ALTERATIONS IN MITOCHONDRIAL MORPHOLOGY WITH
AGE-ASSOCIATED ESTROGEN DEFICIENCY AND ISCHEMIA-
REPERFUSION INJURY

A Thesis in
Physiology

by
Nicole Christine Aurigemma

© 2015 Nicole Christine Aurigemma

Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Master of Science

December 2015
The thesis of Nicole Aurigemma was reviewed and approved* by the following:

Donna Korzick  
Chair, Intercollege Program in Physiology  
Professor of Physiology and Kinesiology  
Thesis Advisor

Gustavo Nader  
Associate Professor of Kinesiology

Joshua Lambert  
Associate Professor of Food Science

*Signatures are on file in the Graduate School
ABSTRACT

Limited research has been conducted to elucidate age-related changes in the female rat heart. Further, no studies to date have analyzed mitochondrial morphology in the aged estrogen (E2)-deficient female rat heart. To examine the mitochondrial morphological alterations associated with age and E2 loss, we studied F334 female rats (n=40) subjected to coronary artery ligation (CAL) to induce ischemia-reperfusion (I/R) injury. In order to offer a more physiological model of estrogen deficiency with aging, we also utilized bilateral ovariectomy (OVX) in a subset of rats at 15 mo to be studied at 22-23 mo (MO OVX, n=16). Both adult and MO OVX rats underwent 31 min ischemia with reperfusion times 10 min, 6 hr, and 24 hr. 7 day reperfusion was studied in a subset of adult (n=2) and ovary-intact aged rats (n=2). Transmission electron microscopy was used to analyze mitochondrial area, density, and circularity. Western blot analysis was conducted on a subset of samples to assess changes in mitochondrial fission and fusion proteins dynamin-related protein 1 (Drp1), mitochondrial fission 1 (Fis1), mitofusin 1 (Mfn1), and optic atrophy 1 (OPA1). Aging with E2 deficiency resulted in smaller, more numerous mitochondria compared to adult. Increases in mitochondrial area and decreases in density were seen in adult and MO OVX within 6 hr of reperfusion, while reperfusion over 24 hr seemed to indicate recovery from ischemic injury, as mitochondrial areas returned to baseline in adult hearts. Mitochondrial Drp1 significantly increased with I/R, while no significant changes were seen in Fis1, Mfn1 or OPA1. For the first time, our mitochondrial morphological data suggests a temporal resolution for I/R injury in the female rat heart.
TABLE OF CONTENTS

LIST OF FIGURES ..................................................................................................... v
LIST OF TABLES ....................................................................................................... vi
ACKNOWLEDGEMENTS ......................................................................................... vii

Chapter 1  Introduction .......................................................................................... 1

Chapter 2  Review of Literature ........................................................................... 8

  Mitochondrial Quality Control and Dynamics ................................................. 8
    Biogenesis ........................................................................................................ 8
    Autophagy ....................................................................................................... 10
  Mitochondrial Dynamics .............................................................................. 14
    Breast Cancer ............................................................................................... 17
    Exercise ........................................................................................................ 18
  Cardiovascular and Mitochondrial Morphological Alteration ...................... 20
    Aging ............................................................................................................ 20
    Estrogen Deficiency ................................................................................... 21
    Ischemia-Reperfusion Injury .................................................................... 23
    Aging and I/R Injury .................................................................................. 26
  Mitochondrial Dysregulation and Cell Death Mechanisms ......................... 27
    Apoptosis .................................................................................................... 28
    Necrosis ...................................................................................................... 29
    Cell Death and I/R ..................................................................................... 31
    Translational Applications for Targeting Cell Death ................................. 33

Chapter 3  Manuscript .......................................................................................... 35

  Introduction .................................................................................................... 35
  Materials and Methods .................................................................................... 37
  Results ............................................................................................................. 44
  Discussion ....................................................................................................... 55

Chapter 4  Conclusions and Future Directions .................................................. 61

  Limitations ..................................................................................................... 63

Bibliography ....................................................................................................... 64

Appendix: Raw Data ............................................................................................ 81
LIST OF FIGURES

Figure 1: Mitochondrial elimination through autophagy and mitophagy ................. 11

Figure 2: Mechanism of mitochondrial fission, fusion, autophagy .......................... 15

Figure 3: Schematic representing the redistribution of cytochrome c through widening of cristae junctions ................................................................. 30

Figure 4: Schematic illustrating the opening of Mitochondrial Permeability Transition Pore. ......................................................................................... 39

Figure 5: Illustration for TEM sample collection .................................................... 40

Figure 6: Morphological changes for Adult, Aged, MO OVX ............................... 46

Figure 7: Time course of changes with increased reperfusion ............................... 47

Figure 8: 7 day Reperfusion in Adult and Aged ..................................................... 49

Figure 9: Area at Risk vs Remote comparisons in Adult and MO OVX ................. 50

Figure 10: Representative qualitative images for Adult, MO OVX, and Aged rats ... 53

Figure 11: Western blot analysis for 6 hr reperfusion ............................................. 55
LIST OF TABLES

Table 1: Estrogen Response Elements with associated DNA sequences. ............... 18
Table 2: Physiological Characteristics of Animals..................................................... 44
Table 3: Qualitative Characteristics of Adult, MO OVX, and Aged rats. ............... 52
Table 4: Means for mitochondrial characteristics....................................................... 82
Table 5: Adult individual mitochondrial values. ....................................................... 83-84
Table 6: MO OVX individual mitochondrial values. ............................................... 85-86
Table 7: Aged individual mitochondrial values.......................................................... 87
Table 8: Raw densometric values for mitochondrial Drp1 ...................................... 88
Table 9: Raw densometric values for mitochondrial Mfn1 ...................................... 89
Table 10: Raw densometric values for mitochondrial Fis1 ..................................... 90
Table 11: Raw densometric values for mitochondrial OPA1 .................................. 91
ACKNOWLEDGEMENTS

Throughout the past two years, many wonderful individuals have supported me and guided me towards the completion of this project. First, I would like to thank my thesis advisor, Dr. Korzick. Thank you for allowing me the opportunity to work in your lab. I believe this thesis experience has helped shape me into a more mature, thoughtful scientist.

I also thank my thesis committee members, Dr. Nader and Dr. Lambert. Thank you for agreeing to review my work, and for helpful ideas and comments to make a well-rounded thesis project. I have sincerely enjoyed the chance to work with you both.

A special thanks goes to Bobbie and Morgan. I would not be here without your constant moral support and friendship. Bobbie, you took me under my wing and believed in me when I doubted myself. Words cannot express how thankful I am for your guidance. Morgan, thank you for your selfless nature and kind words. It means so much to me to have your help throughout this process.

Finally, I thank my family for their strength and support. Kirsten- your positivity helped me through my toughest days. Dad- thank you for calming my nerves and keeping my focus on the big picture. Mom- I am so proud to follow in your footsteps. I am so thankful for your never-ending encouragement and advice. This one’s for you.
Chapter 1

Introduction

Coronary heart disease (CHD) remains the number one cause of death in men and women\textsuperscript{1–3}, affecting over 27 million people in the United States alone\textsuperscript{4}. CHD can present as acute coronary syndrome (ACS), which includes acute myocardial infarction (MI) or unstable angina. Cardiovascular morbidity and CHD-related mortality increases with age, although gender differences are present. Prior to menopause, women have a decreased risk for CHD compared to age-matched men\textsuperscript{5}. After menopause, this trend reverses and the cardiovascular risk for women rises dramatically. Prevalence of CHD in postmenopausal women increases 2-to-3-fold compared to premenopausal women of the same age\textsuperscript{6,7}. Further, more women over the age of 40 will die within one year of experiencing their first myocardial infarction (MI) compared to men\textsuperscript{7}. These findings demonstrate the likely influence of the menopausal transition and associated loss of endogenous estrogen (E\textsubscript{2}) as contributing factors to the augmented cardiovascular risk and increased incidence of CHD events in postmenopausal women with aging.

The fundamental definition of cardiac aging is the progressive degeneration and functional decline of the heart, augmenting the sensitivity to stress and damage. The increased generation of reactive oxygen species (ROS)\textsuperscript{8,9} contributes to oxidative stress on the heart, affecting mitochondrial function. Excess ROS disturbs oxidative phosphorylation by disrupting the proton gradient across the inner mitochondrial membrane, compromising the mitochondrial ability to produce ATP and ultimately
leading to an increase in mitochondrial dysfunction with age\textsuperscript{10,11}. Normal myocardial aging is associated with increased susceptibility to damage following ischemic injury\textsuperscript{12}. E\textsubscript{2}-deficiency exacerbates damage to the already deteriorating function of the aged heart. Following ovariectomy (OVX), adult (5-6 month) and aged (22-24 month) female animals show reduced ischemic tolerance\textsuperscript{13–15}. Data from our laboratory demonstrates that following ischemia and reperfusion (I/R), aged OVX rats experienced functional impairment and greater infarct size than that found in natural aging and OVX in adult\textsuperscript{15}. Therefore, these results suggest that reduced ischemic tolerance and increased I/R injury are influenced by aging and E\textsubscript{2}-deficiency combined.

Significant alterations in mitochondrial morphology are likely to contribute to age-associated decrements in mitochondrial function. Alterations in mitochondrial DNA expression have been associated with age and stress\textsuperscript{16,17} with deletions, in particular, leading to irregularities in mitochondrial cristae\textsuperscript{18}. While most research regarding the effects of age on mitochondrial morphology has been conducted in male animals, important findings may or may not be relevant in understanding age-associated changes in mitochondrial morphology in females. Increased mitochondrial volume with decreased density are seen with aging in male mice\textsuperscript{19–21}, rats\textsuperscript{22}, and hamsters\textsuperscript{23}. In aging male mice, mitochondria become more round and enlarged, potentially due to swelling\textsuperscript{16,21}. More research is necessary to determine whether these findings are also prevalent in aging female models.

Acute myocardial infarction (MI) also results in significant modifications to cardiac mitochondrial ultrastructure which impact function. Early studies examining the effect of ischemia on cardiovascular mitochondria in dogs reveal that damage is time-
dependent. It has been shown that ischemic periods of 15 min or less resulted in reversible damage, such as the occasional loss of mitochondrial cristae, whereas ischemic insults of 30 min or longer present irreversible damage, including abnormally clear matrices, severe cristae disruption and the presence of intramitochondrial granules\textsuperscript{24–26}. Time-dependent damage has also been demonstrated in adult male rats\textsuperscript{27,28}. Ultrastructure damage presents earlier in rats compared to dogs, with the separation of cristae, clearing of mitochondrial matrices, and occasional intramitochondrial granules seen as early as 5-10 min after the onset of ischemia\textsuperscript{29}. Reperfusion adds insult to ischemic injury, as the surge of ROS can contribute to loss of mitochondrial matrix, lysis of cristae, and disorganization of the myofibers in tissues at the periphery of the infarct lesion\textsuperscript{30}.

To the best of our knowledge, there is only one study that has looked at the effects of both aging and I/R injury on mitochondrial morphology. Adult (6-8 month) and aged (22-24 month) male C57BL/6 mice underwent 45-min of ischemia with varying reperfusion times, ranging from 15 min to 24 hr\textsuperscript{31}. Damage to aged tissues after 45 min ischemia without reperfusion was more severe compared to adult, with aged displaying more mitochondria with disrupted cristae\textsuperscript{31}. While 4 hr of reperfusion resulted in swelling, clearing of mitochondrial matrix and loss of cristae in both adult and aged, mitochondria were preserved in both age groups by 24 hr, with decreased resolution with age\textsuperscript{31}. Whether the resolution of ischemic damage occurs within a similar timeframe in the aged female heart following I/R injury remains unknown.

E\textsubscript{2} has been shown to play a key role in mitochondrial biogenesis\textsuperscript{32}, mitochondrial gene transcription\textsuperscript{33} and vascular function\textsuperscript{34}. Its antioxidant and anti-apoptotic functions in cardiomyocytes have also been implicated in protection against cell death\textsuperscript{35}. 
Cardioprotection through E_2 is likely to occur by preserving both mitochondrial function and structure. Chronic E_2 replacement in the form of 17β-estradiol, the main estrogenic hormone in women, has been reported to preserve contractile function of the heart after global ischemia\textsuperscript{36}, as well as reduce infarct size in male\textsuperscript{37} and female\textsuperscript{38} rabbit models of I/R injury.

Adult models of E_2 deficiency have been implicated in models of global and regional ischemia as the cause of severe mitochondrial ultrastructure damage, with increased presence of intramitochondrial granules, as well as swollen and fragmented mitochondria found in OVX rats\textsuperscript{39} and rabbits\textsuperscript{38}, when compared to sham animals. While OVX studies in adult animals provide insight into changes occurring with E_2 loss, these alterations may not be representative of those occurring in an aged female model. Adult rats experience a more robust response to OVX and loss of estrogenic negative feedback on the hypothalamic-pituitary-gonadal axis\textsuperscript{40–42}. In contrast, middle-aged and aged rats have delayed response to OVX as circulating steroid hormones can be detected months after OVX and therefore require more time to demonstrate the resultant increase in gonadotropins in response to lack of estrogenic feedback\textsuperscript{40–42}. Therefore, studies using adult OVX models may not serve as a model for menopause in aging women.

With the limited breadth of research in female aging and I/R injury, more focused research on E_2-deficient female models is necessary to attain a more definitive understanding of the physiology of the aged and E_2-deficient heart, especially at the mitochondrial level.
Statement of Problem

Following the menopausal transition, cardiovascular morbidity and mortality increases in women, associating the loss of E2 with the decrease in ischemic tolerance. Conflicting results regarding timing, duration and type of E2 used for hormone replacement therapy only accelerate the need to find alternative targets for novel treatments of ischemic heart disease, particularly in at-risk aged females. The combined affects both aging and E2-deficiency on cellular effectors of ischemic damage in the aged female heart are understudied. Mitochondrial quality control may be targeted with aging and E2 loss. The resultant decrements in ischemic tolerance and cardioprotection in the aged female heart may be closely related to mitochondrial ultrastructure damage impacting the ability to produce ATP, leading to dysregulated mitochondrial quality control which could potentially contribute to cell death through necrosis and apoptosis. Therefore, studying the singular and combined effects of aging and I/R injury on cardiac mitochondrial morphology may provide important insight into targets for therapeutic interventions to combat ischemic heart disease in the aged and E2-deficient female heart.
Specific Aims and Hypothesis

Specific Aim #1: To determine the combined effects of advancing age and physiologically relevant E2 deficiency on mitochondrial morphology in female F344 rats. Changes in mitochondrial morphology are seen in male species with age. Increases in mitochondrial volume, as well as decreases in numerical density (number of mitochondria per given area) are seen in aged male rodents\textsuperscript{19-23}. While mitochondrial research in aging females is lacking, it has been shown that OVX rabbits undergoing I/R experienced more mitochondrial swelling and cristae disruption as well as sarcomere disarray, compared to rabbits pre-treated with E2\textsuperscript{38}.

Hypothesis\textsubscript{1}: Average mitochondrial size will increase, with concurrent decrease in numerical density in aged vs adult.

Hypothesis\textsubscript{2}: Greater mitochondrial damage such as swelling, cristae disruption and intramitochondrial Ca\textsuperscript{2+} granules will be seen with age-associated E2 deficiency vs adult.

Hypothesis\textsubscript{3}: Myofibrillar fragmentation, z-line streaming and contracture band necrosis damage will be greater in aged E2-deficient cardiac tissue vs adult.
**Specific Aim #2**: To determine the effects of ischemia with varying reperfusion times on mitochondrial morphology in adult and aged F344 rats. Ischemia times under 15 min (without reperfusion) have been found to induce minimal and reversible changes to mitochondria.\(^24,26,29\). Irreversible damage, including mitochondrial matrix clearing, cristae disruption, swelling, accumulation of intramitochondrial Ca\(^{2+}\) granules is seen after 30 min of ischemia only.\(^24,25,27,29,43\). With reperfusion, increasing severity of mitochondrial alterations, including loss of matrix and lysis of cristae\(^30\) occur. Reperfusion also leads to calcium overload in the mitochondrial resulting in mitochondrial membrane permeabilization, the influx of water and thus swelling.\(^44\) Cardiomyocyte apoptosis, associated with mitochondrial fragmentation and fission\(^45\), increases early after MI\(^46\) and peaks at 4.5 hr, whereas necrotic cell death peaks at 24 hr.\(^47\).

**Hypothesis 1**: With increasing reperfusion time up to 6 hr, larger mitochondria with will be seen. Additionally, aged E\(_2\)-deficient tissues will show increased number of swollen, disrupted mitochondria containing intramitochondrial Ca\(^{2+}\) granules with increasing reperfusion times compared to adult tissues. In a subset of animals, 7 day reperfusion will show less mitochondrial alterations in adult vs aged tissues.

**Hypothesis 2**: Remote regions will be unaffected by increased reperfusion.

**Hypothesis 3**: Increased fission proteins, Drp1 and Fis1, will be associated with numerous small mitochondria at 6 hr reperfusion.
Chapter 2
Review of Literature

Following the menopausal transition, cardiovascular morbidity and mortality increases in women, associating the loss of E₂ with the decrease in ischemic tolerance. Despite this fact, the combined affects both aging and E₂-deficiency on cellular effectors of ischemic damage in the aged female heart remains understudied. Mitochondrial quality control (QC) may be targeted with aging and E₂ loss. In the heart, a fine balance of QC and mitochondrial dynamics allows for cardiomyocyte homeostasis. Mitochondrial biogenesis compliments basal autophagy, while individual mitochondria undergo the ebb and flow of fission and fusion. Factors contributing to the added stress of the aged heart include I/R injury and E₂ loss, which may ultimately lead to poorly modulated mitochondrial QC resulting in the induction of death mechanisms of necrosis and apoptosis. The purpose of this review is to address normal cellular operations and elaborate on the impact of I/R and E₂ deficiency on mitochondria as well as the potential induction mechanisms of cellular death with dysregulated QC in the aging female heart.

I. Mitochondrial Quality Control and Dynamics

Biogenesis

Regular cycles of biogenesis and degradation by autophagy, together deemed mitochondrial turnover, permit the maintenance of functional mitochondrial networks within the cardiomyocyte. Early studies report mitochondrial turnover occurs with a half-
life of 17.8 days in the rat heart\textsuperscript{48}, while others suggest a shorter half-life of 5-6 days\textsuperscript{49}. The high energy demands of the heart necessitate a greater concentration of mitochondria, and thus greater mitochondrial turnover, to meet these needs. This is observed in rat cardiomyocytes, where it is estimated that one mitochondria is replaced every 40 minutes\textsuperscript{50}.

The key transcription factor regulating mitochondrial biogenesis is peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1\textalpha), a member of the nuclear receptor superfamily responsible for macrocomplex assembly\textsuperscript{50}. PGC-1\textalpha controls the expression of nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), which in turn direct the transcription of nuclear-encoded mitochondrial proteins, cofactors for assembly of electron transport chain complexes, as well as mitochondrial transcription factor A (Tfam). Tfam is essential for mitochondrial gene transcription which includes the 13 subunits of the respiratory chain as well as mitochondrial DNA (mtDNA) copy number\textsuperscript{51}.

The two isoforms of PGC1, \textalpha and \textbeta, are abundantly expressed in tissues with high metabolic demands including the heart\textsuperscript{52}. External stimuli, such as cold temperature and exercise, can increase PGC-1\textalpha expression leading to augmented expression of mitochondrial oxidative enzymes ATP synthase, and cytochrome oxidase subunits COXII and IV\textsuperscript{53}. PGC-1\textalpha also increases expression of superoxide dismutase (SOD) and glutathione biosynthesis\textsuperscript{54}, thus allowing cardiomyocytes to maintain normal redox status in response to changes in oxidative capacity. Under normal conditions, the enzymes SOD, catalase and glutathione peroxidase scavenge the ROS created in the cell, mainly from the mitochondrial electron transport chain.
Additionally, PGC-1α has been implicated in energy homeostasis maintenance. In a mouse model null for cardiac PGC-1α, a 30-50% reduction in oxidative phosphorylation, fatty acid oxidation and ATP synthesis gene expression is seen compared to wild-type mice, with an additional 10% reduction in functional measures of dP/dt and LVSP\(^{55}\). Despite maintaining normal tissue structure, PGC-1α deficient hearts demonstrate decreased oxidative capacity as well as lessened contractile function\(^{55,56}\), suggesting the importance of PGC-1α for cardiovascular energy homeostasis. Furthermore, PGC-1α has been linked to the regulation of mitochondrial dynamics\(^{57}\).

**Autophagy**

Autophagy plays an important role in cellular health, allowing for the disposal of excess or damaged organelles within the cell. The process of autophagy compliments biogenesis, as a balance of mitochondrial turnover is necessary for the maintenance of cellular homeostasis. Autophagy consists of three main pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy\(^{58}\). Macroautophagy, the most common form of autophagy, progresses to the autophagosomal-lysosomal pathway, permitting the selective degradation of dysfunctional and impaired components of the cell, seen in Figure 1. Pre-autophagosomal structures form around cellular material and encloses to form the autophagosome, which is detectable by electron microscopy based on their double-membrane structures\(^{59}\). The autophagosomal outer membrane then fuses with lysosome to form autophagolysosome, ensuing in the digestion of the inner contents and thus their removal from the cell\(^{60}\).
**Figure 1:** Mitochondrial elimination through autophagy and mitophagy. (Taken from Ikeda⁶¹)
Individual damaged mitochondria can be targeted for degradation through mitophagy, a process regulated by PTEN-induced kinase1 (PINK1) and Parkin. Damage to mitochondria with inner membrane potential loss causes the stabilization of PINK1 to the outer mitochondrial membrane\(^61,62\). As PINK1 accumulates, it recruits E3-ubiquitin ligase Parkin, which ubiquitinates voltage-dependent anion channels and mitofusin\(^2\)\(^61,62\). Parkin also promotes the clearance of damaged mitochondria through the recruitment of ubiquitin binding HDAC6 and p62, which binds LC3 to promote autophagosome formation to remove damaged mitochondria\(^61,62\). If mitophagy fails, damaged mitochondria can be removed by general autophagy, but this process is less selective and may engulf more than just a single mitochondrion for degradation.

Cell survival is dependent on the integrity of mitochondria within the cell. Dysfunctional mitochondria may produce excessive ROS and apoptogens leading to further damage in neighboring mitochondria\(^60\). While Lon proteases and AAA+ proteases degrade small aggregated peptides in order to maintain protein QC\(^63\), removal of damaged mitochondria occurs through autophagy, allowing for a reduction in the oxidative burden\(^64\). In such case, eliminating a few unstable mitochondria could potentially preserve the neighboring mitochondria allowing for maintenance of cellular homeostasis. Under normal conditions, autophagy occurs at low basal levels in the heart\(^65\). Numerous studies examining acute stress such as I/R injury demonstrate increased number of autophagosomes in response to ischemia\(^66-69\) and reperfusion\(^70,71\). Although still debated, upregulated autophagy with ischemia may serve to be cardioprotective. As AMP-activated protein kinase (AMPK) is needed for autophagic activation during I/R, mice that underwent 20 min of ischemia showed increased phosph-
orylation (activation) of AMPK, but not mTOR activity \(^{68}\), which is a negative regulator of autophagy. Hypoxia-inducing factor 1 (HIF1) upregulation and GSK-3β activation also result from ischemia. Activation of GSK-3β inhibits mTOR, allowing for autophagy and potentially leading to cellular protection, while inhibition of GSK-3β during ischemia leads to increased infarct size and number of necrotic cells \(^{72}\). Conversely, inhibition of GSK-3β during 24 hr reperfusion decreased infarct size by activating mTOR and promoting cell survival mechanisms and mitochondrial biogenesis \(^{72}\). Thus GSK-3β activation (and ultimately upregulated autophagy) is cardioprotective during ischemia, while GSK-3β inactivation is cardioprotective during reperfusion.

While augmented autophagy during ischemia may be protective, reperfusion may cause a greater, potentially detrimental, increase in autophagy. The additional surge of ROS, as well as the upregulation of Beclin1 during reperfusion can lead to added cardiovascular damage. Mice with 20 min of ischemia and 20 min of reperfusion demonstrated mTOR activation, which was indicated by phosphorylation of downstream target p70, as well as Beclin1 upregulation \(^{68}\). Reducing Beclin 1 expression in cardiac myocytes reduced I/R-induced autophagy and enhanced cell survival \(^{69}\). Ultimately, an upregulation of autophagy with reperfusion can lead to induction of cell death, discussed in section III. Additionally, a reduction in basal autophagy can result in disarray within the cell. Autophagy and mitophagy decrease with age \(^{73-76}\), as aging leads to accumulation of dysfunctional mitochondria \(^{64}\). It has been postulated that a buildup of lipofuscin or a greater energy requirement to clear larger mitochondria may cause this decrease in autophagy \(^{77}\).
While E2 may play a role in the regulation of autophagy, the mechanism in the heart remains unclear. It has been previously shown that E2 stimulates autophagy\textsuperscript{78}, as well as the suppression of mTOR activation\textsuperscript{79} in non-cardiomyocytes. In contrast, E2 has been shown to attenuate LPS-induced autophagy in cardiomyocytes\textsuperscript{80} as well as in BNIP3-induced cardiac autophagy\textsuperscript{81}. More research is necessary to determine the exact mechanism of E2-regulated autophagy in the heart.

**Mitochondrial Dynamics**

Mitochondrial dynamics are essential for growth and development, as mice germ-line deletions of fission or fusion proteins are embryonic lethal\textsuperscript{57}. Fission and fusion represent the dynamic processes occurring in mitochondria between the time of creation and destruction, as seen in Figure 2. Fission is the division of mitochondria and occurs mainly through the interaction between two proteins, dynamin-related protein 1 (Drp1) and human fission protein 1 (Fis1). Localized in the cytoplasm, the 85-kDa GTPase Drp1, translocates to the mitochondrial outer membrane to play a central regulatory role for mitochondrial fission. The GTPase activity allows for the scission of the outer mitochondrial lipid bilayer in order to initiate the process of fission. The 17-kDa mitochondrial membrane-anchored protein Fis1 acts as a receptor Drp1 on the outer membrane\textsuperscript{82}. More recent studies also demonstrate Fis1 also acts as a docking site for newly identified mitochondrial protein TBC1D15, associated with mitochondrial fission\textsuperscript{83}.
**Figure 2:** Mechanisms of mitochondrial fission, fusion and mitophagy. Initiation of fission and fusion (A), damaged mitochondria removed through fission and mitophagy (B). Drp1, dynamin related protein 1; Mfn1, mitofusin 1; Mfn2, mitofusin 2; Opa1, optic atrophy 1; OMM, outer mitochondrial membrane. (Taken from Dorn and Kitsis62)
Two types of fission can occur—physiological and pathological. Physiological fission has been shown to occur in response to increased intracellular Ca\(^{2+}\) in non-cardiovascular cells\(^{84}\). Fission is closely associated with mitophagy, allowing for smaller mitochondria to be consumed by autophagosomes\(^{85}\). While fission is a necessary part of the normal cell cycle, pathological fission may lead to fragmentation occurring in the early stages of apoptosis\(^{86}\) or autophagy as a consequence of upregulated damage associated-molecular patterns (DAMPs) due to stress/disease states.

Mitochondrial fusion is defined as the tethering and joining of two mitochondria. It involves interplay between two outer mitochondrial membrane proteins mitofusin1 (Mfn1) and mitofusin2 (Mfn2), as well as the optic atrophy 1 (OPA1) found in the intermembrane space or inner mitochondrial membrane (IMM). The 85-kDa mitofusins are GTPase isoforms required for the tethering of two mitochondria\(^{87}\), while OPA1 is a larger 100-kDa GTPase necessary for tethering and fusing of the IMM\(^{88}\). OPA1 may also sequester cytochrome c, as changes in its activity can lead to induction of cell death\(^{89}\).

I/R injury can impact mitochondrial dynamics with the potential to cause cardiovascular damage. Inappropriate mitochondrial fission has been shown to arise from I/R injury in the heart\(^{90}\). I/R injury can lead to the detrimental increase in autophagy. Bnip3, a BH3-domain protein that is a hypoxia-induced death gene, has been shown to promote mitochondrial fission during ischemic injury with a mechanism involving Drp1\(^{91,92}\).
Breast Cancer

While E$_2$ may play a protective role in the cardiovascular system, it may also be carcinogenic in models such as breast cancer. Breast cancer is an E$_2$-dependent malignancy prominent in postmenopausal women. Previous research has identified five estrogen response elements (ERE) in human mitochondrial DNA (mtDNA), four located in the D-loop and one located in the coding region$^{93}$, associated with OXPHOS gene expression of cytochrome oxidase (COX) subunits COXI-IV displayed in Table 1$^{94}$. It is suggested that mtDNA may be a direct target for E$_2$ acting primarily through estrogen receptor β (ERβ), as ERβ antibody has been shown to interfere with the binding of breast cancer cell mitochondrial extract to EREs in human mtDNA$^{95}$. Further, research from the Oliver-Pilar laboratory has shown that the ERα/ERβ ratio is a major determinant of oxidative stress and mitochondrial functionality in various breast cancer cell lines$^{96–99}$. High ERα/ERβ ratio in MCF-7 cell line is associated with increased proliferation and oxidative damage with E$_2$ treatment. Increases in antioxidant and uncoupling protein gene expression are also increased in MCF-7 cells, although increased oxidative stress may lead to post translational modifications that cause inactivation or down regulation, as seen in decreases antioxidant and uncoupling protein activity and protein levels$^{96}$. Low ERα/ERβ ratio in T47D cell line demonstrates decreased ROS as well as increased uncoupling protein levels with E$_2$ treatment$^{96}$. These data suggest that E$_2$ may act as either an antioxidant or pro-oxidant, depending on the ERα/ERβ ratio of the tissue.

Additionally, work from the Oliver-Pilar laboratory has demonstrated the effects of E$_2$ on mitochondrial dynamics. MCF-7 cells treated with E$_2$ for 4 hr and 12 hr
demonstrated increased expression of fusion genes (Mfn1, Mfn2, and Opa1) and fission protein Drp1, while decreased Fis1 was seen at each treatment time point\textsuperscript{100}. Concurrent decreases in mitochondrial respiratory chain complex I-IV were seen in these MCF-7 cells with 24 hr E\textsubscript{2} treatment compared to vehicle treated cells\textsuperscript{100}. This group further explored the relevance of ER\textbeta on mitochondrial dynamics of T47D-ER\textbeta tetracycline-inducible cell line. ER\textbeta repression through doxycycline treatment increased Mfn1, Mfn2, Opa1, PGC1\alpha and Tfr genes. Further, mitochondria function decreased in the absence of ER\textbeta, suggesting the protective role of ER\textbeta in cancer mitochondria\textsuperscript{98}.

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Loop ERE I</td>
<td>5'-XAGCTTATATAAGACCAA-3'</td>
</tr>
<tr>
<td>D-Loop ERE II</td>
<td>5'-AGCTTCCCCAGACA-3'</td>
</tr>
<tr>
<td>D-Loop ERE III</td>
<td>5'-XAGCTTGCGGTGACCAA-3'</td>
</tr>
<tr>
<td>D-Loop ERE IV</td>
<td>5'-AGCTTAGGCATAAGCCTAA-3'</td>
</tr>
<tr>
<td>CO II ERE</td>
<td>5'-AGCTTAGGTCATCCCTA-3'</td>
</tr>
</tbody>
</table>

**Table 1**: Estrogen Response elements with associated DNA sequences. (Adapted from Chen\textsuperscript{101})

*Exercise*

The impact of exercise on mitochondrial dynamics in skeletal muscle may be dependent on the type of training performed. Endurance (aerobic) training has been shown to upregulate Mfn1, Mfn2, and Fis1\textsuperscript{102}. Chronic contractile activity may favor
mitochondrial fusion as increased interfibrillar mitochondrial area and simultaneous increases in Opa1 and Mfn2 are seen in male rats\textsuperscript{103}. Strength (resistance) exercise has been shown to increase mitochondrial biogenesis through increases in PGC1\(\alpha\) in both acute\textsuperscript{104} and chronic\textsuperscript{105} strength training. This augmentation of PGC1\(\alpha\) and Tfam mRNA with strength training is seen independent of aging\textsuperscript{106}. Further, the combination of endurance and resistance exercise (concurrent training) results in 2-fold greater increases in mitochondrial biogenesis proteins PGC1\(\alpha\), PRC, and co-activator PDK4 than with endurance exercise alone\textsuperscript{107}. Exercise can be protective in aging subjects as the slight increase in ROS generated promotes phosphorylation of proteins involved in signaling responses involved in mitochondrial function, antioxidant capacity, and growth\textsuperscript{108}.

Less research has been conducted regarding the effects of exercise on cardiomyocyte mitochondrial dynamics. Of the limited research, it is suggested that chronic exercise training may not impact left ventricular mitochondria and skeletal mitochondrial in a similar fashion. In a 2011 study conducted by Li et al., 12 weeks of treadmill training induced increases in gastrocnemius PGC1\(\alpha\) mRNA and protein content with subsequent activation, while these changes were not seen in the exercised LV\textsuperscript{109}. A recent study demonstrates that aerobic interval training following coronary artery ligation increases in mitochondrial biogenesis, fusion and respiratory function in male rats\textsuperscript{110}. Additionally, exercise training restored mitochondrial quality control in post-myocardial infarction-induced heart failure rat model, as seen by a restoration of respiration, MPTP sensitivity, and reduction of ROS\textsuperscript{111}. More research is necessary to determine a more comprehensive understanding the impact of exercise on the cardiomyocyte mitochondrial dynamics.
II. Cardiovascular and Mitochondrial Morphological Alterations

Aging

The fundamental definition of cardiac aging is the progressive degeneration and functional decline of the heart, augmenting the sensitivity to stress and damage. The increased generation of reactive oxygen species (ROS)\(^8,^9,^{112,113}\) contributes to oxidative stress on the heart, affecting mitochondrial function. Excess ROS disturbs oxidative phosphorylation, disrupting the proton gradient across the inner mitochondrial membrane, compromising the mitochondrial ability to produce ATP, which can be seen with associated increase in oxidative stress in cardiac interfibrillar mitochondria\(^114,115\). Ultimately, augmented ROS increases mitochondrial dysfunction with age\(^10,11\).

Because mtDNA is located in close vicinity to electron transport machinery, mitochondria are highly susceptible to ROS-mediated damage, as seen by the accumulation of mutations with age\(^17\), contributing to further mitochondrial dysfunction\(^116\). Microarray analysis comparing genes coding for mitochondrial proteins in adult and aged rats revealed down regulation of complex I and II activities of oxidative phosphorylation corresponding to decreased energetic efficiency\(^117\). Further, senescence has been shown to result in greater damage following I/R injury, as aged rats displayed a 50% increase in infarct size compared to adult\(^118\).

While most research regarding the effects of age on mitochondrial morphology has been conducted in male animals, important findings may or may not be relevant in understanding the role of mitochondrial morphology in dysregulated QC in the aging female model. Increases in mitochondrial average volume with concurrent decreases in
density measurement are seen with aging in male mice\textsuperscript{19–21}. Consistent with aged mice, aged male rats show an increased mitochondrial volume, although a change in numerical mitochondrial density is not observed\textsuperscript{22}. An increased volume density is seen in aged Syrian hamsters\textsuperscript{23}. In aging male mice, mitochondria become more round and enlarged, potentially due to swelling\textsuperscript{16,21}, while aged male rats display a larger percentage of elongated mitochondria\textsuperscript{22}. Cristae disruption and vacuolization are seen in aged mitochondria in murine models\textsuperscript{114,119}. More research is necessary to determine whether these findings are also prevalent in aging female models.

\textbf{Estrogen-Deficiency}

The potent steroidal hormone, E\(_2\), highly present in females up to menopause and to a lesser extent in males, performs a multitude of beneficial functions. E\(_2\) increases antioxidant gene expression, as well as eNOS activity\textsuperscript{34}. E\(_2\) serves anti-apoptotic functions in rat cardiomyocytes, as it indirectly regulates HSP72, which stabilizes the mitochondrial membrane and thus prevents apoptosome formation\textsuperscript{35}. Increases mtDNA gene transcription have been shown in association with E\(_2\) treatment, particularly of genes related to electron transport chain activity and oxygen consumption\textsuperscript{33}. Although E\(_2\) treatment has been implicated in mitochondrial biogenesis of cancer cell lines by upregulating the expression of NRF1 and Tfam\textsuperscript{32}, research is necessary to confirm whether this is also applicable to cardiomyocytes.

Adult models of E\(_2\) loss may vary compared to aged models. Adult rats experience a more robust response to OVX, displaying relatively undetectable levels of
steroid hormones and a maximal concentration of gonadotropins (gonadotropin-releasing hormone, luteinizing hormone and follicle stimulating hormone) in response to the loss of estrogenic negative feedback on the hypothalamic-pituitary-gonadal axis. In contrast, middle-aged and aged rats may still have detectable levels of circulating steroid hormones months after OVX and therefore require more time to demonstrate the resultant increase in gonadotropins in response to lack of estrogenic feedback. This delayed response to loss of feedback may be attributed to a decrease in pituitary sensitivity to stimulation with age. Therefore, studies using adult OVX models or those studying aged rats too early post-OVX may not capture the loss of estrogen in order to serve as a strong and relevant model for menopause in aging women.

OVX has been shown to induce at least two-fold increases in genes associated with inflammation, extracellular matrix and cell-cell interactions in the adult rat heart. While aged ovariectomized (OVX) Brown Norway rats also resulted in increased pro-inflammatory and pro-fibrotic genes, a decrease in soluble guanylyl cyclase (associated with impaired vascular function) was seen left ventricle tissue. This is in contrast to unchanged soluble guanylyl cyclase in adult OVX Sprague-Dawley rats. While OVX studies in adult animals provide insight into changes occurring with E2 loss, these alterations may not be representative of those occurring in an aged female model.

E2 may be cardioprotective against I/R injury by preserving both mitochondrial function and structure. A reduction in inflammation is seen in ovary-intact female mice at the border zone of infarct compared to males. Chronic E2 replacement in the form of 17β-estradiol, the main estrogenic hormone in women, has been reported to preserve contractile function of the heart after global ischemia. Administration of 17β-estradiol
reportedly reduces the infarct size in rabbit\textsuperscript{37,38} and mouse\textsuperscript{123} models of I/R injury. Similarly, 17$\beta$-estradiol has been shown to reduce the infarct size in dogs after 90 min ischemia and 6 hr of reperfusion, by increasing nitric oxide production and opening Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels\textsuperscript{124}. E\textsubscript{2}-deficiency has been implicated in models of global and regional ischemia as the cause of severe mitochondrial ultrastructure damage, with increased presence of intramitochondrial granules, as well as swollen and fragmented mitochondria found in ovariectomized rats\textsuperscript{39} and rabbits\textsuperscript{38}, when compared to sham animals. The antioxidant functionality of E\textsubscript{2}\textsuperscript{34}, including reducing ROS generation through decreasing NAD(P)H oxidase subunits, increasing ROS scavenging through augmented MnSOD expression, and increasing nitric oxide production by activating eNOS and its cofactor BH\textsubscript{4}\textsuperscript{125}, may be lost with OVX. Alterations in mitochondrial morphology may be partially due to this loss of antioxidant characteristics of E\textsubscript{2}. Additionally, 17$\beta$-estradiol treatment has been shown to be cardioprotective, by preventing OVX-induced cardiomyocyte apoptosis in rats\textsuperscript{126}. With the limited breadth of research in female aging and I/R injury, more focused research on E\textsubscript{2}-deficient female models is necessary to attain a more definitive understanding of the physiology of the aged and E\textsubscript{2}-deficient heart, especially at the mitochondrial level.

\textbf{Ischemia-Reperfusion Injury}

A myriad of complications arise from ischemia, contributing to cardiovascular damage. Increased ROS production occurs during ischemia, produced primarily from complex I\textsuperscript{127} and complex III\textsuperscript{128,129} of the mitochondrial electron transport chain causes a
detrimental oxidative burden. The low oxygen, high Ca^{2+} environment resulting from ischemia leads to a harmful decrease in pH, dropping from 7.2 to 6.3^{130}. Reperfusion intensifies the already present injury, as an additional surge of ROS creates progressive tissue damage secondary to initial ischemic insult^{131}. This damage worsens over time, as time dependent decrements in myocardial function with concomitant increase in infarction volume have been shown to occur up 7 days post I/R in mice^{132}. In adult C57/BL6 male mice receiving 60 min of ischemia, significant decrements in left ventricular functional parameters were seen post I/R, as stroke volume, cardiac output, and ejection fraction decreased at every time point compared to baseline^{132}. With a ~50% increase in end diastolic volume, 340% increase in end systolic volume, 25% decrease in stroke volume, 32% decrease in cardiac output, 47% decrease in ejection fraction, and 38% increase in left ventricular mass, the greatest damage was observed after 7 days post I/R^{132}. Additionally, decreased function with I/R is not limited to the heart, as mitochondria show increased respiratory dysfunction with I/R^{133}.

Acute myocardial infarction (MI) also presents significant modifications within cardiac mitochondria, as seen through changes in mitochondrial morphology and ultrastructure. Early studies examining the effect of ischemia on cardiovascular mitochondria in dogs reveal time-dependent damage. Studies conducted by Jennings and colleagues demonstrated the progression of mitochondrial damage with ischemic insults ranging from 5 to 240 min of ischemia^{24,25,134}. Ischemic periods of 15 min or less resulted in reversible damage, such as the occasional loss of mitochondrial cristae, whereas ischemic insults of 30 min or longer present irreversible damage, including
swollen mitochondrial with abnormally clear matrices, severe cristae disruption and the presence of intramitochondrial granules\textsuperscript{24–27}.

Time-dependent damage has also been demonstrated in rats\textsuperscript{27,28}. Ultrastructure damage presents earlier than in rats compared to dogs, with the separation of cristae, clearing of mitochondrial matrices, and occasional intramitochondrial granules seen as early as 5-10 min after onset of ischemic events\textsuperscript{29}. Mitochondrial swelling is also prominent 30 min to 1 hr after permanent ischemia\textsuperscript{29,43,135}. Reperfusion adds insult to ischemic injury, as the resumption of blood flow contributes to loss of mitochondrial matrix\textsuperscript{30,136} lysis of cristae\textsuperscript{30}, presence of Ca\textsuperscript{2+} granules\textsuperscript{29} and disorganization of the myofibers in tissues at the periphery of the infarct lesion\textsuperscript{30}.

As mitochondrial swelling is a prominent morphological characteristic seen with ischemia, it is important to understand the mechanism. After an ischemic insult, the cells of anoxic tissue resort to anaerobic metabolism. This creates an acidic environment in the cell that usually is handled by the Na\textsuperscript{+}/H\textsuperscript{+} antiporter, but the cell becomes loaded with Na\textsuperscript{+} which cannot be pumped out of the cell\textsuperscript{137}. As a result, the Na\textsuperscript{+}/Ca\textsuperscript{2+} antiporter reverses its activity and the cell now becomes loaded with calcium\textsuperscript{138}. The mitochondrial membrane then permeablates allowing the influx of water, thus swelling the mitochondria\textsuperscript{44}. Alternatively, swelling may result due to decreased potassium uptake and the opening of the mitochondrial K\textsubscript{ATP} channel, resulting in the contraction of mitochondrial matrix and thus increasing the intermembrane space\textsuperscript{139,140}. This compensatory expansion may be cardioprotective as the mitochondria can provide a reservoir for excess water upon exposure to stress\textsuperscript{140}. It must be noted that mitochondrial
swelling as a result of necrotic cell death upon reperfusion is maladaptive and occurs as a worsening of membrane permeabilization, discussed in section III.

**Aging and I/R Injury**

Normal myocardial aging is associated with increased susceptibility to damage following ischemic injury\textsuperscript{12,141} and ischemic intolerance\textsuperscript{142}. Previous work in our laboratory demonstrates that impaired ischemic intolerance in aged male rats is associated with increased PKC\(\delta\) expression, which can be reversed by acute PKC\(\delta\) inhibition\textsuperscript{143} upon reperfusion. Additionally, our laboratory has shown that the ischemic-induced reduction of PKC\(\varepsilon\) and increase in GSK-3\(\beta\) at the mitochondria can be improved by acute PKC\(\varepsilon\) activation\textsuperscript{144}, while ER-\(\beta\) has no effect on ischemic tolerance in aged female rat heart\textsuperscript{145}. Increased susceptibility to damage from greater Ca\(^{2+}\) overload is also seen in aged hearts\textsuperscript{146}, although evidence suggests that Ca\(^{2+}\) overload may develop as early as middle age in male and female mice\textsuperscript{147}.

To the best of our knowledge, only one study has examined the effects of both aging and I/R injury on mitochondrial morphology. Adult (6-8 month) and aged (22-24 month) male C57BL/6 mice underwent 45 min of ischemia with varying reperfusion times, ranging from 15 min to 24 hr\textsuperscript{31}. Damage to aged tissues after 45 min ischemia without reperfusion was more severe compared to adult tissues, with aged displaying more mitochondria with disrupted cristae\textsuperscript{31}. While 4 hr of reperfusion resulted in swelling, clearing of mitochondrial matrix as well as loss of cristae in both adult and aged tissues, 24 hr of reperfusion showed better preserved mitochondria in tissues from adult
vs aged. Whether these results reflect what could happen in a female rat model of aging and I/R remains unknown.

The amalgamation of I/R injury with age and E2-deficiency has additive effects leading to detrimental consequences. Following OVX, adult and aged female animals show reduced ischemic tolerance. Data from our laboratory demonstrates that following I/R, aged OVX Fischer 344 rats experienced functional impairment and greater infarct size than that found in natural aging and OXV alone. While these results suggest that reduced ischemic tolerance and increased I/R injury may result from the combined effects of aging and E2 deficiency, it remains to be seen whether the additive effects impact mitochondrial morphology.

III. Mitochondrial Dysregulation and Cell Death Mechanisms

Dysregulation and abnormalities in mitochondrial QC, including autophagy and turnover, can ultimately lead to cell death mechanisms of apoptosis and necrosis. Compromised mitochondrial QC results in the accrual of damaged mitochondria that are less efficient at generating ATP, but also creates higher levels of oxidative stress by generating more ROS. When autophagy/mitophagy no longer functions properly, such as with aging and reperfusion following ischemic insult, cell death mechanisms ensue.
Apoptosis

Apoptosis is the process of cell death occurring through a genetically programmed sequence of biochemical stages without inducing a secondary inflammatory response. Classic morphological characteristics of apoptosis in the heart include chromatin condensation, nuclear fragmentation, shrinkage of cytoplasm, and sarcolemmal blebbing, all while still maintaining membrane integrity\textsuperscript{148}. Apoptosis can be detected biochemically through in situ end-labeling (ISEL) of nuclei containing 180 to 200 base pair fragments of internucleosomal DNA\textsuperscript{149}, as well as through DNA laddering\textsuperscript{148}.

The initiation of apoptosis occurs through two distinct pathways: extrinsically or intrinsically. The extrinsic, or death ligand-mediated pathway, is initiated by the binding of a death ligand, such as FAS ligand, tumor necrosis factor (TNF)-\(\alpha\), or TRAIL to cell surface receptor\textsuperscript{150}. The formation of the death-inducing signaling complex (DISC) follows suit and leads to procaspase-8 activation. Caspase-8 cleaves procaspase-3, leading to cell death. In contrast, the intrinsic mitochondrial pathway is activated when damage to the cell caused by stress, toxins, or ROS incites the translocation of proteins Bid and Bax to the mitochondrial outer mitochondrial membrane, resulting in permeabilization of the membrane. Membrane permeabilization results in the release of apoptogens from the mitochondria, including cytochrome c (cyt c), Smac/DIABLO, apoptosis-inducing factor (AIF), and endonuclease G (Endo G) to be released from the mitochondria\textsuperscript{151}. The apoptosome assembles, including Cyt C, dATP, Apaf-1, and
procaspase-9 leading to the eventual procaspase-9 activation. Ultimately, Caspase-9 leads to procaspase-3 activation.

Morphological alterations due to apoptosis are not limited to the whole-cell level, as apoptosis remolds the structures of the mitochondrial inner cristae. Under normal conditions, only 15% of cyt c is located in the intermembrane space and available for release\textsuperscript{152}, while the rest remains housed in the cristae. By widening cristae junctions, apoptosis redistributes cyt c from the cristae to the intermembrane space, while also increasing cristae interconnectivity\textsuperscript{153,154} thus increasing the overall availability of cyt c in the intermembrane space (Figure 3). Mitochondrial fission is also associated with apoptosis\textsuperscript{155,156}, further demonstrating that apoptosis affects tissues not only the cell as a whole, but also the organelles within.

**Necrosis**

Unlike apoptosis, necrosis is characterized by disruption of the plasma membrane, and organelle swelling\textsuperscript{66,150}, leading to secondary inflammatory response within the cell. Numerous factors may contribute to the initiation of necrosis including ROS, death receptors, Ca\textsuperscript{2+} overload and ultimately the opening of the mitochondrial permeability transition pore (MPTP). The MPTP is a Ca\textsuperscript{2+}-mediated, non-selective pore in the IMM which allows for molecules <1.5 kDa to pass through, inclusive of water and protons\textsuperscript{157,158}. The MPTP, which was closed during ischemia due to low pH, opens during reperfusion upon the reestablishment of physiological pH\textsuperscript{159}. Once the pore opens, proteins which are unable to cross through the MPTP, exert colloidal osmotic pressure
Figure 3: Schematic representing the redistribution of cytochrome c through the widening of cristae junctions. CI, complex I; cyt c, cytochrome c; OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane; OPA1, optic atrophy 1; MPP+, 1-methyl-4-phenyl-pyridinium; ROS, reactive oxygen species; Bax, Bcl2-associated X protein. (Taken from Ramonet et al.\textsuperscript{154})
resulting in mitochondrial swelling\textsuperscript{137}. This influx of water leads to swelling of matrix, without disrupting the IMM. On the other hand, the OMM is not able to withstand the influx of water and will therefore rupture, releasing cyt c and initiating the apoptotic cascade. As a result, the proton gradient and electrical potential across the IMM collapse, causing the uncoupling of oxidative phosphorylation. If the MPTP remains open, necrotic cell death will ensue, as seen in Figure 4.

While necrosis is generally considered unregulated, evidence suggests that some necrosis may actually be controlled. Necroptosis (referred to as programmed necrosis) is the regulated form of cell death with morphological and physiological outcomes distinctly different from apoptosis. While apoptosis is initiated by caspase activation, necroptosis requires receptor-interacting protein 1 (RIP1) and RIP3 to activate pseudokinase MLKL in order to carry out regulated cell death\textsuperscript{160}.

\textbf{Cell Death and I/R Injury}

Although a majority of cell death associated with myocardial I/R occurs through necrosis, more recent studies have shown that apoptosis plays a larger role early in the injury. Apoptosis has been identified as the predominant form of cell death occurring in both rats and humans after ischemia\textsuperscript{148}, occurring as early as 2.25 hr post- myocardial ischemia\textsuperscript{161} and peaking at 4.5 hr\textsuperscript{47}. Necrosis occurs later than apoptosis, as it has been shown to peak at 24 hr\textsuperscript{47}. Additionally, the decrease in pH/ intracellular acidosis during
Figure 4: Schematic illustrating the opening of the Mitochondrial Permeability Transition Pore. MPT, mitochondrial permeability transition. (Adapted from Halestrap).
ischemia has been shown to play a role not only in apoptotic signaling\textsuperscript{162–165}, but also in the prevention of MPTP opening\textsuperscript{137} While reperfusion may restore intracellular ATP in ischemic cardiomyocyte, it also appears to accelerate apoptotic processes, as ISEL staining can be detected after only 1 hr of reperfusion following a 45 min coronary occlusion in male rats\textsuperscript{161}. Further, the increased surge of ROS and Ca\textsuperscript{2+} resulting from reperfusion, can lead to the opening of the MPTP, suggesting that reperfusion may be the main constituent of lethality in I/R injury.

Preventing the opening of the MPTP can protect the heart, specifically against necrosis as a result of I/R injury. Perfused rat heart pretreated with sanglifehrin-A, an inhibitor of MPTP opening, resulted in a significant reduction in infarct following 35 min ischemia with 2 hr reperfusion\textsuperscript{166}. Comparably, necrostatin-1 (nec-1) has been shown to protect the heart against ischemia reperfusion injury. Working to specifically inhibit necroptosis, nec-1 has been shown to reduce infarct size in mice subjected to 30 min of ischemia followed by 2 hr of reperfusion\textsuperscript{167}.

**Translational Applications for Targeting Cell Death**

Recent studies have identified potential mechanisms for attenuation of cell death targeting modulators of mitochondrial dynamics and morphology. Mitochondrial fusion mitigates apoptosis by preventing cyt c release\textsuperscript{168} and the permeability transition\textsuperscript{169}. Alternatively, Drp1 inhibitor mdivi-1 has been shown to confer cardioprotection by inhibiting fission. A single intravenous dose of mdivi-1 significantly reduced the infarct size in an in vivo murine model\textsuperscript{9}. Similarly a new inhibitor, P110, has been shown to
inhibit mitochondrial fission while improving mitochondrial bioenergetics and heart function after I/R injury. By targeting proteins responsible for mitochondrial morphology and dynamics, these studies demonstrate novel and promising interventions for cardioprotection. While these aforementioned results are encouraging, more research is necessary regarding the prevention of apoptotic cell death through drug treatments targeting mitochondrial morphology and dynamics.
Chapter 3
Manuscript

Introduction

Coronary heart disease (CHD) is the number one cause of death in men and women\textsuperscript{1-3}, affecting over 27 million people in the United States alone\textsuperscript{4}. Cardiovascular morbidity and CHD-related mortality increases with age, although gender differences are present. Prevalence of CHD in postmenopausal women increases 2-to-3-fold compared to premenopausal women of the same age\textsuperscript{6,7}. Further, more women over the age of 40 will die within one year of experiencing their first myocardial infarction (MI) compared to men\textsuperscript{7}. These findings demonstrate the likely influence of the menopausal transition and associated loss of endogenous E\textsubscript{2} as contributing factors to the augmented cardiovascular risk and increased incidence of CHD events in postmenopausal women with aging.

While most research regarding the effects of age an I/R on mitochondrial morphology has been conducted in male animals, important findings may or may not be relevant in understanding age-associated changes in mitochondrial morphology with I/R in females. Increased mitochondrial volume with decreased density measurement are seen with aging in male mice\textsuperscript{19-21}, rats\textsuperscript{22}, and hamsters\textsuperscript{23}. In aging male mice, mitochondria become more round and enlarged, potentially due to swelling\textsuperscript{16,21}. Additionally, ischemic insults of 30 min or longer present irreversible damage, including abnormally clear matrices, severe cristae disruption and the presence of intramitochondrial granules in rats\textsuperscript{26} and dogs\textsuperscript{24-26}. Reperfusion adds insult to ischemic
injury, as the surge of ROS and pH reversal can initiate the opening of mitochondrial permeability pore transition (MPTP)\textsuperscript{159,171,172}, potentially contributing to loss of mitochondrial matrix, lysis of cristae, and disorganization of the myofibers in tissues at the periphery of the infarct lesion\textsuperscript{30}. To the best of our knowledge, there is only one study that has looked at the effects of both aging and I/R injury on mitochondrial morphology, in male mice\textsuperscript{31}. Although these researches found age-associated difference in the extent of I/R injury over time, mitochondrial morphological data did not demonstrate age-related changes at 4 hr and 24 hr reperfusion\textsuperscript{31}. Further, it was reported that slight improvement of mitochondrial ultrastructure was seen in both adult and aged mice at 24 hr R, although it remains unclear what component of ultrastructure improved\textsuperscript{31}.

In order to provide a more physiological relevant model of E\textsubscript{2}-deficiency with aging, the present study uses a novel method of ovariectomy (OVX). In a subset of rats (15 months), we utilized bilateral OVX and studied the middle aged-to-old rats (MO OVX) at 22-23 months. By performing OVX at the rat’s chronological equivalent of middle age and allowing a 7 month post-OVX period, this model allows for the proper decline of steroid hormones while also allowing the maximal gonadotropin release in response to the removal of estrogenic negative feedback.

The purpose of this study is to determine whether mitochondrial morphological changes are also prevalent in adult and aged E\textsubscript{2} deficient female models, as the resolution of ischemic damage in the aged female heart following I/R injury remains unknown.
Materials and Methods

Animal care

Adult (5-6 mo, n=20) and aged (22-23 mo, n=20) female Fischer 344 rats were supplied by Charles River (Baltimore, MD). Rats were housed under a 12:12-hr light-dark cycle and received food and water ad libitum. All animal handling and utilization protocols were reviewed and approved by the Penn State University Institutional Animal Care and Use Committee and adhere to American Physiological Society’s Guiding Principles in the Care and Use of Animals. Previously, we have demonstrated age-associated reductions in E2 levels \(^{118,173,174}\). In order to provide a more physiological model of E2-deficiency with aging, we also utilized bilateral ovariectomy (OVX) in a subset of aged rats at 15 mo and studied them at 22-23 mo (middle aged-to-old OVX, MO OVX, n=16). Successful surgery was confirmed at the time of death by measuring uterine weight. Ovary-intact animals were used as age-matched controls.

Coronary artery ligation

Coronary artery ligation (CAL) was used to generate I/R injury (31 min I with 10 min, 6 hr, 24 hr, or 7 day R) as previously described. Rats were randomly assigned to receive I/R surgery or sham surgery. Briefly, rats were anesthetized using a combination of ketamine [40 mg/kg body wt ip] and xylazine [12mg/kg body wt ip]. Rats were intubated and ventilated on Harvard Rodent Ventilator Model 638 (Holliston, MA, USA). A 1.5 cm incision was made in the left lateral third intercostal space to expose the heart
and the pericardium was opened. The left coronary artery was ligated with 6-0 prolene 3mm from its origin. Both ends of the suture were fed through PE-90 tubing and secured with a hemostat for the duration of ischemia\textsuperscript{175,176}. Blanching of the tissue below the ligation indicated that the coronary artery was successfully occluded. Following the 31 min ligation, the hemostat was removed, releasing the ligature, the chest cavity was closed, and the animal was allowed to recover for the appropriate reperfusion time. Adipamizole [1 mg/kg body wt ip] and Buprenorphine [0.05mg/kg body wt ip] were administered in the cases of 6 hr, 24 hr, 7 day R. Ventilation was maintained until the rats were able to breathe on their own and allowed to recover on a heating pad.

Sham surgeries followed similar protocol, with minor changes. Thoracotomy and placement of suture were performed in identical fashion but the suture was not tightened during the 31 min of “ischemia” in order to provide sham ischemic period. In a small subset of adult (n=2) and aged (n=2) animals, hearts were harvested without I/R protocol to serve as baseline controls.

\textit{Heart preparation}

When appropriate reperfusion time was reached, animals were anesthetized by injection of sodium pentobarbital (40 mg/kg body wt ip), hearts were rapidly removed by midline thoracotomy and immediately submerged in saline (4°C). Within 60 seconds of excision, hearts were secured via aortic cannulation to a Langendorff apparatus and perfused at constant pressure (85 mmHg), temperature (37°C), and pH (7.4) with a modified Krebs-Henseleit bicarbonate buffer as previously described\textsuperscript{118,144,174}. The suture
around the ligated artery was then re-tightened and Evan’s blue dye was injected into the hanging heart to visualize the ischemic tissue (viable reperfused areas of the myocardium stained blue, while non-reperfused areas remained pale). The heart was then removed from the Langendorff apparatus and collected for either transmission electron microscopy (TEM) analysis or western blotting.

**TEM tissue sample preparation**

After injection with Evan’s blue dye, the heart was sliced in four equal transverse sections, from apex (slice 1) to the top of the heart (slice 4), as illustrated in Figure 5A. For analysis of mitochondrial morphology, 1mm x1mm samples were taken from both the border of the ischemic zone (deemed “area at risk” or AAR) and areas located remotely from infarct, within slice 2 (Figure 5B). Samples were then immersed in fixative consisting of 16% paraformaldehyde, 25% glutaraldehyde. Samples were rinsed in 0.1M cacodylate buffer. Post-fixation in 1% osmium tetroxide for 1hr, was followed by en Bloc staining with 2% uranyl acetate for 1 hr. The samples were dehydrated through a series of graded alcohol washes before overnight incubation in 50:50 solution of Spurrs Resin and acetone. The following day, the samples were incubated in 100% Spurrs resin before being embedding in fresh Spurrs to polymerize at 60°C overnight. Samples were trimmed and 70nm thick sections of the sample were cut using a Leica Ultracut UCT Microtome (Buffalo Grove, IL, USA) with diamond razor\(^{177}\) and placed on copper TEM sample grid (2 per region per animal). The sections were double stained in
Figure 5: Illustration for TEM sample collection. A) Demonstrates location of transversely cut heart slices after Langendorff hang. TEM samples taken from slice 2, underneath the ligature. B) Demonstrates specific locations within slice 2- Area at Risk (AAR) taken from pale tissue on the border of white infarcted region; Remote tissues taken from region located opposite to infarction.
uranyl acetate and lead citrate for contrast. Once completely dry, the samples were randomly imaged on the JEOL 1200 EXII transmission electron microscope (Peabody, MA, USA) with camera. For each animal, 4-6 images per AAR and Remote region (totaling 8-12 images for each animal), were taken of longitudinal myofiber cross sections using Gatan Digital Image software. ImageJ (NIH) software was used to analyze the mitochondria in four randomly chosen images per region. For each image, every individual mitochondria was traced and counted, in order to determine mitochondrial area (μm²), mitochondrial density (number of mitochondrial per μm² of the image), and mitochondrial circularity. Circularity is calculated by a ratio of mitochondrial length to width, with values closer to 1 representing more circular mitochondria, while values closer to 0 represent elongated mitochondria.

**Qualitative Analysis**

Qualitative analysis of the ultrastructure damage of cardiomyocyte mitochondria was assessed according to a modified scoring system previously described⁴⁰, for prevalence of electron densities, crista disruption, and myofibrillar disarray. Cristae disruption is characterized by transition from dense tightly packed concentric crista to the presence of tortuous crista with matrix clearing. Myofibrillar disarray is characterized by Z-line streaming, increased spacing between myofilaments and distortion of the contractile system. For each image, a grade was given for each qualitative characteristic: (+) present in 0-25% of the image; (+++) present in 26-50% of the image; (++++) present in 51-75% of the image; (++++++) present in 76-100% of image.
**Western Blot tissue sample preparation**

For Western blot analysis, mitochondrial subcellular protein fractions were prepared as described previously\(^\text{118}\). Using glass-glass grinder, frozen LV samples were briefly homogenized in 10 vol of buffer A containing (in mM) 250 sucrose; 10 Tris•HCl, pH 7.4, 1 EDTA, pH 7–8, 1 orthovanadate; 1 NaF; 0.3 PMSF; 5 μg/ml each of leupeptin and aprotinin; and 0.5 μg /ml pepstatin A. After resuspension the sample was subjected centrifugation at 10,000 \(\times g\). The resultant pellet was washed in buffer A and recentrifuged at 10,000 \(\times g\). The final pellet was resuspended in buffer B containing (in mM): 150 NaCl; 20 Tris•HCl, pH 7.4, 10 EDTA, pH 7–8, 1 orthovanadate; 1 NaF; 0.3 PMSF and 0.5 μg /ml pepstatin A, 5 μg /ml each of leupeptin and aprotinin, and 1% NP-40 and centrifuged at 21,000 \(\times g\). The resultant supernatant was defined as the mitochondrial fraction. All protein concentrations were determined by the method of Bradford\(^\text{178}\).

**Western Blot analysis**

Western blot analysis was executed according to well-established procedures in our laboratory\(^\text{179}\). Equal amounts of total and mitochondrial protein sample were electrophoresed on Criterion SDS-polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Millipore). After transfer, membranes were incubated in blocking buffer (6% nonfat milk) and probed with primary antibody (1:1000) against mitofusin 1 (Mfn1), dynamin-related protein 1 (Drp1), optic atrophy 1 (OPA1) (Santa Cruz Biotechnology), or human fission 1 (Fis1) (Biovision), as previously
described\textsuperscript{180}. Membranes were incubated wash buffer with appropriate horseradish peroxidase-linked secondary antibody (1:20,000): anti-rabbit IgG (GE Health) for Mfn1, Drp1 and Fis1; mouse anti-goat IgG (Santa Cruz Biotechnology) for OPA1. Enhanced chemiluminescence (GE Amersham) was used to visualize immunoreactive bands. Densitometry analysis was performed using Scion Image (NIH).

\textit{Statistics}

Group comparisons for mitochondrial area and density in response to age, reperfusion, and region of tissue were analyzed using two-way ANOVA with \textit{post hoc} Tukey test for comparisons with significant interactions. Statistical differences for comparisons involving two groups were determined using Student’s \textit{t}-test. All variables expressed as mean± standard error (SE). Significance was defined as P<0.05.
Results

Physiological Characteristics of Animals

The physiological characteristics of rats used in this study are provided in Table 2. As expected, adult body weight was significantly lower than aged (P=0.006) and MO OVX (P<0.001). Additionally, MO OVX body weight was 14% greater than that of the aged (P=0.026). Adult left ventricle (LV) weight was significantly lower than both aged (P=0.006) and MO OVX (P<0.001). MO OVX LV/body weight ratio was significantly lower than both adult (P<0.001) and aged (P=0.011). E2 deficiency decreased uterine weight of MO OVX animals, which displayed weights 41% lower than adult (P=0.011) and 55% lower than aged (P=0.024). Compared to adult, adipose weight in aged increased almost 90% (P=0.02), while the MO OVX increased almost 170% (P<0.001). Additionally, MO OVX animals gained 40% more adipose weight than the aged (P=0.002).

<table>
<thead>
<tr>
<th>Group</th>
<th>LV (mg)</th>
<th>BW (g)</th>
<th>LV/BW (mg/g)</th>
<th>Uterine Weight (mg)</th>
<th>Adipose Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult (n=18)</td>
<td>482.5 ± 9.2</td>
<td>204.1 ± 2.3</td>
<td>2.313 ± 0.04</td>
<td>469.7 ± 71</td>
<td>6.769 ± 0.50</td>
</tr>
<tr>
<td>Aged (n=4)</td>
<td>660.8 ± 70*</td>
<td>278.8 ± 13*</td>
<td>2.367 ± 0.22</td>
<td>620.0 ± 147</td>
<td>12.69 ± 2.1*</td>
</tr>
<tr>
<td>MO OVX (n=16)</td>
<td>646.6 ± 12*</td>
<td>316.7 ± 6.1**</td>
<td>2.048 ± 0.04**</td>
<td>277.2 ± 33**</td>
<td>17.82 ± 0.64**</td>
</tr>
</tbody>
</table>

Table 2: Physiological Characteristics of Animals. Values are means ± SE. LV, left ventricle weight. BW, body weight. LV/BW, left ventricle-to-body weight ratio. *Significantly different from Adult, †Significantly different between MO OVX and Aged, P<0.05.
**Aging**

In the absence of ischemia, aging and E$_2$ deficiency produced changes in mitochondrial area and density (Figure 6). Aged mitochondria demonstrated a tendency for increased area and density measures, although changes did not reach statistical significance. Conversely, MO OVX tissues demonstrated a 8% decrease in mitochondrial area (P=0.011) with concurrent 11% increase in mitochondrial density (P=0.011) compared to adult. In comparison to aged mitochondria, MO OVX displayed 19% smaller mitochondria (P=0.008). Mitochondrial circularity was unaffected by age or the combination of age and E$_2$ deficiency.

**Reperfusion and Age**

Reperfusion resulted in changes in adult and MO OVX mitochondrial area (Figure 7). Mitochondria isolated from adult sham were 10 % larger than those of MO OVX sham (P=0.013). At 10 min reperfusion, adult I/R mitochondria increased 26% in size compared to sham (P=0.004), and 23% larger mitochondria compared to MO OVX (P<0.001). Reperfusion time of 6 hr produced increases in mitochondrial area for both adult and MO OVX, 20% and 25% respectively, compared to shams (P<0.001). A significant age x reperfusion interaction (P=0.011) was revealed by ANOVA at 24 hr reperfusion. MO OVX increased 18% compared to sham (P=0.018), whereas adult decreased 10% compared to sham. Age and reperfusion also produced changes in mitochondrial density of adult and MO OVX I/R tissues. In the absence of ischemia, there were significantly greater mitochondria in MO OVX compared to adult.
Figure 6: Morphological characteristics of Adult, Aged and MO OVX. (A) Mitochondrial Area (μm²), Mitochondrial Density (# mitochondria/μm²), Mitochondrial Circularity (AU) (B) Representative images. Adult Sham n=2, Aged No Surgery n=2, MO OVX Sham n=2. * significant difference Adult vs MO OVX † significant difference Aged vs MO OVX (P<0.05).
Figure 7: Time course of changes with increased reperfusion. (A) Mitochondrial area (μm²) and density (#mitochondria/μm²) (B) Representative images for adult I/R and MO I/R. * significant age effect, † significant I/R effect P<0.05
ANOVA indicated a significant age x reperfusion interaction (P=0.032) at 10 min reperfusion. Adult I/R density decreased 28% (P=0.005), while MO OVX density increased 17% (P=0.006) compared to adult sham. Further, with 10 min R, adult tissues displayed 65% less mitochondria than MO OVX (P<0.001). At 6 hr, a significant reduction in density was seen in MO OVX (P<0.001). With 24 hr R, ANOVA indicated a significant age x reperfusion interaction (P=0.011). MO OVX mitochondria were significantly larger than sham (P=0.018) and adult mitochondria (P=0.013).

In a subset of animals, I/R with 7 days of reperfusion impacted both adult and aged mitochondrial area and density (Figure 8). Both adult tissues and aged tissues demonstrated decreases in mitochondrial area, 33% and 44% respectively, compared to no surgery controls (P<0.001). Conversely, both adult and aged mitochondrial density increased 36% and 17% compared to respective control animals (P=0.032).

**Reperfusion and Region**

The region from which the tissues were harvested produced changes in adult mitochondrial area (Figure 9). Specifically, ANOVA revealed a significant reperfusion x region interaction (P<0.001) with 10 min reperfusion. Compared to shams, increased mitochondrial area was seen in AAR tissues, while remote tissues displayed a 19% reduction in size (P=0.003). Additionally, the AAR region displayed 35% larger
Figure 8: 7 day Reperfusion in Adult and Aged. Mitochondrial area (μm²) (A) and Density (# mitochondria/ μm²) (B). n=2 for each group. † Significant I/R effect (P<0.05)
Figure 9: Area at Risk vs Remote comparison for Adult and MO OVX. Area at Risk, AAR. †significant I/R effect ‡ significant region effect (P<0.05).
mitochondria compared to remote (P<0.001). With 6 hr reperfusion, ANOVA revealed another significant interaction of reperfusion x region (P<0.001) in adult mitochondria. Adult AAR mitochondria were significantly larger than shams (P<0.001), as well as 25% larger than remote mitochondria (P<0.001) at 6 hr reperfusion. Again, ANOVA revealed a significant reperfusion x region interaction (P=0.045) at 24 hr, where remote tissue displayed mitochondria 18% larger than in AAR (P=0.011). Reperfusion time of 7 days resulted in decreases in both AAR and remote mitochondria of 15% and 16%, respectively, although differences were not significant.

Regional and reperfusion differences in mitochondrial area values were less pronounced in MO OVX tissues (Figure 9). 10 min reperfusion produced no change in MO OVX AAR or remote tissues. However, ANOVA revealed a significant reperfusion x region interaction (P=0.005) at 6 hr reperfusion. MO OVX AAR tissues exhibited 26% larger mitochondria compared to remote (P=0.001) and sham tissues (P=0.001). At 24 hr, there was a trend for larger mitochondria in AAR region compared to remote (P=0.054).

Qualitative Characteristics

Qualitative characteristics of electron densities, disrupted cristae, and myofibrillar disarray are displayed in Table 3, with representative images in Figure 10. Slight increases in electron densities (++) were seen with all reperfusion time points in adult samples, 6 hr and 24 hr R in MO OVX, and 7 day R in aged compared to controls (+) for each age group. Increased cristae disruption (+++) was seen with 6 hr R in adult, and
with both 10 min and 6 hr R in MO OVX. Increased myofibrillar disarray (+++) was seen with 6 hr and 24 R in MO OVX.

<table>
<thead>
<tr>
<th>Reperfusion Time</th>
<th>Electron Densities</th>
<th>Disrupted Cristae</th>
<th>Myofibrillar Disarray</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10 min</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>6 hr</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>24 hr</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>7 day</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>MO OVX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>10 min</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>6 hr</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>24 Hr</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Aged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>7 day</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 3: Qualitative Characteristics for Adult, MO OVX and Aged rats.

Expression of Mitochondrial Fission-Fusion Proteins in the Heart

At 6 hr reperfusion fission protein Drp1 was significantly increased by I/R (Figure 11A) in both adult and MO OVX tissues (P=0.002). Mitochondrial Fis1 (Figure 11B) and Mfn1 (Figure 11D) showed a tendency to decrease with I/R, although not significant. Fusion protein Opa1 (Figure 11C) demonstrated the tendency to increase with age and I/R, although it was not significantly different at the 6 hr reperfusion time point.
Figure 10: Representative qualitative images for Adult, MO OVX and Aged rats. (1000x mag)
Figure 11: Western Blot analysis for 6 hr reperfusion. Mitochondrial Drp1 (A), Mitochondrial Fis1 (B), Mitochondrial OPA1 (C), Mitochondrial Mfn1 (D). Representative western blot above each graph: lanes 1 and 2 (Adult sham), lanes 3 and 4 (Adult I/R), lanes 5 and 6 (MO OVX sham), Lanes 7 and 8 (MO OVX I/R). Adult sham (n=3), Adult I/R (n=4), MO OVX Sham (n=4), MO OVX I/R (n=4). † significant I/R effect (P=0.002)
Discussion

The purpose of this investigation was to determine the effects of age-associated E\textsubscript{2} deficiency and I/R injury on mitochondrial morphology. In vivo model of CAL was used in conjunction with TEM techniques on adult, MO OVX, and aged rats, totaling 40 animals and \~18,000 mitochondria. Aged-associated E\textsubscript{2} deficiency resulted smaller, more numerous mitochondria, contrary to our original hypothesis. Additionally, increased reperfusion time up to 6 hr displayed larger mitochondria with increased mitochondrial damage, with exacerbated damage in MO OVX tissues. Finally, we found increased fission proteins expression at 6 hr in both adult and MO OVX mitochondrial fractions. Our findings demonstrated the effects of age-associated E\textsubscript{2} deficiency and various reperfusion times on mitochondrial area and density. This investigation provides the first evidence for significant alterations in mitochondrial morphology and potential resolution of ischemic damage following I/R in the female rat heart.

Effects of Aging on Mitochondrial Morphology

Previous studies have demonstrated the effects of advancing age mitochondrial morphology of male rodent models\textsuperscript{16,19,21–23,119,181,182}. We now extend these findings to the female adult and aged E\textsubscript{2} deficient rat heart. Our results indicate a tendency for increasing mitochondrial area in aged ovary-intact animals, while the combination of age and OVX lead to the significant prevalence of many small mitochondria. Previous studies demonstrate an increase in mitochondrial volume with concurrent decrease in density in
the hearts of aged mice\textsuperscript{19,20} and rats\textsuperscript{22}. No studies to date have examined the integrated effects of both aging and E\textsubscript{2} deficiency on mitochondrial morphology of the female heart. For the first time, our data demonstrate the combination of aging and E\textsubscript{2} deficiency in the female heart may impact mitochondrial morphology differently than in the aged male heart.

As no other mitochondrial morphology studies have been conducted in aged E\textsubscript{2} deficient rat heart, we can only compare our results to previous studies conducted in aged males. Our results demonstrate that the decreased mitochondrial area and increased mitochondrial density (\textbf{Figure 6}) opposes the results found in the literature regarding the effects of age on male cardiac mitochondria. Sex differences would be the obvious choice to explain these alterations, but as both aged males and aged OVX females are both lacking in E\textsubscript{2}, our results are puzzling. One possible mechanism for morphological differences in MO OVX may be dysregulation of mitochondrial quality control in combination with age-associated inflammation. It is well documented age is linked to decreases in autophagy\textsuperscript{64,73,74,77,183,75,76} and increases in inflammation\textsuperscript{184–186}. Because damaged mitochondria are no longer cleared from the cells, these impaired and inefficient mitochondria produce excess ROS, triggering NF\textsubscript{x}B inflammatory pathway. ROS triggers inflammasome formation leading to the production of inflammatory cytokines TNF\textalpha, IL-6, IL-1\beta, and IL-18\textsuperscript{184}. Additionally, it has been shown that adipocytes release pro-inflammatory cytokines. As such, the drastic 170\% increase in subcutaneous adipose tissue seen in our MO OVX compared to adult (\textbf{Table 2}), may contribute to age-associated inflammation\textsuperscript{187,188}. Ultimately impaired autophagy, ROS and inflammation may result in the inability to remove mitochondria. Although, our
results demonstrate the increase number of smaller mitochondria in MO OVX hearts, the small sample sizes requires more studies to provide definitive evidence for change with age and OVX.

**Effects of Reperfusion on Mitochondrial Morphology**

While effects of cardiac ischemia on mitochondria morphology have been extensively studied in dogs and rats, less research has been conducted on the impact of reperfusion (following ischemia) on mitochondrial morphology, particularly in female models. A limited number of studies using female rat models examine the effects of ischemia and I/R injury on cardiac mitochondrial morphology, although the latter study examined only the loss of mitochondrial matrix and granules at 60 min of reperfusion. To the best of our knowledge, only one study has examined the combined effects of age and I/R on mitochondrial morphology in aged male mice, while these factors have yet to be examined in the female heart. For the first time, we have demonstrated that aging and increased reperfusion time results in alterations in mitochondrial area and density in both the adult-ovary intact and aged- E2 deficient rat heart.

The present study demonstrates a time course of changes in mitochondrial area and density, with reperfusion increasing from 10 min to 7 days. The greatest change in area and density was seen at 10 min reperfusion, with adult displaying more pronounced changes compared to MO OVX (Figure 7). A possible cause for increased mitochondrial area at 10 min reperfusion may be mitochondrial swelling due to the mitochondrial
permeability transition pore (MPTP) opening. It is well known that the MPTP is closed during ischemia due to increased pH, but opens with reperfusion once pH returns back to a physiologic level\textsuperscript{159,171,172}. Additionally, the first few minutes after the opening of MPTP results in colloid-osmotic swelling of the mitochondria\textsuperscript{191}. As 10 min reperfusion is the closest time point to the initiation of reperfusion measured in this study, the increased size and decreased density of adult mitochondria may be swelling as a result of MPTP opening. Further, increased cristae disruption at 6 hr in both adult and MO OVX mitochondrial (Table 3) may represent the onset of cell death mechanisms.

It has been shown that mitochondrial fission is associated with apoptotic cell death\textsuperscript{45}, which peaks at 4.5 hr in models of permanent ischemia\textsuperscript{47}, and may occur earlier with reperfusion\textsuperscript{148}. We hypothesized that apoptotic mitochondrial fission with occurrence of increased fission proteins would be associated with the 6 hr reperfusion time point. Western blot analysis supported this hypothesis, as mitochondrial Drp1 increased significantly with I/R for both adult and aged tissues (Figure 11A). Surprisingly, our TEM data showed that mitochondrial area remains increased at 6 hr reperfusion for both adult and MO OVX tissues (Figures 7 and 9). One interpretation is that increased fission proteins seen at 6 hr may not be actively involved in fission at that time. As increases in the number of small mitochondria become prevalent at later reperfusion times of 24 hr and 7 day in adult tissues (Figure 7), this may indicate a potential delay of mitochondrial fragmentation seen after fission protein upregulation. To the best of our knowledge, no studies have been conducted to determine the time necessary for Drp1 activation to result in mitochondrial fission. For the first time, we have demonstrated the potential temporal nature of Drp1 upregulation and its resultant
manifestation of fragmented mitochondria. Whether fragmented mitochondria appear earlier than 24 hr remains to be studied.

Previous research has reported that improvement in mitochondrial morphology can be seen at 24 hr reperfusion in both adult and aged mice\textsuperscript{31}, although researchers fail to go into further detail regarding the actual improvement. Our data demonstrate adult mitochondrial area at 24 hr returning to baseline values (\textbf{Figure 4}), while MO OVX mitochondrial area remains increased, and displayed increased myofibrillar disarray (\textbf{Table 3}). It may be possible that the adult hearts are better suited for removal of swollen mitochondrial through autophagy which is upregulated with ischemia\textsuperscript{66–69} and reperfusion\textsuperscript{70,71} as mitochondrial area return to baseline values after enduring 24 hr of reperfusion. Because autophagy decreases with age\textsuperscript{73,74}, it may be possible that enlarged mitochondria seen at 24 hr R in the MO OVX might be a result the age related decrement in autophagy. Further, aged- E2 deficient heart may not be able to rid itself of larger mitochondria, as it has been postulated that the energy requirement to remove these enlarged mitochondria may be too great\textsuperscript{77}. Therefore, our data shows that adult mitochondria may experience a quicker morphological resolution to their I/R injury, while MO OVX mitochondria remain large potentially due to decreased autophagy and the inability to meet the energy requirement to remove big mitochondria from the cell. As the sample size at 24 hr is small, more research is necessary to develop a more in depth analysis at this reperfusion time point.
Effects of Region on Mitochondrial Morphology

Previous studies have examined the progression of necrosis and the extension of infarct with reperfusion$^{30,192–195}$. While transmural extension of infarct within the area at risk is seen with reperfusion times of 1 hr$^{30}$ and 24 hr$^{192}$, these studies did not analyze tissues located remotely to the ischemic insult. As no previous studies have specifically compared the mitochondrial morphology of both AAR and remote tissues, while few appear to show no evidence of cell death in the remote/normal tissues with reperfusion$^{196}$, we hypothesized that mitochondrial morphology in remote regions would be unaffected by increasing reperfusion times. Our data demonstrated that while remote mitochondria of MO OVX remained unaffected with increasing reperfusion times (Figure 9), adult remote mitochondria displayed a significant decrease in area compared to shams at 10 min reperfusion. It is unlikely that damage had spread to these remote tissues, as organelle swelling would be expected with necrotic cell death$^{66,150}$, although our results state otherwise. Despite this alteration, remote mitochondria were significantly smaller that AAR mitochondria, at 10 min in adult and 6 hr reperfusion in both adults and MO OVX hearts.
Chapter 4

Conclusions and Future Directions

This study provides the first morphological evidence that age-associated E₂ deficiency and I/R wielded a variety of effects on cardiomyocyte mitochondrial morphology from female Fischer 344 rats. Major findings are as follows:

Specific Aim #1:

Hypothesis₁: Average mitochondrial size increased, without concurrent decrease in numerical density in aged vs adult.

Hypothesis₂: Greater mitochondrial damage such as swelling, cristae disruption and intramitochondrial Ca²⁺ granules were seen age-associated E₂ deficiency vs adult.

Hypothesis₃: Myofibrillar fragmentation, z-line streaming and contracture band necrosis damage following I/R injury were greater in aged E₂-deficient cardiac tissue vs adult, particularly at 24 hr.

Specific Aim #2:

Hypothesis₁: With increasing reperfusion time up to 6 hr, larger mitochondria were seen. Reperfusion time of 10 min significantly altered mitochondrial area and density in adult and MO OVX hearts. Adult hearts displayed greatest changes in mitochondrial area and density compared to MO OVX. Further, decreases in mitochondrial area with simultaneous increases in density are seen in adults at 24
hr and 7 day reperfusion. Additionally, aged E\textsubscript{2}-deficient tissues showed increased number of swollen, disrupted mitochondria containing intramitochondrial Ca\textsuperscript{2+} granules with increasing reperfusion times compared to adult tissues. In a subset of animals, 7 day reperfusion showed less mitochondrial alterations in adult vs aged tissues.

**Hypothesis 2:** Remote regions were unaffected by increased reperfusion.

**Hypothesis 3:** Increased fission protein, Drp1, but not Fis1, were associated with 6 hr reperfusion. Numerous small mitochondrial were not evident until 24 hr in adult hearts.

These findings indicate that the MO OVX model requires further investigation better determine how age-associated E\textsubscript{2} deficiency impacts mitochondrial morphology in conjunction with I/R injury, as well as whether mitochondrial morphology may become alternative indication for apoptotic and necrotic cell death mechanisms. Several future directions are as follows:

1. Study mitochondrial area and density measures in MO OVX at 7 day reperfusion in order compare to adult and aged 7 day OVX.

2. Perform western blot at each of the various reperfusion times to develop a better understanding of how mitochondrial fission and fusion protein regulation is impacted with age-associated E\textsubscript{2} deficiency and I/R.

3. Perform DNA laddering and TUNEL staining to determine the onset and severity of apoptotic and necrotic cell death with age-associated E\textsubscript{2} deficiency in conjunction with I/R.
Several limitations were apparent while conducting the present study. The limitations are as follows:

1. **Small sample sizes:** Small sample sizes ($n=2$) for controls studies in adult, aged and MO OVX rats prevented definitive interpretation on the effect of aging alone on mitochondrial morphology.

2. **Western Blotting:** Small sample size ($n=3-4$) used to examine mitochondrial proteins resulted in insignificant values due to reduced statistical power. Due to time constraints, western blotting data remained uncorrected. Therefore, western blot data presented herein was lacking correction for loading inconsistencies.

3. **Sample availability:** Due to limited western blotting samples, the only time point used to analyze changes in mitochondrial morphological changes was 6 hr R. This time point may not have captured these changes to fullest extent.

4. **Sample preparation:** Because hearts were hung and perfused with Krebs-Heinseleit buffer at $37^\circ$C and subsequently immersed in ice cold fixative, it is possible that hypercontraction of myofibers could be a potential artifact and not a proper indication of how the muscle truly responded to I/R injury.
Bibliography


## Appendix

### Raw Data

<table>
<thead>
<tr>
<th>Reperfusion Time</th>
<th>Region</th>
<th>n</th>
<th>Mean Area (μm²)</th>
<th>Mean Density (# mitochondria/μm²)</th>
<th>Mean Circularity (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No surgery</td>
<td>No Surgery</td>
<td>2</td>
<td>0.5148 ±0.02</td>
<td>0.5831 ± 0.04</td>
<td>0.6993 ± 0.01</td>
</tr>
<tr>
<td>Sham</td>
<td>Sham</td>
<td>7</td>
<td>0.4334 ± 0.01</td>
<td>0.6935 ± 0.02</td>
<td>0.7154 ± 0.01</td>
</tr>
<tr>
<td>10 min</td>
<td>AAR</td>
<td>3</td>
<td>0.5634 ± 0.05</td>
<td>0.4784 ± 0.06</td>
<td>0.7190 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td></td>
<td>0.3610 ± 0.01</td>
<td>0.7862 ± 0.06</td>
<td>0.7372 ± 0.01</td>
</tr>
<tr>
<td>6 hr</td>
<td>AAR</td>
<td>4</td>
<td>0.5388 ± 0.03</td>
<td>0.6560 ± 0.03</td>
<td>0.7579 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td></td>
<td>0.4081 ± 0.02</td>
<td>0.6521 ± 0.03</td>
<td>0.7242 ± 0.01</td>
</tr>
<tr>
<td>24 hr</td>
<td>AAR</td>
<td>2</td>
<td>0.4067 ± 0.01</td>
<td>0.6921 ± 0.03</td>
<td>0.7699 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td></td>
<td>0.4823 ± 0.03</td>
<td>0.5791 ± 0.02</td>
<td>0.76701 ± 0.01</td>
</tr>
<tr>
<td>7 day</td>
<td>AAR</td>
<td>2</td>
<td>0.3440 ± 0.02</td>
<td>0.7984 ± 0.04</td>
<td>0.7235 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td></td>
<td>0.4012 ± 0.03</td>
<td>0.7465 ± 0.04</td>
<td>0.7224 ± 0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reperfusion Time</th>
<th>Region</th>
<th>n</th>
<th>Mean Area (μm²)</th>
<th>Mean Density (# mitochondria/μm²)</th>
<th>Mean Circularity (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>Sham</td>
<td>7</td>
<td>0.4013 ± 0.01</td>
<td>0.7732 ± 0.03</td>
<td>0.7108 ± 0.01</td>
</tr>
<tr>
<td>10 min</td>
<td>AAR</td>
<td>3</td>
<td>0.4338 ± 0.04</td>
<td>0.7908 ± 0.05</td>
<td>0.6934 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td></td>
<td>0.4155 ± 0.02</td>
<td>0.8456 ± 0.05</td>
<td>0.6968 ± 0.01</td>
</tr>
<tr>
<td>6 hr</td>
<td>AAR</td>
<td>4</td>
<td>0.5005 ± 0.04</td>
<td>0.6718 ± 0.04</td>
<td>0.6998 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td></td>
<td>0.3704 ± 0.02</td>
<td>0.8179 ± 0.04</td>
<td>0.7217 ± 0.01</td>
</tr>
<tr>
<td>24 hr</td>
<td>AAR</td>
<td>2</td>
<td>0.4718 ± 0.03</td>
<td>0.6445 ± 0.05</td>
<td>0.7355 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td></td>
<td>0.4307 ± 0.02</td>
<td>0.7864 ± 0.03</td>
<td>0.7101 ± 0.01</td>
</tr>
</tbody>
</table>
### Table 4: Means for Mitochondrial Characteristics. All values means ± SE.

<table>
<thead>
<tr>
<th>Reperfusion Time</th>
<th>Region</th>
<th>n</th>
<th>Mean Area (μm²)</th>
<th>Mean Density (# mitochondria/μm²)</th>
<th>Mean Circularity (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Surgery</td>
<td>No surgery</td>
<td>2</td>
<td>0.4974 ± 0.04</td>
<td>0.7305 ± 0.07</td>
<td>0.7362 ± 0.01</td>
</tr>
<tr>
<td>7 day</td>
<td>AAR</td>
<td>2</td>
<td>0.2775 ± 0.01</td>
<td>0.8604 ± 0.13</td>
<td>0.7065 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td>2</td>
<td>0.4142 ± 0.02</td>
<td>0.7417 ± 0.05</td>
<td>0.6897 ± 0.01</td>
</tr>
<tr>
<td>Rat #</td>
<td>Group</td>
<td>Region</td>
<td>Adult Mitochondrial Values</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>--------</td>
<td>---------------------------</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Area (μm²)</td>
<td>Density (# mitochondria/μm²)</td>
<td>Circularity (AU)</td>
</tr>
<tr>
<td>402</td>
<td>No Surgery</td>
<td></td>
<td>0.5484 ± 0.03</td>
<td>0.5952 ± 0.05</td>
<td>0.7293 ± 0.02</td>
</tr>
<tr>
<td>578</td>
<td>No Surgery</td>
<td></td>
<td>0.4896 ± 0.03</td>
<td>0.5740 ± 0.07</td>
<td>0.6768 ± 0.01</td>
</tr>
<tr>
<td>820</td>
<td>Sham</td>
<td></td>
<td>0.4590 ± 0.01</td>
<td>0.5320 ± 0.02</td>
<td>0.7649 ± 0.02</td>
</tr>
<tr>
<td>1651</td>
<td>Sham</td>
<td></td>
<td>0.4680 ± 0.02</td>
<td>0.6130 ± 0.03</td>
<td>0.7111 ± 0.01</td>
</tr>
<tr>
<td>1673</td>
<td>Sham</td>
<td></td>
<td>0.3555 ± 0.02</td>
<td>0.9679 ± 0.08</td>
<td>0.7142 ± 0.01</td>
</tr>
<tr>
<td>822</td>
<td>Sham</td>
<td></td>
<td>0.5133 ± 0.02</td>
<td>0.4948 ± 0.02</td>
<td>0.7661 ± 0.01</td>
</tr>
<tr>
<td>1680</td>
<td>Sham</td>
<td></td>
<td>0.4376 ± 0.02</td>
<td>0.7490 ± 0.05</td>
<td>0.7070 ± 0.01</td>
</tr>
<tr>
<td>1687</td>
<td>Sham</td>
<td></td>
<td>0.4305 ± 0.01</td>
<td>0.7425 ± 0.02</td>
<td>0.6854 ± 0.01</td>
</tr>
<tr>
<td>1691</td>
<td>Sham</td>
<td></td>
<td>0.3803 ± 0.03</td>
<td>0.7111 ± 0.03</td>
<td>0.7040 ± 0.01</td>
</tr>
<tr>
<td>1698</td>
<td>10 min AAR</td>
<td></td>
<td>0.5250 ± 0.02</td>
<td>0.4240 ± 0.2</td>
<td>0.7578 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>10 min Remote</td>
<td></td>
<td>0.3501 ± 0.02</td>
<td>0.7757 ± 0.06</td>
<td>0.7297 ± 0.01</td>
</tr>
<tr>
<td>1689</td>
<td>10 min AAR</td>
<td></td>
<td>0.3300 ± 0.03</td>
<td>0.8032 ± 0.05</td>
<td>0.7145 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>10 min Remote</td>
<td></td>
<td>0.3667 ± 0.02</td>
<td>1.041 ± 0.08</td>
<td>0.7511 ± 0.06</td>
</tr>
<tr>
<td>1683</td>
<td>10 min AAR</td>
<td></td>
<td>0.7185 ± 0.03</td>
<td>0.3705 ± 0.02</td>
<td>0.6825 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>10 min Remote</td>
<td></td>
<td>0.3691 ± 0.02</td>
<td>0.6690 ± 0.07</td>
<td>0.7378 ± 0.01</td>
</tr>
<tr>
<td>1657</td>
<td>6 hr AAR</td>
<td></td>
<td>0.4998 ± 0.03</td>
<td>0.6664 ± 0.03</td>
<td>0.7008 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>6 hr Remote</td>
<td></td>
<td>0.4314 ± 0.05</td>
<td>0.6745 ± 0.06</td>
<td>0.7348 ± 0.01</td>
</tr>
<tr>
<td>1613</td>
<td>6 hr AAR</td>
<td></td>
<td>0.4850 ± 0.05</td>
<td>0.6796 ± 0.4</td>
<td>0.7588 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>6 hr Remote</td>
<td></td>
<td>0.3738 ± 0.03</td>
<td>0.6865 ± 0.3</td>
<td>0.7100 ± 0.01</td>
</tr>
<tr>
<td>1609</td>
<td>6 hr AAR</td>
<td></td>
<td>0.4499 ± 0.04</td>
<td>0.7539 ± 0.01</td>
<td>0.8035 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>6 hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>1607</td>
<td>AAR</td>
<td>0.7009 ± 0.02</td>
<td>0.5294 ± 0.04</td>
<td>0.740 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td>0.4229 ± 0.03</td>
<td>0.5764 ± 0.02</td>
<td>0.7291 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>576</td>
<td>24 hr</td>
<td>AAR</td>
<td>0.4315 ± 0.02</td>
<td>0.7314 ± 0.07</td>
<td>0.7617 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td>0.5029 ± 0.06</td>
<td>0.5731 ± 0.04</td>
<td>0.7583 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>821</td>
<td>24 hr</td>
<td>AAR</td>
<td>0.3867 ± 0.02</td>
<td>0.6606 ± 0.04</td>
<td>0.7765 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td>0.4616 ± 0.05</td>
<td>0.5850 ± 0.03</td>
<td>0.7758 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>680</td>
<td>7 day</td>
<td>AAR</td>
<td>0.3269 ± 0.02</td>
<td>0.7404 ± 0.03</td>
<td>0.7384 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td>0.3336 ± 0.03</td>
<td>0.7719 ± 0.04</td>
<td>0.7274 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>681</td>
<td>7 day</td>
<td>AAR</td>
<td>0.3612 ± 0.02</td>
<td>0.8563 ± 0.07</td>
<td>0.7086 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td>0.4688 ± 0.03</td>
<td>0.7210 ± 0.08</td>
<td>0.7173 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

**Total Adult mitochondria analyzed**: 8214

**Table 5**: Adult individual mitochondrial values. All values means ± SE.
<table>
<thead>
<tr>
<th>Rat #</th>
<th>Group</th>
<th>Region</th>
<th>Area (μm²)</th>
<th>Density (# mitochondria/μm²)</th>
<th>Circularity (AU)</th>
<th># Analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1652</td>
<td>MO OVX</td>
<td>AAR</td>
<td>0.3771 ± 0.02</td>
<td>0.9500 ± 0.02</td>
<td>0.6850 ± 0.01</td>
<td>733</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>Remote</td>
<td>0.3747 ± 0.03</td>
<td>0.9433 ± 0.06</td>
<td>0.7175 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>1671</td>
<td>MO OVX</td>
<td>AAR</td>
<td>0.3670 ± 0.02</td>
<td>0.7776 ± 0.05</td>
<td>0.6817 ± 0.02</td>
<td>311</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>Remote</td>
<td>0.4356 ± 0.02</td>
<td>0.7399 ± 0.03</td>
<td>0.714 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>1674</td>
<td>MO OVX</td>
<td>AAR</td>
<td>0.5351 ± 0.07</td>
<td>0.6404 ± 0.05</td>
<td>0.7095 ± 0.01</td>
<td>482</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>Remote</td>
<td>0.4564 ± 0.06</td>
<td>0.7858 ± 0.07</td>
<td>0.6579 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>1686</td>
<td>MO OVX</td>
<td>AAR</td>
<td>0.6990 ± 0.02</td>
<td>0.5340 ± 0.02</td>
<td>0.7008 ± 0.01</td>
<td>245</td>
</tr>
<tr>
<td></td>
<td>6 hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1658</td>
<td>MO OVX</td>
<td>AAR</td>
<td>0.5264 ± 0.07</td>
<td>0.5265 ± 0.05</td>
<td>0.6830 ± 0.03</td>
<td>431</td>
</tr>
<tr>
<td></td>
<td>6 hr</td>
<td>Remote</td>
<td>0.4415 ± 0.04</td>
<td>0.7322 ± 0.05</td>
<td>0.7497 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>1682</td>
<td>MO OVX</td>
<td>AAR</td>
<td>0.3875 ± 0.03</td>
<td>0.8165 ± 0.05</td>
<td>0.6828 ± 0.01</td>
<td>749</td>
</tr>
<tr>
<td></td>
<td>6 hr</td>
<td>Remote</td>
<td>0.3533 ± 0.02</td>
<td>0.8156 ± 0.09</td>
<td>0.6721 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>Group</td>
<td>AAR</td>
<td>Value 1 ± SE</td>
<td>Value 2 ± SE</td>
<td>Value 3 ± SE</td>
<td>Count</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td>6 hr</td>
<td>MO OVX</td>
<td>0.4020 ± 0.03</td>
<td>0.7374 ± 0.05</td>
<td>0.7243 ± 0.03</td>
<td>235</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td>0.3164 ± 0.03</td>
<td>0.9060 ± 0.09</td>
<td>0.7433 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td>MO OVX</td>
<td>0.4719 ± 0.04</td>
<td>0.6509 ± 0.07</td>
<td>0.7369 ± 0.01</td>
<td>590</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td>0.4042 ± 0.03</td>
<td>0.7569 ± 0.04</td>
<td>0.7167 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td>MO OVX</td>
<td>0.4718 ± 0.03</td>
<td>0.6348 ± 0.05</td>
<td>0.7336 ± 0.02</td>
<td>495</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Remote</td>
<td>0.4660 ± 0.01</td>
<td>0.8257 ± 0.04</td>
<td>0.7013 ± 0.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Total MO OVX mitochondria analyzed** 8107

**Table 6:** MO OVX individual mitochondrial values. All values means ± SE.
<table>
<thead>
<tr>
<th>Rat #</th>
<th>Group</th>
<th>Region</th>
<th>Aged Mitochondrial Values</th>
<th>Density (# mitochondria/ μm²)</th>
<th>Circularity (AU)</th>
<th># Analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1663</td>
<td>No Surgery</td>
<td></td>
<td>0.5934 ± 0.04</td>
<td>0.6225 ± 0.10</td>
<td>0.7536 ± 0.03</td>
<td>268</td>
</tr>
<tr>
<td>777</td>
<td>No Surgery</td>
<td></td>
<td>0.4014 ± 0.02</td>
<td>0.8384 ± 0.09</td>
<td>0.7187 ± 0.01</td>
<td>381</td>
</tr>
<tr>
<td>678</td>
<td>Aged 7 day</td>
<td>AAR</td>
<td>0.2750 ± 0.01</td>
<td>0.6747 ± 0.09</td>
<td>0.7052 ± 0.01</td>
<td>421</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remote</td>
<td>0.4275 ± 0.01</td>
<td>0.7467 ± 0.11</td>
<td>0.6825 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>677</td>
<td>Aged 7 day</td>
<td>AAR</td>
<td>0.2814 ± 0.04</td>
<td>1.139 ± 0.15</td>
<td>0.709 ± 0.004</td>
<td>364</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remote</td>
<td>0.4054 ± 0.03</td>
<td>0.7385 ± 0.06</td>
<td>0.6944 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total Aged mitochondria analyzed</td>
<td>1434</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 7:** Aged individual mitochondrial values. All values means ± SE.
## Mitochondrial Drp1
### Gel 1- 6/8/15

<table>
<thead>
<tr>
<th>Lane</th>
<th>Group</th>
<th>Rat #</th>
<th>AU</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Adult Sham</td>
<td>1703</td>
<td>11562.551</td>
</tr>
<tr>
<td>3</td>
<td>Adult Sham</td>
<td>1680</td>
<td>19434.472</td>
</tr>
<tr>
<td>4</td>
<td>Adult Sham</td>
<td>1687</td>
<td>21460.886</td>
</tr>
<tr>
<td>5</td>
<td>MO OVX I/R</td>
<td>1612</td>
<td>20962.179</td>
</tr>
<tr>
<td>6</td>
<td>MO OVX I/R</td>
<td>1608</td>
<td>21904.35</td>
</tr>
<tr>
<td>7</td>
<td>MO OVX I/R</td>
<td>1693</td>
<td>26941.765</td>
</tr>
<tr>
<td>8</td>
<td>MO OVX I/R</td>
<td>1658</td>
<td>27891.794</td>
</tr>
<tr>
<td>9</td>
<td>MO OVX Sham</td>
<td>1694</td>
<td>15720.38</td>
</tr>
<tr>
<td>10</td>
<td>MO OVX Sham</td>
<td>1654</td>
<td>13535.602</td>
</tr>
<tr>
<td>11</td>
<td>MO OVX Sham</td>
<td>1692</td>
<td>13533.551</td>
</tr>
<tr>
<td>12</td>
<td>MO OVX Sham</td>
<td>1667</td>
<td>17130.551</td>
</tr>
<tr>
<td>13</td>
<td>Adult I/R</td>
<td>1609</td>
<td>27483.38</td>
</tr>
<tr>
<td>14</td>
<td>Adult I/R</td>
<td>1613</td>
<td>44082.229</td>
</tr>
<tr>
<td>15</td>
<td>Adult I/R</td>
<td>1607</td>
<td>23793.915</td>
</tr>
<tr>
<td>16</td>
<td>Adult I/R</td>
<td>1701</td>
<td>30554.25</td>
</tr>
</tbody>
</table>

**Table 8:** Raw densometric values for mitochondrial Drp1.
<table>
<thead>
<tr>
<th>Lane</th>
<th>Group</th>
<th>Rat #</th>
<th>AU</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Adult Sham</td>
<td>1703</td>
<td>9192.974</td>
</tr>
<tr>
<td>3</td>
<td>Adult Sham</td>
<td>1680</td>
<td>11476.338</td>
</tr>
<tr>
<td>4</td>
<td>Adult Sham</td>
<td>1687</td>
<td>17537.702</td>
</tr>
<tr>
<td>5</td>
<td>MO OVX I/R</td>
<td>1612</td>
<td>9836.581</td>
</tr>
<tr>
<td>6</td>
<td>MO OVX I/R</td>
<td>1608</td>
<td>10673.045</td>
</tr>
<tr>
<td>7</td>
<td>MO OVX I/R</td>
<td>1693</td>
<td>16234.702</td>
</tr>
<tr>
<td>8</td>
<td>MO OVX I/R</td>
<td>1658</td>
<td>6720.56</td>
</tr>
<tr>
<td>9</td>
<td>MO OVX Sham</td>
<td>1694</td>
<td>12758.652</td>
</tr>
<tr>
<td>10</td>
<td>MO OVX Sham</td>
<td>1654</td>
<td>12326.187</td>
</tr>
<tr>
<td>11</td>
<td>MO OVX Sham</td>
<td>1692</td>
<td>11567.409</td>
</tr>
<tr>
<td>12</td>
<td>MO OVX Sham</td>
<td>1667</td>
<td>11910.995</td>
</tr>
<tr>
<td>13</td>
<td>Adult I/R</td>
<td>1609</td>
<td>12004.874</td>
</tr>
<tr>
<td>14</td>
<td>Adult I/R</td>
<td>1613</td>
<td>8465.924</td>
</tr>
<tr>
<td>15</td>
<td>Adult I/R</td>
<td>1607</td>
<td>3550.711</td>
</tr>
<tr>
<td>16</td>
<td>Adult I/R</td>
<td>1701</td>
<td>8188.631</td>
</tr>
</tbody>
</table>

**Table 9:** Raw densometric values for mitochondrial Mfn1.
<table>
<thead>
<tr>
<th>Lane</th>
<th>Group</th>
<th>Rat #</th>
<th>AU</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Adult Sham</td>
<td>1703</td>
<td>10062.238</td>
</tr>
<tr>
<td>3</td>
<td>Adult Sham</td>
<td>1680</td>
<td>11569.137</td>
</tr>
<tr>
<td>4</td>
<td>Adult Sham</td>
<td>1687</td>
<td>19340.522</td>
</tr>
<tr>
<td>5</td>
<td>MO OVX I/R</td>
<td>1612</td>
<td>5393.167</td>
</tr>
<tr>
<td>6</td>
<td>MO OVX I/R</td>
<td>1608</td>
<td>7523.238</td>
</tr>
<tr>
<td>7</td>
<td>MO OVX I/R</td>
<td>1693</td>
<td>16398.279</td>
</tr>
<tr>
<td>8</td>
<td>MO OVX I/R</td>
<td>1658</td>
<td>11985.38</td>
</tr>
<tr>
<td>9</td>
<td>MO OVX Sham</td>
<td>1694</td>
<td>11171.894</td>
</tr>
<tr>
<td>10</td>
<td>MO OVX Sham</td>
<td>1654</td>
<td>14457.501</td>
</tr>
<tr>
<td>11</td>
<td>MO OVX Sham</td>
<td>1692</td>
<td>11582.48</td>
</tr>
<tr>
<td>12</td>
<td>MO OVX Sham</td>
<td>1667</td>
<td>9831.581</td>
</tr>
<tr>
<td>13</td>
<td>Adult I/R</td>
<td>1609</td>
<td>12784.915</td>
</tr>
<tr>
<td>14</td>
<td>Adult I/R</td>
<td>1613</td>
<td>9286.723</td>
</tr>
<tr>
<td>15</td>
<td>Adult I/R</td>
<td>1607</td>
<td>4896.51</td>
</tr>
<tr>
<td>16</td>
<td>Adult I/R</td>
<td>1701</td>
<td>11845.258</td>
</tr>
</tbody>
</table>

**Table 10:** Raw densometric values for mitochondrial Fis1.
## Mitochondrial OPA1

### Gel 4- 6/24/15

<table>
<thead>
<tr>
<th>Lane</th>
<th>Group</th>
<th>Rat #</th>
<th>AU</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Adult Sham</td>
<td>1703</td>
<td>1815.376</td>
</tr>
<tr>
<td>3</td>
<td>Adult Sham</td>
<td>1680</td>
<td>3915.196</td>
</tr>
<tr>
<td>4</td>
<td>Adult Sham</td>
<td>1687</td>
<td>5532.752</td>
</tr>
<tr>
<td>5</td>
<td>Adult I/R</td>
<td>1609</td>
<td>6602.338</td>
</tr>
<tr>
<td>6</td>
<td>Adult I/R</td>
<td>1613</td>
<td>4659.953</td>
</tr>
<tr>
<td>7</td>
<td>Adult I/R</td>
<td>1607</td>
<td>1299.012</td>
</tr>
<tr>
<td>8</td>
<td>Adult I/R</td>
<td>1701</td>
<td>3217.125</td>
</tr>
<tr>
<td>9</td>
<td>MO OVX I/R</td>
<td>1612</td>
<td>8748.359</td>
</tr>
<tr>
<td>10</td>
<td>MO OVX I/R</td>
<td>1608</td>
<td>8943.652</td>
</tr>
<tr>
<td>11</td>
<td>MO OVX I/R</td>
<td>1693</td>
<td>12041.915</td>
</tr>
<tr>
<td>12</td>
<td>MO OVX I/R</td>
<td>1658</td>
<td>4588.146</td>
</tr>
<tr>
<td>13</td>
<td>MO OVX Sham</td>
<td>1694</td>
<td>6365.388</td>
</tr>
<tr>
<td>14</td>
<td>MO OVX Sham</td>
<td>1654</td>
<td>8826.187</td>
</tr>
<tr>
<td>15</td>
<td>MO OVX Sham</td>
<td>1692</td>
<td>3358.539</td>
</tr>
<tr>
<td>16</td>
<td>MO OVX Sham</td>
<td>1667</td>
<td>3846.66</td>
</tr>
</tbody>
</table>

**Table 11:** Raw densometric values for OPA1.