* 2014; Ascione *et al.,* 2002). Hence, these experiments provide a good translational model that can be used to test additions to cardioplegic solutions used in human studies. However, additional studies in higher animals are essential before G1 can be used in humans. Since, the older population is most likely to undergo cardiac surgery, these results could help improve outcomes of cardiac surgeries, especially in older men and women.

4.6 Limitations

The Langendorff-perfused mouse heart model used in this study has some limitations, although it is a widely used model for preclinical cardiovascular studies. It is an *ex vivo* study where the heart is isolated from the body of an animal. This could be an advantage, as the effects of drugs can be observed directly on the heart and blood vessels. On the other hand this could be a disadvantage, as the heart is isolated from other systems of the body which could potentially influence drug effects on the heart. Also, in this model of heart perfusion it is difficult to study the heart at more clinically relevant post-ischemic timepoints, like 12 or 24 hours post-reperfusion. Another limitation in this model is the use of relatively "healthy" animal hearts. In clinical studies, cardiac surgery is performed on diseased hearts, whereas in this study hearts were taken from mice with no known cardiovascular disease. Therefore, use of a diseased heart model could be more relevant to clinical practice. Lastly, the analysis of infarct area was challenging. When pictures of the heart slices were taken, some infarct areas were difficult to analyze. The infarcts were neither distinct white nor completely red. However, consistency was

maintained while measuring all the infarcts and only tissues that were completely red were considered viable.

4.7 Conclusions

This study, for the first time, demonstrated sex- and age-specific differences in the recovery of cardiac function after hypothermic STH2 cardioplegic arrest, followed by 90 min of ischemia. Moreover, a novel strategy of incorporating the GPER agonist, G1, in cardioplegia was shown to enhance its properties in this model. The results from this thesis demonstrated that there were sex specific differences in the recovery of cardiac function in adult and old male and female mice using STH2 cardioplegia. Adult female mice recovered better than adult male mice in reperfusion, which demonstrated that female hearts were more resistant to ischemic damage than male hearts. However, hearts from old mice of both sexes showed similar recovery in reperfusion. This suggested that hearts from older mice might have better protection with hypothermic STH2 cardioplegia than hearts from younger mice. In addition, older mice were heavier, which may have increased levels of the aromatase enzyme in their body and led to higher estrogen availability for cardioprotection. Also, ischemic preconditioning of the heart during cannulation might have provided certain level of protection. The combination of G1+STH2 also showed interesting age- and sex-specific cardioprotection in reperfusion. Adult female hearts were well protected with G1. This may be due to G1-mediated PI3K/Akt pathway and inhibition of mPTP opening. Also, G1 has vasodilatory effects, which may contribute to the higher coronary flow in reperfusion in adult female hearts. By contrast, adult male hearts were not protected by G1, although old hearts from both

sexes were protected by this treatment. This suggests that old hearts might have some prior cardioprotection, potentially from endogenous estrogen levels. Overall, incorporation of G1 in traditional cardioplegia could be a good strategy to improve outcomes of cardiac surgery, at least in adult females and old patients of both sexes. However, further studies are required to elucidate the underlying mechanism for this G1 mediated protection.

4.8 Future work

A key focus of future studies would be to identify the mechanism of G1 mediated cardioprotection in this experimental model. For that, a cellular model previously developed in our lab, could be used to evaluate effects of G1 added to cardioplegia on Ca^{2+} homeostasis (Egar *et al.,* 2014; O'Blenes *et al.,* 2011). In this model, myoctyes are superfused with buffer and exposed to simulated cardioplegia (with sodium dithionite, an O_2 scavenger) with or without drugs. A gas containing 90% N_2/10% CO_2 is directed over the chamber to reduce pO_2 to 12 mmHg and pH to 6.8 to simulate tissue conditions during cardioplegic arrest (Flaherty *et al.,* 1980; Wilson *et al.,* 1980). After exposure of cells to cardioplegia with or without drug, myocytes can be reperfused and recordings of contractions and Ca^{2+} transients can be made. Following this procedure we could determine whether Ca^{2+} overload is implicated in the cardioprotective effects of G1. Also, freezing some G1-treated hearts could be useful for molecular studies of potential pathways implicated in the effects of G1 on the heart.

Understanding the male-female differences in response to G1 in young adults would also be of interest. It is possible that increasing reperfusion time could reveal

beneficial effects of G1 in adult male hearts. Also, higher concentrations of G1 (1 μM) were not tested in adult male mouse hearts. Hence, it would be interesting to know whether male hearts could respond to a 1 μM concentration of G1. Furthermore, the sample size could be increased in a few of the experimental groups, if additional aged mice become available.

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Sent: Monday, July 23, 2018 8:51 PM
To: Anjali Ghimire
Subject: Re: Copyright permission for using a figure in my dissertation

Hi Anjali,

Yes, feel free to use it! And good luck :)

Monique

On Jul 23, 2018, at 11:32 AM, Anjali Ghimire <Anjali.Ghimire@Dal.Ca> wrote:

Dear Monique Guilderson,

I hope you are good. I am Anjali Ghimire, MSc 2nd year student at Dalhousie University. I am working in Prof. Susan Howlett's lab in an aging project. Presently, I am writing my thesis and will be defending very soon. I am particularly writing this email to ask your permission to use one of the pictures you created to keep in my dissertation. The picture from Keller and Howlett's (2016) paper of adult vs. aged heart appropriately describes the physiology I would like to show the readers of my thesis. I understand you are the copyright holder for this picture (attached along with this email) and I would like to request you to provide me your permission to use this picture in my dissertation.

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Thank you so much.

With best regards,
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Graduate Student
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