

INVESTIGATING THE ANOXIC PROTECTIVE EFFECTS OF
SEX HORMONES AND EPHRINA1

Benjamin Juhl

A Signature Honors Project Presented to the

Honors College

East Carolina University

In Partial Fulfillment of the

Requirements for

Graduation with Honors

by

Benjamin Juhl

Greenville, NC

Spring 2024

BACKGROUND

CARDIOVASCULAR DISEASE IN THE UNITED STATES

It is estimated that every thirty-three seconds, someone in the United States dies of cardiovascular disease (Centers for Disease Control and Prevention, 2023). Moreover, while significant scientific advancements have been made in the 20th and 21st centuries to combat various cardiovascular diseases, they remain the leading cause of death both nationally and globally (World Health Organization, 2020). Cardiovascular disease (CVD) is characterized by any functional or structural defect of the heart or accompanying vasculature (Cleveland Clinic, 2021). Those of the structural variety most commonly involve the heart valves and chambers and can be congenital or developed during a person's lifetime, usually due to unmanaged hypertension (Yale Medicine, 2024).

Those of the functional variety can be subdivided into diseases that block or obstruct blood flow to the heart's "plumbing system" or those characterized by abnormal electrical activity. This dichotomy of plumbing vs. electrical can help us understand how cardiovascular function can be inhibited and the direct consequence of each type of inhibition. Arterial disease is characterized by the presence of calcific plaque in either the arteries that supply the heart (coronary artery disease), the brain (carotid artery disease), or the rest of the body (peripheral artery disease) (Bauersachs et al., 2019). In each case, atherosclerotic plaque can develop and grow within the arteries, leading to partial and, in some cases, total occlusion of blood flow (Bauersachs et al., 2019). The significance of these effects is highly dependent on their specific location, as severe peripheral artery disease may cause cramping in your legs. In contrast, severe coronary artery disease significantly predisposes you to myocardial infarction and potentially sudden cardiac death (Bauersachs et al., 2019).

Electrical disorders of the heart, similar to structural disorders, can also occur at various stages of development (Desai & Hajouli, 2024). Such conditions are characterized by electrical conduction flaws that lead to abnormal heart rates or rhythms (Desai & Hajouli, 2024). In a healthy human heart, electrical impulses originate in the sinoatrial (SA) node and then subsequently stimulate the upper chambers of the heart (atria) to contract, relay at the atrioventricular (AV) node, before eventually conducting through the bundle of His and Purkinje fibers to stimulate a contraction of the heart's lower chambers (ventricles) (Chang & Li, 2021). This cycle then continuously repeats to create a normal sinus rhythm, the rhythm of an otherwise healthy heart (Desai & Hajouli, 2024). However, if any step of this pathway is inappropriately interrupted or delayed, there can be consequences. The mildest heart rhythm disorders or arrhythmias are premature atrial or ventricular contractions, PACs, or PVCs. In most cases, premature beats are benign and can be quickly suppressed with medications (Suba et al., 2021). Other arrhythmias, such as atrial fibrillation, carry significant stroke risks in addition to ventricular tachycardia and fibrillation, which can lead to sudden cardiac death if not treated promptly (Chang & Li, 2021).

While all of these diseases can vary significantly in etiology, severity, and mechanism, most have the potential to be exacerbated by a few select lifestyle choices, primarily drug and alcohol abuse, consumption of processed foods, and sedentary lifestyle, which then develop into significant risk factors such as hypertension, hyperlipidemia, and type 2 diabetes (Desai & Hajouli, 2024). As these lifestyle factors play such an important role, CVD tends to disproportionately affect lower-income communities where access to modern medical treatment and healthy food options is significantly reduced (Jilani et al., 2021).

CORONARY ARTERY DISEASE AND MYOCARDIAL INFARCTION

Out of all the cardiovascular diseases previously discussed, coronary artery disease (CAD) is, in many ways, the most ruinous. Colloquially referred to as “heart disease,” coronary artery disease is characterized by the development of atherosclerotic plaque within the arteries that supply oxygenated blood to the heart (Libby & Theroux, 2005). Ischemia is when these plaques grow to the extent that they diminish the amount of blood the myocardial tissue receives (Severino et al., 2020). As these plaques grow, they become at higher risk for rupture, which then subsequently results in a blood clot that blocks the entire artery, causing a severe ischemic event known as an acute myocardial infarction or "heart attack" (Libby & Theroux, 2005).

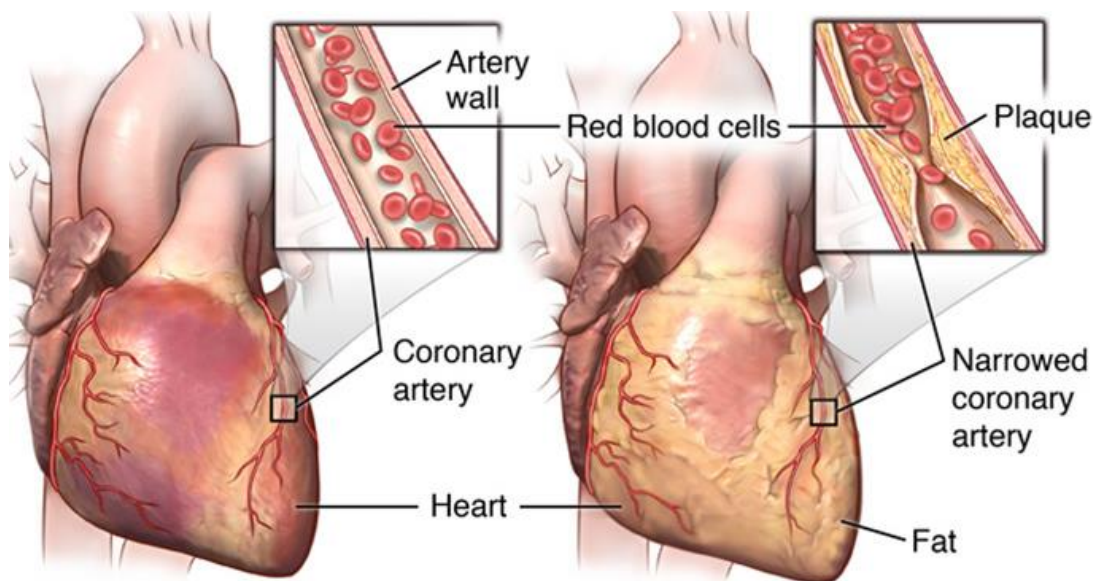


Figure 1: Coronary artery disease. The progression of atherosclerotic plaque buildup constricting arterial blood flow (UChicago Medicine, 2024)

The localization of the atherosclerotic disease is essential for understanding the danger posed. The left main (LM) coronary artery is the primary channel that branches off into the left anterior descending (LAD) artery and left circumflex artery (LCx). This means that if it were to become completely obstructed, the left side of the heart would quickly become hypoxic and would undergo likely fatal myocardial injury (Raja et al., 2019). Specific coronary arteries, like the LAD, commonly develop atherosclerotic disease and experience plaque ruptures more than others (Chatzizisis et al., 2007). While the specifics of why this occurs are not entirely understood, many attribute it to the sharp hemodynamic pressure changes within the LAD due to its specific anatomy (Chatzizisis et al., 2007). Patient outcomes with CAD, like many other chronic diseases, are dependent on early diagnosis, adequate risk factor mitigation, and proper medicinal and possibly surgical management (Libby & Theroux, 2005). The traditional risk factors for CAD are uncontrolled blood pressure (hypertension) and elevated blood cholesterol levels (hyperlipidemia) (Powell-Wiley et al., 2021). The combination of hypertension and hyperlipidemia creates the perfect storm to build high-risk atherosclerotic plaque within arteries throughout the body. This mechanism can be generally understood by thinking of the cholesterol in your blood slowly starting to attach to the walls of your arteries and accumulating. Then, this process is accelerated by elevated blood pressure (Powell-Wiley et al., 2021). Once high-risk plaques are developed, continued high levels of blood cholesterol can lead to eventual total artery occlusion. At the same time, uncontrolled hypertension also vastly increases the risk that one of these plaques may rupture, causing an ischemic event (Severino et al., 2020).

The dangers that uncontrolled hypertension and hyperlipidemia pose are why most medical management of CAD is focused on stabilized blood pressure and cholesterol (Powell-Wiley et al., 2021). Medications like angiotensin-converting enzyme (ACE) inhibitors or

angiotensin II receptor blockers (ARBs) are commonly used for hypertension. At the same time, HMG-CoA reductase inhibitors, also known as statins, are commonly used in hyperlipidemia, all of which have been shown to provide significant mortality benefits in patients with known coronary artery disease (Powell-Wiley et al., 2021).

However, what if patients do not know they have CAD? While screening of those with risk factors and development of symptoms is standard, some do not realize they have the disease until they are in the midst of a severe ischemic event (Libby & Theroux, 2005). In these situations, nitroglycerin is commonly used as an emergent therapy to increase blood flow and relieve anginal chest pain. However, it does not reopen arteries or significantly protect myocardial cells from hypoxic injury (Nakayama et al., 2022). Many cases of advanced CAD can also warrant surgical interventions such as angioplasty or bypass surgery to restore proper blood flow to the myocardium. While these procedures can significantly reduce long-term symptoms and sometimes increase mortality, they also cannot reinvigorate already damaged tissue (Libby & Theroux, 2005).

SEX DIFFERENCES

Coronary artery disease does not affect biological males and females equally (Nyström et al., 2022). While males suffer a greater incidence of myocardial infarction and fatal coronary artery disease at all ages, the discrepancy between sexes becomes significantly smaller for women over the age of 50 due to a reduction in estradiol levels due to menopause (Moolman, 2006). Symptom onset of cardiovascular diseases can significantly vary between men and women, leading to changes in American Heart Association guidelines within the last decade (Mehta et al., 2016). For example, both sexes commonly experience a radiating form of chest

pain and dyspnea, but females solely tend to experience more atypical symptoms such as nausea and vomiting (Mehta et al., 2016). The cardioprotective effects of estrogen are believed to be achieved by reducing the development of atherosclerotic disease and preserving myocardial tissue during and after an ischemic event (Moolman, 2006). However, the molecular mediators of these effects are yet to be understood entirely (Moolman, 2006).

On average, the male heart is larger and has a greater cardiac output, or liters of blood pumped by the heart per minute. (5.9 vs 4.6 L/min) (St. Pierre et al., 2022). However, the female heart has a 7% larger ejection fraction, 10-14% greater contractility, and is richer in cardiomyocytes on average (St. Pierre et al., 2022). By these metrics, female hearts are more efficient on average despite their smaller relative size, furthering that the hearts of males and females are different, which may clarify why their cardiovascular disease outcomes vary (Nyström et al., 2022). Despite these differences, there is minimal treatment specialization for cardiovascular disease between males and females (Nyström et al., 2022).

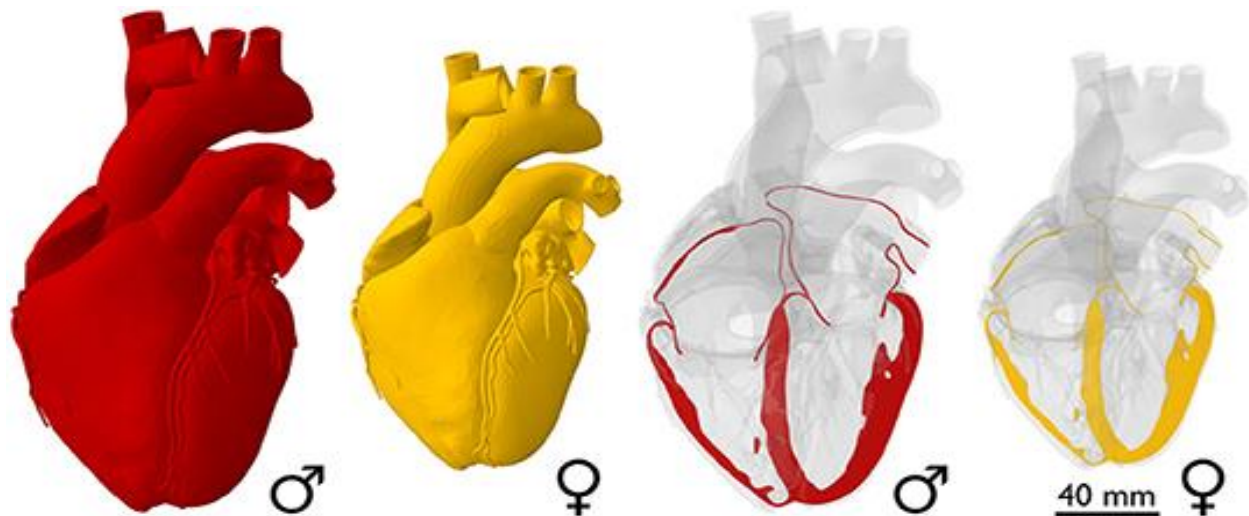


Figure 2: Human male and female hearts. Differences in structure are critical to understand the observed disparities in clinical outcomes between biological males and females (St. Pierre et al., 2022).

EPHRINA1

The Ephrin family of receptor tyrosine kinases is one of the largest found in humans (Darling & Lamb, 2019). While they have been extensively studied in a developmental context, we continue to better understand their various functions from a general physiologic standpoint (Arvantis & Davy, 2008). EphrinA1 has been a protein of interest within our lab based on its demonstrated cardioprotective effects, explicitly demonstrating the ability to preserve cardiac tissue in acute coronary injury. In prior mice-model trials, those who received an intramyocardial injection of recombinant EphrinA1 had less severe infarcts than those who received a control. We hypothesize that the influence of sex hormones on Ephrin signaling contributes to the observed differences in cardiovascular disease outcomes between biological males and females.

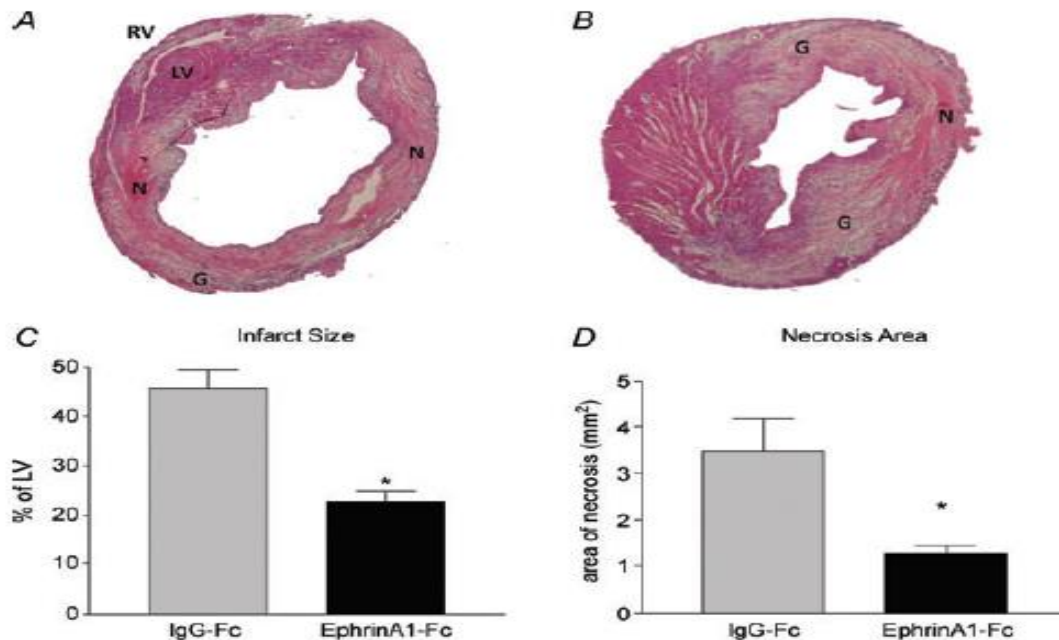


Figure 3: *EPHRINA1*-fc. Treatment groups were found to significantly outperform control in facilitating cardioprotection, thus maintaining greater levels of viable tissue (Virag Lab).

METHODS

CELL CULTURE

Human umbilical vein endothelial cells (HUVECs) and human inducible pluripotent stem cells (h-iPSC-CM) were utilized to observe the role of sex hormones and EphrinA1 on apoptosis and anoxic stress. HUVECs served as a standard and easily cultured in-vitro model, allowing us to imitate the effect potential treatments may have on the endothelial cells surrounding the heart. H-iPSCs were utilized to observe the results of our treatments on cardiomyocytes, thus allowing us to directly gauge to what extent sex hormones and EphrinA1 could preserve contractile cardiac cells from death.

THAWING & PLATING HUVECs

1. QUICKLY thaw HUVECs in bead bath until only a small ice pellet is visible
2. (In the hood) If not already added to the stock bottle of media, add 1X pen-strep to the aliquot of media (10uL of antibiotic per 1mL of media)
3. Add 500uL-1mL of fresh media to the cell solution.
4. Spin in the swinging bucket centrifuge at 1,000 rpm for 3 minutes
5. (In the hood) Aspirate the supernatant and resuspend the cell pellet in fresh media. The amount of media will depend on the number of cells in solution and your desired plating density.
6. Add fresh media to each well/flask/dish and then add your cell solution.
7. Incubate at 37°C in normal oxygen (bottom incubator).
8. Replace media every 2-3 days or as needed.

FEEDING IPSCs

Materials

- mTeSR (50 ml aliquots in cell culture fridge, get one and initial so only you use that conical of media)
- pen-strep
- DPBS
- Serological pipet
- Micro pipettor
- Conical
- a. Pipet fresh mTeSR into clean conical (2 ml per well of 6 well plates, 4 ml per well if overfeeding)
- b. Add pen-strep to fresh mTeSR and let media warm to room temp (stock is 100x, need 1x in media)
- c. Aspirate old media from wells
- d. Wash each well with ~1 ml of DPBS
- e. Add fresh mTeSR + pen-strep
- Pen-strep stock is 100X amount needed in media is 1x (12.5 ml of mTeSR means you need 125 μ l pen-strep)
- Always let media get to room temp before adding to cells
- Always get 0.5 ml extra of mTeSR to account for the volume lost during pipetting
- Pipet media/DPBS into wells at an angle so cells are not washed from the plate

PASSAGING IPSCs

Materials for Passaging

- mTeSR (use only conical with your initials, if you run out, get another and label with your initials)
- Pen-strep
- Versene
- Y drug/ROCK inhibitor (refer to ROCK aliquoting document)
- DMEM/F12
- Matrigel
- DPBS
- Ice
- Serological pipette
- Micropipette
- Conicals
- New unopened cell culture plates

Materials for Cell Counting

- Trypan blue
- Cells suspended in mTeSR + ROCK
- Piece of Parafilm
- Micro pipettor
- Countess cell counting chamber slides

Passaging

- a. Cells must be at 70%-80% confluency to passage
- b. Make Matrigel plates
 - One tube of Matrigel makes two 6 well plates, one 24 well plates, or one 10cm plate
 - KEEP MATRIGEL ON ICE! It will solidify if allowed to get to room temp
 - Get 12.5 ml of DMEM/F12; keep this on ice as well
 - Add ~150 μ l of DMEM/F12 to the tube of Matrigel, pipette up and down
 - Pipette contents of Matrigel tube into conical containing DMEM/F12
 - Mix by pipetting up and down with serological pipet
 - Add 1 ml of DMEM/F12-Matrigel mix to each well of a 6 well plate or add 0.5 ml to each well of a 24 well plate or add 10 ml to a 10cm plate
 - If needed immediately, put Matrigel-coated plates in the incubator for at least 30 mins
 - If not needed immediately, wrap Matrigel-coated plates in parafilm and keep them in the 4°C fridge for up to one week. Plates will be ready for use after sitting in the fridge overnight. Make sure to warm plates for 30 mins in the incubator before use.

- c. Gather reagents needed.
 - 750 μ l Versene per well of a six well plate
 - mTeSR + 1x ROCK inhibitor (Y drug) to resuspend single cells
 - mTeSR + 1x pen-strep + 1x μ M ROCK inhibitor for new plates
 - DPBS
- d. Once new Matrigel plates are ready, get cells from the incubator
- e. Aspirate old media and wash with DPBS
- f. Add 750 μ l Versene to each well and let incubate at room temp for 3-5 minutes. Check cells under microscope at 3 mins. **Once edges of cell colonies round up, aspirate Versene IMMEDIATELY!**
- g. Add resuspension media (mTeSR + 1x ROCK) to cells. Volume added to each well will depend on cell density and split ratio
- h. **GENTLY** run a cell lifter over the bottom of each well to help break up colonies and get cells off the plate
- i. Get out new Matrigel plates. Aspirate DMEM/F12 and wash with DPBS.
- j. Add mTeSR + 1x pen-strep + 1x ROCK to new plates. 2 ml per well of a 6 well plate
- k. Tilt plate containing cells toward you and use a micro pipettor to wash the remainder of cells into the suspension media for collection. **Do not wash cells too many times!** Limit washes to 3-5 titrations per well
- l. Cells in resuspension media can be collected in a conical or directly pipetted onto new plates from the old plates if needed
 - If collecting cells in a conical, make sure to pipet up and down to evenly distribute cells before counting or plating
 - Counting cells is only necessary if planning to differentiate
- m. Media **MUST** be replaced the next day with mTeSR + 1x pen strep. **ROCK inhibitor should not be left on cells for longer than a day.**
- n. Feed cells daily or every other day depending on the color of media (yellow=change now!) and the amount of cell debris present.
- o. Cells will reach confluency in 3-5 days depending on density seeded.



Figure 4: Human induced pluripotent stem cell derived cardiomyocytes (hIPSC-CM). Cluster of mature hIPSC-CMs captured during diastole. Once at this stage of development, clusters beat together in a rhythmic manner consistent with human myocardium.

TREATMENT GROUPS

Concentrations of sex hormone treatments were derived based on human physiologic standards and prior in-vitro studies both elsewhere and within our lab (Smeeth et al., 2021) (Yu et al., 2020). Solutions were initially prepared within EtOH, DMSO, and ddH₂O to determine if solvent variability had any influence on treatment outcomes.

Trials were conducted exposing the cells for either 15 minutes or 24 hours to observe whether exposure duration significantly influenced cardioprotective ability. Estradiol (E2) and testosterone (T) groups were dosed at 0, 0.1, 1.0, 10, 100, or 1,000 nM, respectively.

EFNA1 groups were dosed at 0, 0.1, 0.2, 2, 10, 20 $\mu\text{g/mL}$, respectively. Oxyrase was deployed within these trials to induce hypoxic states.

PROTEIN QUANTIFICATION

Western blot analysis was utilized to measure the specific levels of apoptosis mediators as well as EphrinA1. Specifically, we probed for Akt and mTOR based on their known influence on regulating programmed cell death. Bicinchoninic Acid (BCA) assay was used to quantify total protein levels prior to western blot in order to normalize sample volumes. Pierce BCA Protein Assay Kit and protocol were deployed for these trials.

BCA PROTOCOL

Preparation of the BCA working reagent (WR)

1. Use the following formula to determine the total volume of WR required:

$$(\# \text{ of standards} + \# \text{ of unknowns}) \times (\# \text{ of replicates})$$

$$\times (\text{volume of WR per sample}) = \text{total volume of WR required}$$

- 200 uL of WR is required for each sample in the microplate procedure
2. Prepare the WR by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1, Reagent A:B)

Microplate procedure (sample to WR ratio =1:20)

1. Pipette 25 uL of standard or unknown sample replicate into a microplate well (working range = 20-200 ug/mL)
2. Add 200 uL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37 °C for 30 minutes.
4. Cool plate to room temperature. Measure the absorbance at 650 nm on a plate reader.

WESTERN BLOT PROTOCOL

Starting from cell culture:

- Plate cells in a 12-well plate at a density of 100K/well
- The following day, begin serum starvation with 0.5% FBS in DMEM (can use DMEM/F-12)
- After 24 hours of serum starvation, apply treatment per your experimental design.
- Harvest cells by removing media and lysing with M-PER + 1X HALT protease and 1X HALT phosphatase inhibitors (both are at 100X stock)
 - 200uL/well of a 6WP; 150uL/well of a 12WP; 100uL/well of a 24WP
- Centrifuge lysate for 10 min at 4°C and 14,000 rpm
- Set aside 10uL of each sample for BCA; add 6X Laemmli buffer w/ BME to the rest of the lysate.

Western Blot:

- Run:
 - Load gels w/ 2-60ug of total protein and run at 100V for 15min followed by 180V for 40-50 min (or until dye runs off the bottom of the gel)
- Transfer:
 - Transfer either overnight at 20V in 4°C or for 90min at 70V RT.
- Ponceau S Stain:
 - Wash membrane(s) in ddH₂O for 1 min before adding Ponceau S; rock for 5-60 min (or until protein bands are visible)
 - Wash w/ ddH₂O until the background is white; wrap in plastic wrap to keep the membrane moist and image.
 - After imaging, wash w/ 0.1M NaOH for 1 min to remove dye.
 - Wash w/ TBST for 3 minutes to remove any remaining dye.
- Block:
 - Block for 1 hour w/ 5% BSA in TBST
- Primary Ab:
 - Incubate with primary ab (1:1,000 in 5% BSA in TBST) overnight on rocker in 4°C.
- Secondary Ab:
 - Wash 3x5 minutes with TBST
 - Incubate w/ secondary ab (HRP fusion, 1:5,000 in 5% BSA in TBST) for 1 hour on rocker at RT.
 - Wash 3x5 minutes with TBST
 - Rinse briefly with DI water
- Developing:
 - Apply chemiluminescent substrate and image.

RESULTS AND DISCUSSION

DIFFICULTIES IN CELL CULTURE AND WESTERN BLOT

Troubleshooting and error are a natural part of the research process for most individuals. To quote the Roman Philosopher Seneca: "The important thing about a problem is not the solution, but the strength we gain in finding a solution" (Vogt, 2015). Within this project, I and other research team members made various errors and mistakes. All of these allowed me to delve deeper into our methodology and develop our creative problem-solving abilities.

Cell culture was confounded by recurring contamination that significantly limited our ability to grow iPSCs to confluency and differentiate into cardiomyocytes. In response, various concentrations of Penicillin/Streptomycin (Pen/Strep) were trialed to reduce the proliferation of bacteria among our samples, with minimal success. However, HUVECs tended to be more resilient in vitro and could proliferate with Pen/Strep. While our initial goal was to have a large sample size of iPSC-CMs to observe cardiac function in-vitro directly, we had to pivot instead and focus more on the effects treatments would have on endothelial cells. This data was still valuable to us, given the various types of endothelial tissue in and around the heart (Allbritton-King & García-Cardena, 2023).

Once cells were treated and prepared, specific markers of apoptosis and cellular injury were probed in addition to EphrinA1 levels via western blot. Protein kinase B (Akt), mammalian target of rapamycin (mTOR), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and matrix metalloproteinase (MMP) were among those specifically selected. After countless attempts to adequately image and quantify western blot results, minimal correlation could be drawn between differences in hormone exposure due to technical difficulties. These technical difficulties include but are not limited to inconsistent gel polymerization, loss of power during gel running or

transferring steps, excessive reuse of antibody solutions, expired reagents, denatured samples, and otherwise general lack of necessary laboratory skills.

To correct these errors, I sought assistance from Dr. Virag, former lab employees, and other staff within the Physiology department. Per their feedback, Gel polymerization reagents were remade twice. Antibody solutions were made at various dilutions, and the duration and voltage of running and transfer were modified. Multiple chemiluminescent substrates were attempted. Trials were completed using previously harvested animal tissue to refine skills with seemingly protein-rich samples.

Despite these efforts, no significant data were generated regarding the effects of various sex hormone concentrations on cellular resilience or survival.

Near the end of the project, it was discovered that our fume hood's innermost segments were vastly contaminated with bacterial growth. This finding, in part, explains the persistent difficulties with cell culture. In addition, many samples were later discovered to have been improperly prepared for long-term storage. While many factors were confounding western blot success, unawareness that viable protein within many samples had decreased tenfold over months trials were being attempted was a significant impediment.

PRELIMINARY DATA

Despite difficulties acquiring actionable data from in-vitro trials, ongoing projects within our lab using small animal in-vivo models were analyzed to compare cardiac output in males vs. females and EFNA1-FC vs. control groups. As expected, female mice demonstrated higher ejection fractions and fractional shortening than males. Additionally, intramyocardial administration of EFNA1-fc outperformed various intravenous constructs administered via the mice's tail vein.



Figure 5:. *Sex differences. Comparing cardiac output via fractional shortening and ejection fraction.*

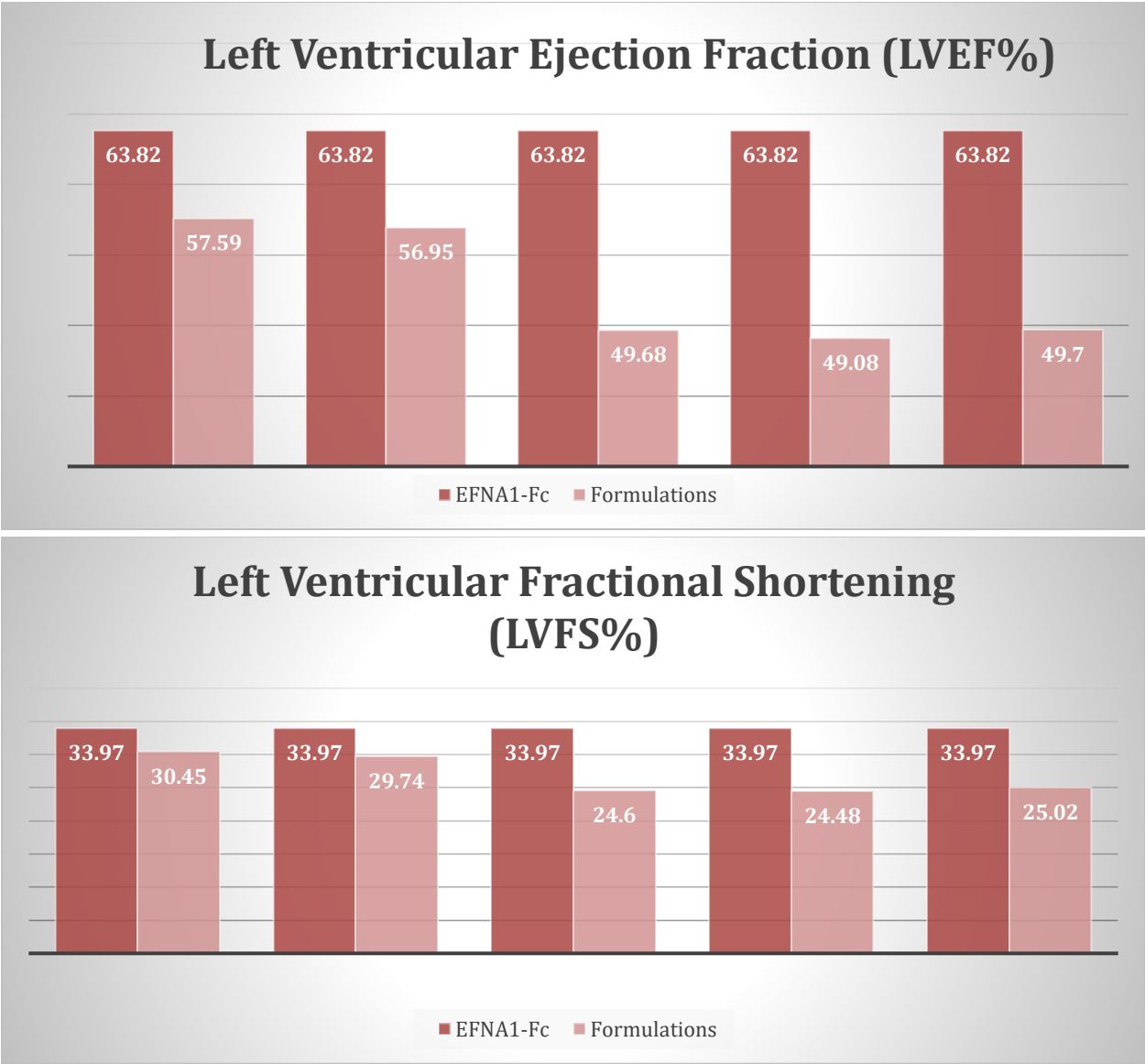


Figure 6: Intramyocardial injection and tail-vein IV formulations. Comparing two methods of EFNA1-fc administration and their effects on cardiac output.

FUTURE PROJECTS AND IMPLICATIONS

If I were to continue this project, I would begin by thoroughly decontaminating the fume hood and surrounding cell culture areas. Additionally, having a more official training program and feeding schedule for lab members should reduce the chance of the errors made previously from being repeated going forward. Once cell culture has reached stable reliability, I will adopt the same mindset of further training and coordination in protein and RNA quantification methods. At this point, I would be confident that the abovementioned methods would be much more successful and yield significant results.

Other ideas for further understanding the interplay between sex hormones and EphrinA1 that were not attempted during my project included, but are not limited to, staining cells post-treatment with Oil Red O to measure lipotoxicity and using RT-PCR to directly measure differential expression of EFNA1 between male and female-induced treatments.

Despite the various difficulties detailed above, based on the analysis outlined in this paper and previous findings within our lab, EphrinA1 has the potential to be a cardioprotective therapy for patients post-myocardial infarction through the optimal means of its administration and dosing specific to males and females have yet to be defined. As coronary heart disease continues to kill Americans indiscriminately and, specifically, North Carolinians at disgustingly high rates, the need for such a therapy is as high as ever.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Jitka Virag for her continued mentorship and guidance throughout the project. I worked in her lab for nearly my entire undergraduate career at East Carolina University and learned an immense amount. Additionally, I would like to thank Taylor Byers, Cameron Taylor, Emily Newsome, Dr. Robert Hughes, Dr. Bobbie Garvin, and the entire Department of Physiology for their significant contributions to the execution of this project. An East Carolina University Undergraduate Research and Creativity Award partially funded this project.

REFERENCES

- Allbritton-King, J. D., & García-Cardena, G. (2023). Endothelial cell dysfunction in cardiac disease: driver or consequence? *Frontiers in Cell and Developmental Biology*, 11(25). <https://doi.org/10.3389/fcell.2023.1278166>
- Arvanitis, D., & Davy, A. (2008). Eph/ephrin signaling: networks. *Genes & Development*, 22(4), 416–429. <https://doi.org/10.1101/gad.1630408>
- Bauersachs, R., Zeymer, U., Brière, J.-B., Marre, C., Bowrin, K., & Huelsebeck, M. (2019). Burden of coronary artery disease and peripheral artery disease: A literature review. *Cardiovascular Therapeutics*, 2019(1), 1–9. <https://doi.org/10.1155/2019/8295054>
- Centers for Disease Control and Prevention. (2023, May 15). *Heart disease facts*. Centers for Disease Control and Prevention. <https://www.cdc.gov/heartdisease/facts.htm>
- Chang, W., & Li, G. (2021). Clinical review of sick sinus syndrome and atrial fibrillation. *Herz*, 47. <https://doi.org/10.1007/s00059-021-05046-x>
- Chatzizisis, Y. S., Giannoglou, G. D., Parcharidis, G. E., & Louridas, G. E. (2007). Is left coronary system more susceptible to atherosclerosis than right? A pathophysiological insight. *International Journal of Cardiology*, 116(1), 7–13. <https://doi.org/10.1016/j.ijcard.2006.03.029>
- Cleveland Clinic. (2021, May 10). *Cardiovascular Disease: Symptoms, Types, Causes, Management & Prevention*. Cleveland Clinic. <https://my.clevelandclinic.org/health/diseases/21493-cardiovascular-disease>
- Darling, T. K., & Lamb, T. J. (2019). Emerging Roles for Eph Receptors and Ephrin Ligands in Immunity. *Frontiers in Immunology*, 10. <https://doi.org/10.3389/fimmu.2019.01473>

Desai, D. S., & Hajouli, S. (2024). *Arrhythmias*. PubMed; StatPearls Publishing.

<https://www.ncbi.nlm.nih.gov/books/NBK558923/#:~:text=Arrhythmia%20entails%20a%20broad%20spectrum>

Jilani, M. H., Javed, Z., Yahya, T., Valero-Elizondo, J., Khan, S. U., Kash, B., Blankstein, R., Virani, S. S., Blaha, M. J., Dubey, P., Hyder, A. A., Vahidy, F. S., Cainzos-Achirica, M., & Nasir, K. (2021). Social Determinants of Health and Cardiovascular Disease: Current State and Future Directions Towards Healthcare Equity. *Current Atherosclerosis Reports*, 23(9). <https://doi.org/10.1007/s11883-021-00949-w>

Libby, P., & Theroux, P. (2005). Pathophysiology of Coronary Artery Disease. *Circulation*, 111(25), 3481–3488. <https://doi.org/10.1161/circulationaha.105.537878>

Mehta, L. S., Beckie, T. M., DeVon, H. A., Grines, C. L., Krumholz, H. M., Johnson, M. N., Lindley, K. J., Vaccarino, V., Wang, T. Y., Watson, K. E., & Wenger, N. K. (2016). Acute Myocardial Infarction in Women. *Circulation*, 133(9), 916–947. <https://doi.org/10.1161/cir.0000000000000351>

Moolman, J. (2006). Unravelling the cardioprotective mechanism of action of estrogens. *Cardiovascular Research*, 69(4), 777–780. <https://doi.org/10.1016/j.cardiores.2006.01.001>

Nakayama, N., Yamamoto, T., Kikuchi, M., Hanada, H., Mano, T., Nakashima, T., Hashiba, K., Tanaka, A., Matsuo, K., Nomura, O., Kojima, S., Yamaguchi, J., Matoba, T., Tahara, Y., Nonogi, H., Force, for the J. R. C. (JRC) A. C. S. (ACS) T., Emergency, the G. E. C. on behalf of the J. C. S. (JCS), & Committee, C. C. (2022). Prehospital Administration of Aspirin and Nitroglycerin for Patients With Suspected Acute Coronary Syndrome. *Circulation Reports*, 4(10), CR–22-0060. <https://doi.org/10.1253/circrep.CR-22-0060>

- Nyström, A., Strömberg, S., Jansson, K., Faresjö, Å. O., & Faresjö, T. (2022). Cardiovascular risks before myocardial infarction differences between men and women. *BMC Cardiovascular Disorders*, 22(1). <https://doi.org/10.1186/s12872-022-02555-3>
- Powell-Wiley, T. M., Poirier, P., Burke, L. E., Després, J.-P., Gordon-Larsen, P., Lavie, C. J., Lear, S. A., Ndumele, C. E., Neeland, I. J., Sanders, P., & St-Onge, M.-P. (2021). Obesity and Cardiovascular disease: a Scientific Statement from the American Heart Association. *Circulation*, 143(21). <https://doi.org/10.1161/cir.0000000000000973>
- Raja, J. M., Nanda, A., Pour-Ghaz, I., & Khouzam, R. N. (2019). Is early invasive management as ST elevation myocardial infarction warranted in de Winter's sign?—a “peak” into the widow-maker. *Annals of Translational Medicine*, 7(17), 412–412. <https://doi.org/10.21037/atm.2019.07.19>
- Severino, P., D'Amato, A., Pucci, M., Infusino, F., Adamo, F., Birtolo, L. I., Netti, L., Montefusco, G., Chimenti, C., Lavalle, C., Maestrini, V., Mancone, M., Chilian, W. M., & Fedele, F. (2020). Ischemic Heart Disease Pathophysiology Paradigms Overview: From Plaque Activation to Microvascular Dysfunction. *International Journal of Molecular Sciences*, 21(21). <https://doi.org/10.3390/ijms21218118>
- Smeeth, D. M., Kourouzidou, I., Duarte, R. R. R., Powell, T. R., & Thuret, S. (2021). Prolactin, Estradiol and Testosterone Differentially Impact Human Hippocampal Neurogenesis in an In Vitro Model. *Neuroscience*, 454, 15–39. <https://doi.org/10.1016/j.neuroscience.2019.12.021>
- St Pierre, S. R., Peirlinck, M., & Kuhl, E. (2022). Sex Matters: A Comprehensive Comparison of Female and Male Hearts. *Frontiers in Physiology*, 13(1), 831179. <https://doi.org/10.3389/fphys.2022.831179>

- Suba, S., Fleischmann, K. E., Schell-Chaple, H., Prasad, P., Marcus, G. M., Hu, X., & Pelter, M. M. (2021). Diagnostic and prognostic significance of premature ventricular complexes in community and hospital-based participants: A scoping review. *PLOS ONE*, 16(12), e0261712. <https://doi.org/10.1371/journal.pone.0261712>
- UChicago Medicine. (2019). *Coronary Artery Disease and Chest Pain*. Uchicagomedicine.org. <https://www.uchicagomedicine.org/conditions-services/heart-vascular/coronary-artery-disease>
- Vogt, K. (2015). *Seneca (Stanford Encyclopedia of Philosophy)*. Stanford.edu. <https://plato.stanford.edu/entries/seneca/>
- Willemars, M., Nabben, M., Verdonchot, J., & Hoes, M. F. (2022). Evaluation of the Interaction of Sex Hormones and Cardiovascular Function and Health. *Current Heart Failure Reports*, 19(4), 200–212. <https://doi.org/10.1007/s11897-022-00555-0>
- World Health Organization. (2020). *Global health estimates: Leading causes of death*. World Health Organization. <https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-leading-causes-of-death>
- Yale Medicine. (2024). *Structural Heart Disease*. Yale Medicine. <https://www.yalemedicine.org/conditions/structural-heart-disease#:~:text=It>
- Yu, X., Zheng, J., Cai, T., Wang, Z., & Zhu, G. (2020). Testosterone antagonizes paraquat-induced cardiomyocyte senescence via the mIGF-1/SIRT1 signaling pathway. *Brazilian Journal of Medical and Biological Research*, 53(10). <https://doi.org/10.1590/1414-431x20209849>