The Physiological Effects of Various Vasoactive Agents on Mouse Ear Microvasculature
(Technical Paper)

Ethical Considerations of Genetic Engineering
(STS Paper)

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Technical Prospectus

I.SPECIFIC AIMS

A vasoactive agent is an administered drug that either constricts or dilates the blood vessels (Hollenberg, 2011). Vasoactive agents are utilized by plastic surgeons in transplantations and reconstructive surgeries. A successful reconstruction or transplantation depends partly on ensuring the vascular vessels, or pedicles, supplying the tissue are active and attached well. The rapidly acting vasoactive agents chosen for this study generally are applied to the pedicle vessels that need to be reattached to the functional vasculature. The slower longer lasting agents chosen for this study are applied to the peripheral vessels in the entire tissue flap to ensure they are open to provide adequate blood flow while healing.

We will specifically be studying the magnitude and timeframe of activity of the vasoactive agents nitroglycerin, diltiazem, papaverine, and lidocaine. These come in liquid solution forms, for fast action, as well as paste-like gel forms, for slower longer action. Nitroglycerin is converted to nitric oxide in the body. Nitric oxide activates a phosphorylation pathway that ultimately results in the dephosphorylation of myosin light chains in smooth muscle fibers, causing the relaxation of smooth muscle within blood vessels (Kim and Schaller, 2019). Lidocaine acts on sodium ion channels and locks the channels in the open state, preventing nerve depolarization (Beecham and Goyal, 2019). Diltiazem is a calcium channel blocker, and specifically it inhibits the influx of extracellular calcium ions across the myocardial and vascular smooth muscle cell membranes during depolarization (PubChem). Papaverine acts on cardiac and smooth muscle cells as a nonxanthine phosphodiesterase inhibitor and relaxes smooth muscle cells (PubChem). Nitroglycerin and diltiazem are more frequently used topically for tissue flaps, while papaverine and lidocaine are usually used for pedicle treatment. However, the

exact kinetics of their action in their use in reconstructive surgery is not known. It would be valuable to know more of the effects on microvasculature as well as how long agents circulate in the system, their optimum base dosage, and cytotoxic dosage. Decisions of which drug to use also need to be based on patient specifics, such as whether or not the patient has a history of smoking, or another condition that could negatively couple with a vasoactive agent to create a harmful effect to the patient. Deeper understanding of each of these vasoactive agents will aid doctors in making the right, informed decision that is best for their patient's specificities.

To fulfill this research, we are developing a model to observe the vasoactivity of each drug in the microvasculature of a mouse ear; which is a well-established model for viewing microcirculation (Barker et al., 1994). The mouse ear is an established model because its relatively thin structure, about 500 micrometers thick, allows for non-invasive imaging of blood vessels as well as it being almost identical to human skin structure except for sweat ducts and glands (Yousefi et al., 2014). The imaging modalities used in the model will be sidestream dark field microscopy, two-dimensional laser Doppler perfusion imaging, and photography with ImageJ analysis. We are working under the guidance and advice of Dr. Cottler and Dr. Campbell, who both work in UVA's Plastic Surgery Department.

Aim 1: Develop an experimental small animal model with repeated measures of blood perfusion and vessel diameter change.

Motivation: The model will be used to determine the timing, course, and magnitude of the physiologic effects of different vasoactive agents. This model needs to be created and evaluated first to ensure we have an effective tool to observe the effects of each vasoactive agent without modifying the natural state with imaging modality. The model will be evaluated on its ability to non-invasively image the perfusion levels in the ear in

real-time, the vessel prominence by ear region, and the vessel diameters without altering the vessels. We plan to use sidestream dark field microscopy, two-dimensional laser Doppler perfusion imaging, and photography with ImageJ analysis to observe the effect of different vasoactive agents on the microvascular networks within said model.

Output: Mouse ear model imaging system optimized for observing the events before and after a vasoactive agent is applied.

Aim 2: Perform testing and analysis of the perfusion and the vasculature response in mouse ear caused by vasoactive agents.

Motivation: Using the model developed in Aim 1, we will test various clinically used vasoactive agents in the ear of a mouse compared to an untreated ear which will serve as the control. Through sidestream dark field microscopy, two-dimensional laser Doppler perfusion imaging, and photography with ImageJ analysis we will measure tissue perfusion and vessel dilation as a function of dose as well as the active timeframe of each agent as well as how it affects the subject and for what time period. The effects will be measured by change in diameter of the vessels, and perfusion levels.

Output: Quantitative and qualitative characterization of vasoactive agents within the model.

Being able to experimentally document the effects of various vasoactive agents will provide surgeons the necessary information to guide their use clinically. Knowing the effects of different agents assists in doctors picking the correct one for each case. We hope to provide not only more information on these common vasoactive agents, but also to provide a model that can be used to learn more about future and emerging drugs intended for similar applications.

II. SIGNIFICANCE

Tissue flaps can be used in reconstructive surgery to recreate structures such as the breast. A flap is a transfer of tissue that still has a persistent blood supply from a major artery and vein to a recipient area. The transferred tissue can contain multiple types of tissue, and the higher volume of tissue transferred will be compromised if the arterial supply or venous drainage is interrupted during or following the transfer of the tissue (2019a).

During surgery, intraoperative vasospasms can have potentially devastating effects, while after surgery inadequate blood flow can lead to rejection of the newly implanted tissue (Vargas, Iorio, & Lee, 2015). Vasospasms can lead to decreased blood flow or clotting and is estimated to have significant effects on 5-10% of procedures (Afridi & Paletz, 2000). After breast reconstructive surgeries, skin flap necrosis is a common complication and can occur in 5-30% percent of procedures (Guinard, Carpenter, & Morell, 1992; Robertson, Jeevaratnam, Agrawal, & Cutress, 2017). Skin flap necrosis can occur if the network of blood vessels that supply blood to the tissue were damaged and there isn't enough blood flow to the skin, which causes the breakdown of tissue known as necrosis (2019b). Vasoactive agents injected during procedures or topically applied post-operatively are possible ways to pharmacologically prevent these complications. We would like to provide guidance on the use of vasoactive agents to decrease these complications. The injectable vasoactive agents are utilized to decrease vasospasms of the pedicle to ensure newly established flow at the anastomosis is maintained. In addition, the topical agents are used to ensure that perfusion of the tissue flaps distal to the pedicle is maintained. Agents being used today are known to cause vasodilation, however there is very little known of the duration and magnitude of vasodilation effect. Additionally, there is no animal model that has been developed to test and compare such characteristics of each agent.

We will create an experimental animal model that allows for repeated measures of blood flow to determine the time course and effect of a collection of vasoactive agents. This animal model will provide an avenue to determining the specific characteristics of the agents we test and can be further applied to any other vasoactive agents not included in our study or future innovations that enter the field. Our study will provide surgeons with more in-depth understanding of each agent. This will support surgeons in providing patients with individualized care best suited for success in each patient.

There is a lack of research in specific characteristics of vasoactive agents in the reconstructive surgery field. Some studies have been done on preventing vasospasms in animal and in vitro models but few have been in a controlled fashion or on clinical subjects (Vargas et al., 2015). Deeper understanding of each vasoactive agent and their potential to prevent not only vasospasms but also necrosis is vital to providing individualized care. Patient specifics such as age, weight, high blood pressure, or a history of smoking can all have an effect on vessel function and should be taken into account when deciding how much vasodilation is needed to aid blood flow (Stapleton, James, Goodwill, & Frisbee, 2008).

Lidocaine was studied in 1992 to evaluate its effect on percent increase of capillary blood flow in humans over therapeutic and subtherapeutic concentrations and compared to mepivacaine and bupivacaine, two other vasoactive agents popular at the time (Guinard et al., 1992). However, it has not been studied since and compared to more recent agents. Also lacking is studies into its long term effects and exact kinetics.

Deeper understanding of the kinetics of these vasoactive agents is also essential to further studies on possible negative effects. For example, nitroglycerin is a common vasoactive agent used in reconstructive surgeries, but it is also the oldest and the most commonly prescribed short

acting anti-anginal agent (Boden, Padala, Cabral, Buschmann, & Sidhu, 2015). Nitroglycerin has been shown in rat studies to increase the severity of subsequent heart attacks when given for hours as a continuous dose (Affairs, n.d.). While this is not a direct connection to doses given in reconstructive surgeries, this study highlights the need to test each agent and understand any potential downfalls or limits of use.

III. INNOVATION

These probes have not been used in a study like this for this purpose before, nor have mice ears been used to study the effects of these vasoactive agents. This study will utilize two imaging modalities, the Braedius probe and the PeriScan PIM Blood Perfusion Imager, as well as iPhone cameras.

The Braedius probe is a digital handheld video microscopy camera that is coupled with specialized software designed to capture and analyze the video images. The Braedius probe, a product of Braedius Medical, was created to be used for visualization and assessment of microcirculation (Aykut, Veenstra, Scorcella, Ince, & Boerma, 2015). The imaging device consists of a pen-like probe incorporating incident dark field illumination (IDF) illumination with a set of high-resolution lenses projecting images on to a computer controlled image sensor synchronized with very short pulsed illumination light (Aykut et al., 2015). Illumination is provided by concentrically placed light-emitting diodes (Goedhart et al., 2007). In this study, it will be used to assess the peripheral and distal vessel prominence and observe the response of the vessels starting from the main branch of the vessels to the most distal small branches. (Goedhart et al., 2007)

The PeriScan PIM Blood Perfusion Imager is intended for non-invasive two-dimensional imaging of peripheral tissue blood perfusion (Hirai, 2005). The PeriScan PIM System is a blood

perfusion imager and system based on the laser Doppler technology. It has been documented and proven to have linearity between the measured blood perfusion and the true blood perfusion in the tissue being imaged. This technique has two major advantages over the single-probe technique. The first is that blood flow is measured over an area rather than at a single site, which might improve reproducibility. Secondly, the laser beam is non-contact, as opposed to the single probe, which involves direct contact with the skin and could interfere with blood flow by applying pressure. The laser beam, approximately 1 mm in diameter, is scanned across tissue in two dimensions using a moving mirror. The scattered light signal is analysed to provide a two-dimensional image of blood flow (Murray et al., 2004). In this study, it will be used to visualize the overall effect and perfusion changes in the mouse ear, and it will be used to compare the perfusion amount and time course between controls and different applications. Analysis of images will be done with Image J.

IV. APPROACH

Preliminary work has focused mainly on model development and imaging modalities. The optimal mouse orientation for imaging resolution and ear flatness has been explored. The best positioning found was a dorsal orientation, with the mouse laid on its stomach, snout in a cone to stay under anesthesia whilst imaged, and ear flattened on a small petri dish with a small application of Aqua Gel. The modalities have been practiced with and explored to determine which is best suited to measure each parameter of the study. There a few factors that still need to be explored, including how many images to take in one recording, how best to follow one vessel from the base of the ear to the distal edge using the Braedius probe, and how to get the best resolution. Practice tests were run as a baseline for creating an experiment plan and imaging schedule. An injection of 4% concentrated Lidocaine and an application of Nitro-Bid paste, for

nitroglycerin, were the initial testing agents. One mouse was injected at the base of its ear, and a different mouse had Nitro-Bid applied to its ear. The initial observations made from the lidocaine injection were an almost immediate, noticeable to the naked eye, response, after 20 minutes a very noticeable difference in color of the entire ear, as well as more pronounced vessels visible from the base of the ear to the distal edge. Injecting lidocaine into the first mouse's other ear after 20 minutes from the first injection resulted in death of the mouse. In the study each mouse will only have one ear tested on, the other ear will remain as a control. The initial observations made from the Nitro-Bid paste application were no noticeable response initially or after 20 minutes. However, after 24 hours the applied ear was pinker than the control ear. The Tegaderm® bandage covering of the ear, applied to allow the Nitro-Bid to absorb into the ear and mimic clinical application, had come off sometime in the 24 hours after application. This was possibly due to lack of toleration by the mouse or due to the mouse being housed with other mice that may have pulled it off. In the study the mouse with the Tegaderm® covering will be housed separately, and it will be checked after 8 hours to provide a better estimation of when the covering typically comes off. This will not mimic typical patient conditions but will provide better estimation of where the model differs from human conditions.

Aim 1

1.Rationale

The process of optimizing and setting the method into a finalized protocol will include determining the target resolution for each imaging modality, determining the Region of Interest standard to aim for each imaging round, and evaluating the reproducibility of the method and the data itself.

2. Experimental Methods

The Blood Perfusion Imager will be used for large scale observation and analysis. The Region of Interest feature will be used to quantify perfusion levels and compare between time points. The Braedius probe will be used for regional observation and analysis, comparing the proximal, middle, and distal regions of the ear for vessel prominence and diameter change. iPhone cameras will be used to capture total ear color change, vessel prominence, and vessel diameter change with ImageJ analysis.

Aim 2

1.Rationale

The method of testing the agents will differ slightly based on the form of the agent. All mice will be placed under anesthesia for injection or application of drugs and imaging.

2. Experimental Methods

For injectable agents, one ear of each mouse will be used for control and will have no agent injected. The other ear will be used for testing. Pre-injection imaging will be done with the Blood Perfusion Imager, the Braedius Probe, and a camera once the ear is removed of excess hair and wiped down with alcohol. The vasoactive agent will be injected using a small gauge needle inserted into the base of the ear. Immediately post injection imaging will be done with all three same modalities as pre-injection. Then imaging with all 3 modalities will be done 15 minutes post injection. Imaging will be done hourly for 6 hours after the 15 minutes imaging.

For topically applied agents, one ear of each mouse will be used for control and will have no agent applied. The other ear will be used for testing. Pre-application imaging will be done with the Blood Perfusion Imager, the Braedius Probe, and a camera once the ear is removed of excess hair and wiped down with alcohol. The vasoactive agent will be

applied gently to the entire side of one ear using a Q-tip applicator. Immediately post application imaging will be done with all 3 modalities. The ear will then be covered with Tegaderm® and the mouse will be placed in separate housing. After 8 hours, the Tegaderm® covering will be checked and imaging with all 3 modalities will be done. The final imaging will be done 24 hours post application with all 3 modalities. There will be 6 repeated trials for each drug. Each mouse will be given adequate time to fully recover from the agent as well as the anesthesia.

A timeline of the project schedule is included as Appendix 1. The method will continue to be optimized and formed into a protocol through the month of November into December. The trials of the study will begin in December and extend into March. Data analysis and report writing will take place March through April with the conclusion of the project occurring in May.

There are some risks associated with this project. This is the first time these vasoactive agents are being administered to mice, and the appropriate doses are not established. We will address this by running practice experiments to determine the correct dose to observe a response without reaching a lethal dose to the mouse. The topical agents will vary slightly from clinical practice as it will be difficult to keep the Tegaderm® in place covering the area that had the agent applied. This will possibly shorten the time the agent is able to work on the area. The clinical practice is to apply the agent and cover with Tegaderm® before sending the patient home for several days. Then upon patient return the Tegaderm® is removed and the area is observed for response. However, in clinical practice generally by the time the area is checked no response is a good response, meaning as long as the area is not rejecting the transplant or the reconstruction looks to be failing then the agent worked. In this way, it will be more of an advantage to observe the mouse ear area closer to application to witness a response.

Additionally, the mouse ear is much smaller than the clinical area generally worked on, so the difference in application exposure time might not result in significant barriers for drawing analogous conclusions.

Statistical analysis will be done to evaluate the vessel diameter change in the tested ear versus the control and determine whether there is statistical significance in the results or if the method needs to be further optimized. As each mouse will have a tested ear and a control ear a t-test will most likely be run to determine the statistical significance. Statistical analysis may also be run to evaluate how consistent our data collection is for each drug through every trial.

V. APPENDIX

Appendix 1: Timeline

Appendix 2: Budget

Budget Item	Total Cost
Isoflo/O2	$63
Lidocaine	$70
Nitroglycerine	$32
Diltiazem	$26
Mice	$200
Mice Housing	$594
Tegaderm®	$25
Aqua Gel	$13
Applicators	$6
Gloves	$15
Total	$1,044

Appendix 3: Team

Jillian Bracaglia, BME student

Julia Riedy, BME student

Dr. Patrick Cottler, Capstone Advisor

Dr. Christopher Campbell, Capstone Advisor

Price Lab

UVA MR5 Vivarium

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STS Prospectus

What is genetic engineering? Why does it matter? Genetic engineering is one of the many responses from the scientific community to the significant problem of genetic diseases and mutations that lead to various health issues. This prospectus will explore genetic engineering, the implications of patient specificity and human diversity through a technical example, and the technical and social dimensions of both genetic engineering and human diversity.

Before genetic engineering can be discussed, its foundation must be explained. Humans are made up of cells, every cell has DNA, and DNA is what makes our body function. DNA is made up four core molecules that form sequences carrying genetic instructions for development, functioning, growth, and reproduction of all known organisms. The order of our DNA what makes us different from every other person. DNA sequences are unique to each person, however certain things need a specific set up to work properly.

Genetic engineering is defined as the direct manipulation of an organism's genes, in other words DNA, to alter an organism's characteristics in a particular way ("What is genetic engineering?," n.d.). There are various applications of genetic engineering, such as scientific research, agriculture, technology and medical treatments. One avenue of genetic engineering in scientific research is genetically engineering organisms to discover the function of specific genes("Genetic Engineering Products | Boundless Microbiology," n.d.). In agriculture, genetic engineering has been utilized to improve the resilience and nutritional value of crops like potatoes, tomatoes, and rice.

Gene therapy is a potential medical treatment and is defined as introducing DNA into a patient to treat a genetic disease. One treatment method is to introduce new DNA that contains the sequence for a functioning gene that will correct the effects of a disease-causing mutation,

such as cystic fibrosis(“Gene therapy—Mayo Clinic,” n.d.). There are two types of gene therapy, somatic gene therapy and germline gene therapy. Somatic gene therapy is gene therapy that affects cells of the body that don’t produce sperm or eggs, meaning the effects of the gene therapy will not be passed down to the patient’s children. Germline gene therapy affects the cells that produce sperm or eggs, and therefore will pass effects down to the patient’s children and subsequent generations. Another treatment method is genome editing. Genome editing entails making specific changes to the DNA of a cell or an organism and this is done by using an enzyme, which is a biological molecule that can significantly speed up processes. The enzyme cuts the DNA sequence at a specific point and this cut is then repaired by the cell the DNA is in. The specific DNA section that is cut can be chosen to coincide with an error or mutation in the DNA sequence, so when this section gets cut the repair of the cut also results in the correction of the error or mutation that was previously there.

This area of science and medical research is promising for a number of reasons, but one significant reason is the opportunity genetic engineering provides to create medical treatments that are specific to an individual. Every human being is different, with different medical histories, different environments and vocations lived and worked in. These individual details can become important in certain health issue scenarios. Another significant promising reason is genetic engineering’s potential to treat diseases before they occur, or for example stop cancer before it grows. Our current state of medicine is reactively treating issues as they are discovered by symptoms. Often treatment is focused on the downstream events that follow some sort of malfunction of our bodies, not on fixing the original malfunction. Genetic engineering can be a major step forward in the future for proactively treating issues by altering genetics to fix the malfunction before it creates damage.

The details that make a human unique, from a large picture perspective down to the microscopic and nanoscopic level, are of high importance for many reasons. Human diversity is important for many reasons, again on many different levels. Keeping this in mind is essential when considering the technical and social dimensions of genetic engineering.

From a social dimension perspective, human diversity plays a key role in looking towards the future of genetic engineering. The regulations and public opinion of genetic engineering and its effect on human diversity will either be guidelines protecting diversity or barriers blocking damage to diversity as well as potential medical progress.

Genetic engineering is currently only available in clinical trials as a medical treatment. However, in the future the availability and the boundaries of genetic engineering will need to be carefully examined. Conditions such as cost of gene therapy, insurance coverage, germline or somatic effect, and definition of criticality will all factor into how society progresses.

The ethical implications of genetic engineering are widespread and significant. I believe genetic engineering can make a very positive impact throughout the world, but it will require heavy discussion and consideration of the ethics of how it's implemented. I believe the consideration should begin with acquiring as much information as possible, evaluating many alternative actions through various ethical approaches, testing scenarios in each of these approaches, and reflecting on the possible outcomes.

I have begun gathering information, but the questions I would like to focus more on include: What are the current regulations? How has it been applied so far? Where can it go from here? Which diseases/issues can it target or should it target? What diseases would be most benefited? What would the costs associated with treatments be? Would cost lead to inequality or lack of availability? How would insurance handle these cases? Are there proposed future

regulations from the government? Would government or insurance firms define necessary criticality? Would it become standard like vaccines or be elective? What are the ethical considerations for both somatic cell line altering and germ line altering? Who are the stakeholders, who would act as barriers, who would be big influences? Are there belief systems that would go against it?

The approaches I will use and have begun using to consider this are the five ethical approaches proposed by Gorman and Werhane. These approaches are the utilitarian approach, the rights approach, the justice approach, the common good approach, and the virtue approach.

Through using the utilitarian approach, I will consider which option will produce the most good and do the least harm. The option that may produce the most good is allowing any genetic altering ranging from elective to necessary for survival. However, then further consideration is needed for who will have access. For this to truly produce the most good, this should be covered by all insurance firms, fully allowed by the government, and fully available for everyone. This then leads me to consider the rights approach and whether or not this option best respects the rights of all who have a stake. Allowing anyone to have any kind of genetic altering done may not respect the scientists who created the method to alter a specific trait or disease if they cannot be compensated in some way. But if genetic altering followed the price trend cancer therapies have created, the cost of genetic therapy could create barriers for lower income patients. From a justice approach, this option may not treat people equally or proportionately. The common good approach and the virtue approach could also provide new perspectives to consider this topic from. I plan on answering more of the questions I have posed, considering more options from each of these approaches, and creating more scenarios to evaluate the possible outcomes. These next steps from this prospectus will lead to a well-rounded thesis

that considers many potential future scenarios to prepare for as genetic engineering advances in science and moves closer to implementation in our medicine.

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Fig. 5. In vivo imaging of microvasculature in mouse ear. (A) MAP image... (n.d.). Retrieved from ResearchGate website: https://www.researchgate.net/figure/In-vivo-imaging-of-microvasculature-in-mouse-ear-a-MAP-image-of-a-05-mm-12-mm-area_fig5_257533909

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