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The effect of various chemical factors on angiogenesis in the chick chorio-allantoic membrane

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THE EFFECT OF VARIOUS CHEMICAL FACTORS ON ANGIOGENESIS IN THE

CHICK CHORIO-ALLANTOIC MEMBRANE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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LIST OF ABBREVIATIONS

CAM = Chorio-Allantoic Membrane

NO = Nitric Oxide

O_2 = Oxygen

PO_2 = Partial Pressure of Oxygen

CO = Carbon Monoxide

CO_2 = Carbon Dioxide

H_2O_2 = Hydrogen Peroxide

SNAP = A Nitric Oxide Donor (S-Nitroso-N-acetyl-DL-penicillamine)

L-NAME = A Nitric Oxide Synthase Inhibitor (N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride)

PBS = Phosphate Buffered Saline

$CaCl_2$ = Calcium Chloride

RBC = Red Blood Cell

VEGF = Vascular Endothelial Growth Factor

VEGFR2 = Vascular Endothelial Growth Factor Receptor

D_f = Fractal Dimension

GPCR = G-protein Coupled Receptor

AT_1 = Angiotensin II Receptor

ACE = Angiotensin Converting Enzyme

NOS = Nitric Oxide Synthase

eNOS = Endothelial Nitric Oxide Synthase

HIF-1 = Hypoxia Inducible Transcription Factor 1

cGMP = Cyclic Guanosine Monophosphate

MW = Molecular Weight (grams per mole)

mm = Millimeters

µm = Micrometers

mmHg = Millimeters of Mercury

ABSTRACT

THE EFFECT OF VARIOUS CHEMICAL FACTORS ON ANGIOGENESIS IN THE CHICK CHORIO-ALLANTOIC MEMBRANE

By Heather Amanda Hammond, BS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2012

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The chick chorio-allantoic membrane (CAM) contains a complex vascular network commonly used to study angiogenesis. The application of chemical factors and oxygen barrier films onto this tissue can easily influence the process of angiogenesis. In this study, oxygen barrier film patches (Krehalon, polyvinylidene chloride, 12 μm thick, O_2 transmission rate = 2.19 $cm^3 \cdot ml/100\ in^2 \cdot day \cdot atm$) were applied to areas of the CAM. Holes were made in the film and alginate beads incubated in various chemical factors were placed in the holes. After 24 and 48 hours of exposure to the alginate beads, images were

taken of the tissue using a stereomicroscope and then processed using ImageJ software (from the National Institue of Health (NIH)). The images were analyzed with the Fractal Analysis plugin of ImageJ (also from NIH) using four parameters. These parameters are the number of vessel segments, the number of vessel bifurcations, the total length of the vessels, and the complexity of the vascular network. From these parameters, the chemical factors can be identified as promoting angiogenesis (pro-angiogenic), inhibiting angiogenesis (anti-angiogenic), or having no effect on angiogenesis (not angiogenic). For the angiogenic beads, significant results were found in at least one of the four parameters. SNAP and H_2O_2 gave pro-angiogenic responses while Angiotensin II, Losartan, and Adenosine were anti-angiogenic. To test the effect of an oxygen barrier film patch on angiogenesis, images were taken of the tissue under the film patch (virtual holes) and holes exposed to atmospheric oxygen. Analysis of the virtual holes compared to the control holes gave significant results for several of the film patches. These film patches are distinguished by the chemical that was tested on each of the films. The virtual holes containing Angiotensin II, Losartan, Adenosine, and H_2O_2 gave pro-angiogenic results while SNAP and L-NAME virtual holes were anti-angiogenic. Thus, the chemical factors and the oxygen barrier film patches did have an effect on angiogenesis in the CAM.

INTRODUCTION

Overview of the Cardiovascular System

The cardiovascular system is made up of three major components: the heart, blood, and blood vessels. The heart pumps blood through blood vessels to supply the body with nutrients such as oxygen (O_2) and to get rid of wastes like carbon dioxide (CO_2). Deoxygenated blood enters the pulmonary circuit from the right side of the heart and this blood enters the lungs where CO_2 is released from and O_2 is taken up by the blood. The oxygenated blood is then pumped through the systemic circuit by the left ventricle of the heart. This blood supplies O_2 to the body and transports CO_2 and other wastes back to the heart where the cycle repeats. Figure 1 shows a diagram of this cycle.

Angiogenesis

The formation of the blood vessels of the cardiovascular system is called angiogenesis. The way in which these blood vessels form is unique and complex. Blood vessels grow in response to chemicals in their immediate environment. Some chemicals are pro-angiogenic, promoting blood vessel growth, while others are anti-angiogenic, inhibiting blood vessel growth. Depending on which chemicals are present, blood vessels will lengthen and bifurcate into a distinct pattern.

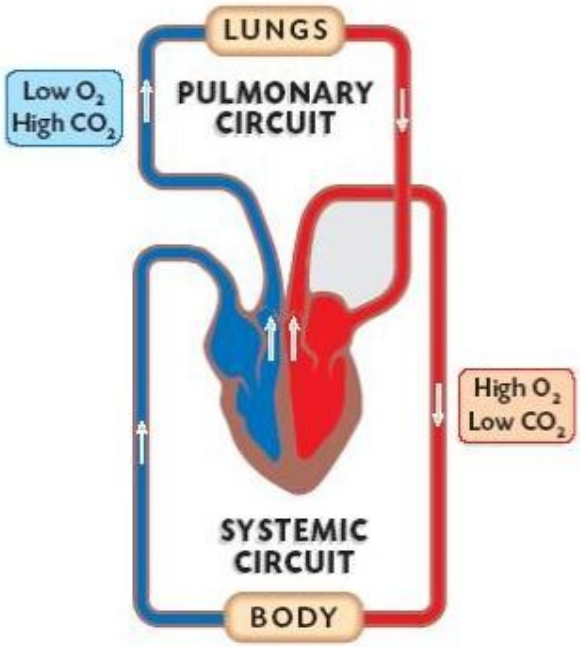

Figure 1. Diagram of Pulmonary and Systemic Circuit of Blood Flow (modified from http://leavingbio.net/CIRCULATORY%20SYSTEM/CIRCULATORY%20SYSTEM. htm)

The dynamic process of angiogenesis continues throughout life. Stimulating angiogenesis can have therapeutic value in a number of diseases such as ischemic heart disease or in wound healing. Excess or uncontrolled angiogenesis can also be detrimental to the body by contributing to diseases such as retinopathy or by facilitating tumor growth (Carmeliet 2003, Carmeliet 2005). Drugs that promote or inhibit angiogenesis are currently being developed, especially those that can be used in cancer treatments (Folkman 2007).

The mechanism of angiogenesis is well studied. According to Adair and Montani (2011), in sprouting angiogenesis, the tip of a developing capillary will move through the extracellular matrix towards a pro-angiogenic chemical such as VEGF (Vascular Endothelial Growth Factor), see Figure 2. The cell on the tip of the developing capillary, a tip cell, secretes proteolytic enzymes to digest the extracellular matrix so that the capillary can move through it. VEGF receptors are present on the filopodia of the tip cells so that the tip cells can distinguish differences in the concentration of VEGF and follow along the highest gradient of VEGF. Actin filaments in the filopodia pull the tip cell towards the VEGF as the endothelial stalk cells proliferate, elongating the capillary. The tip cells of two or more capillaries converge and fuse together to create a lumen through which blood can flow. Microvascular pericytes are then recruited to the site to stabilize the maturing capillary.

The VEGF concentration gradient is crucial for tip cell migration and stalk cell proliferation. As shown in Figure 3, there is not only a gradient for VEGF but also a gradient of the VEGF receptor (VEGFR2). There are more VEGF receptors on the tip

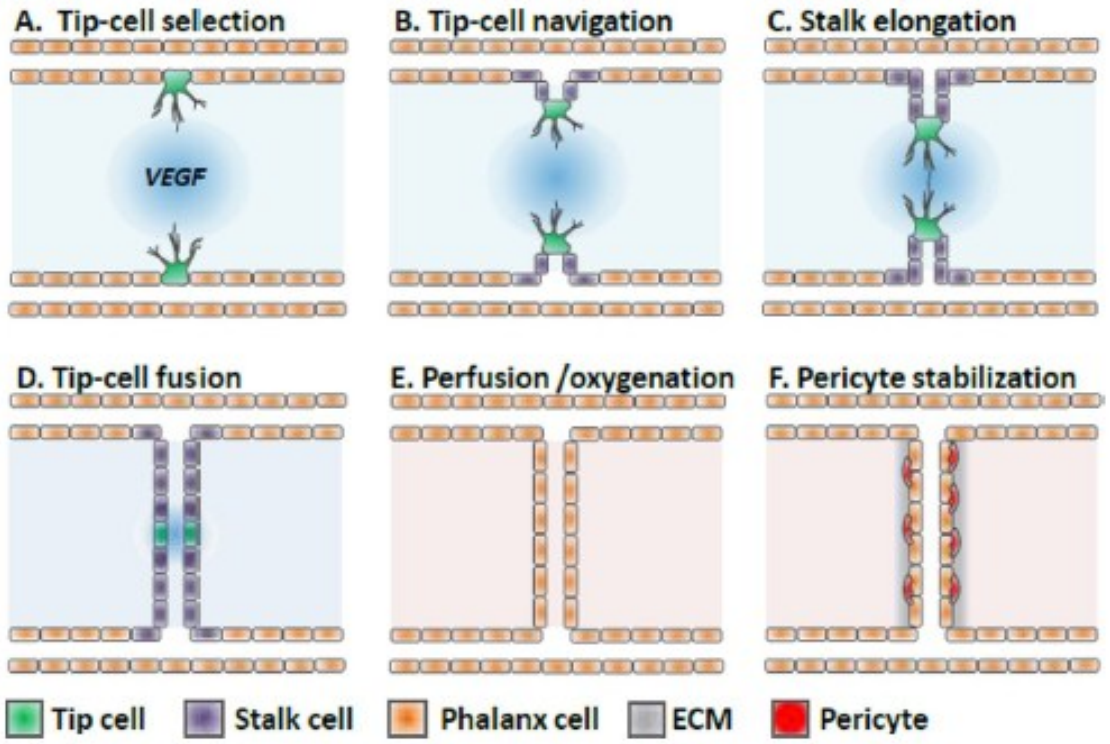

Figure 2. Mechanism of Angiogenesis (Adair and Montani 2011).

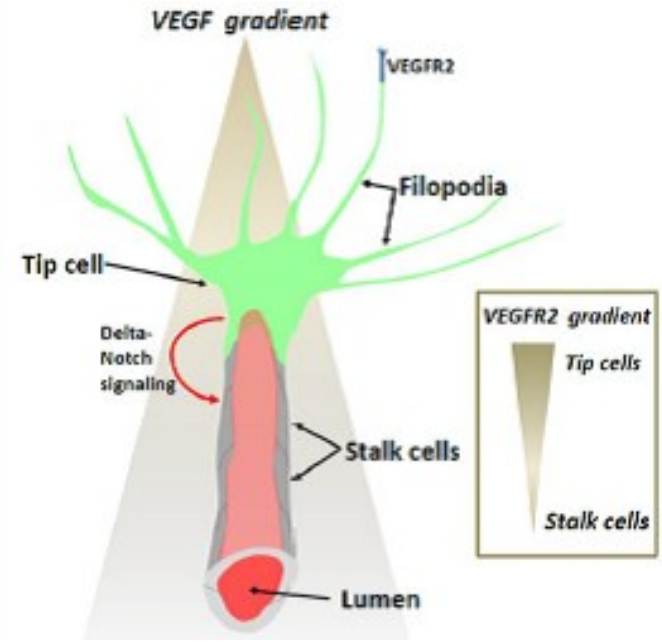

Figure 3. VEGF Receptor (VEGFR2) Gradient (Adair and Montani 2011).

cells than on the stalk cells so that the tip cells can respond to the high concentration of VEGF that they are migrating towards. Delta-Notch signaling also occurs. This ligand-receptor interaction only occurs when the cells are in direct contact. Delta is produced in tip cells and finds its receptor, Notch, in the stalk cells. The activation of Notch then induces proteolysis of one of Notch's domains. This domain can then move into the nucleus to suppress expression of the VEGF receptor in the stalk cell. Migration of the stalk cells is then prevented.

Another recently discovered form of angiogenesis is termed intussusceptive or splitting angiogenesis as described in Adair and Montani (2011). Its mechanism is not completely understood, although it is known that it involves the splitting of existing blood vessels to form new blood vessels. The vessel wall extends into the lumen causing the single vessel to split into two vessels forming artery and vein bifurcations (Adair and Montani 2011).

Chorio-Allantoic Membrane (CAM)

The chorio-allantoic membrane (CAM) of a developing chick embryo has been widely used as a model system for *in vivo* research on angiogenesis (Auerbach et al. 2003, Ribatti et al. 1996, Ribatti 2012, Zijlstra et al. 2006). Use of the CAM preparation is a simple, reproducible, reliable, and low cost approach to investigate the growth of blood vessels. The CAM of the developing chick embryo is easily accessible and can be manipulated once the shell of the egg has been broken. Many changes occur in the embryo

over the course of only a few days. These morphological and functional changes can easily be evaluated. The dense capillary network of the CAM is an ideal model to visually observe and track angiogenesis. Recently, the chick CAM has been used to look at angiogenesis in tumors (Deryugina and Quigley 2008) and also as an indicator of anti- or pro-angiogenic properties of chemicals (Deryugina and Quigley 2008).

However, there are some disadvantages in using the CAM to study angiogenesis. Since the embryo is developing at a rapid rate during the early stages of its incubation, it is difficult to distinguish the effects of test substances on the CAM from that of real neovascularization. Also, existing vessels may change their orientation or location as the CAM develops (Ribatti 2012). Because of this, it is important to establish control sites in the vasculature that can be evaluated without influence from the substances being tested.

The CAM is essential for viability of the developing chick embryo. The CAM first becomes visible on day six of incubation and continues to grow as the chick develops. The rich vascular network of the CAM mainly functions as a gas exchange organ for the developing embryo. The CAM develops against the shell so that it is in close contact with the external environment through pores in the shell. Because of its location, the CAM can provide O_2 to the embryo and remove excess CO_2 by diffusion through the pores of the shell. The CAM can also provide calcium ions to the embryo by absorbing calcium from the shell and transporting it through the bloodstream. It also functions as a reservoir for excretory products such as urea, uric acid, and ammonia. After the chick hatches, the CAM is left attached to the shell (Bellairs and Osmond 2005).

Ex ovo model of the CAM

Two main approaches exist for using the CAM as a model system. An in-shell approach is used when only a portion of the developing embryo is needed. Only part of the shell is removed and the embryo is left to develop inside the shell. Tissue graft experiments typically use this in-shell model (Zijlstra et al. 2006). The shell-less or *ex vivo* model can be used when it is necessary to manipulate a larger part of the embryo during the course of the experiment (Auerbach et al. 1974, Dohle et al. 2009, Fisher 1993, Palen and Thorneby 1975). The entire embryo is removed from the shell and left to develop *ex ovo*. Without the shell, the developing embryo has no protection from the outside environment so it is more prone to bacterial contamination and hyperoxic conditions. Long-term viability is low. Even with these disadvantages, the *ex ovo* model is used extensively because the embryo can be manipulated (Auerbach et al. 2003, Jeong et al. 2011, Magalhaes et al. 2010).

Chick Circulation

Chick red blood cells (RBCs) are different from human red blood cells in that they are much larger and their nucleus remains after they have matured. In chick embryos, primitive RBCs which express primitive hemoglobin are present until day six of development when definitive RBCs can be found. Definitive RBCs express adult hemoglobin. Embryonic blood contains different types of primitive and adult hemoglobin with different oxygen binding properties (primitive hemoglobin has a lower oxygen

binding affinity than adult hemoglobin). It is not known what determines when the embryo switches from embryonic hemoglobin expression to adult hemoglobin expression. Evidence indicates that nuclear transcription factors may regulate the switch or the change could be in response to ambient oxygen pressure changes (Baumann and Meuer 1992).

Hematopoiesis occurs in the yolk sac of the embryo throughout its development (bone marrow hematopoiesis is established late in development). The yolk sac grows over the surface of the yolk and blood vessels develop connecting the yolk sac with the heart of the embryo. Blood cells first begin to circulate on day two of incubation, driven by a primitive U-shaped heart. The heart pumps the blood cells through arteries that supply both the vitelline and the allantoic circulation. The vitelline circulation is responsible for supplying nutrients from the yolk to the developing embryo and, up to day six of incubation, it functions as the respiratory gas exchange organ. Around day six of incubation, the allantoic sac fuses with the chorion and respiratory function is transferred to the blood vessels of the CAM (Baumann and Meuer 1992, Baumann and Dragon 2005).

Oxygen is important to the developing embryo, but may not be essential during its initial development. Experiments have shown that four day old chick embryos can survive in short-term incubation with 1% carbon monoxide (CO) over four hours, but for six day old chick embryos mortality to this CO exposure is increased to 30%. Also, if a major blood vessel is ligated for a few hours in three and four day old embryos so that oxygen transport is impaired, there is no alteration in oxygen consumption or embryonic development. But when four day old chick embryos are kept under hypoxic conditions (13.5% ambient oxygen), their growth is retarded by day six. Once a closed circulatory

system has been established (day 5-6), the chick dependence on O_2 is increased (Baumann and Dragon 2005). Other effects of oxygen on RBCs have been extensively studied (Khorrami et al. 2008, Phu et al. 1986, Tazawa et al. 1976).

Analyzing Angiogenesis

Angiogenesis can be quantified at the microscopic level (Adair et al. 1994, Strick et al. 1991). Morphological effects on angiogenesis caused by various chemicals can be evaluated by observing magnified images of the blood vessels through a microsope. Pro-angiogenic chemicals should increase the vascularity of the affected region and one should observe an increase in the number of vessels segments, the number of vessel bifurcations, the total length of the vessels, and the complexity of the vascular network (i.e., how much of the available space is taken up by the new vessels). Anti-angiogenic chemicals should have the opposite effect on these parameters. Blood vessel growth would be decreased with anti-angiogenic chemicals. Chemicals which are angiogenic-neutral should have no effect on these parameters when compared to a similar control region that does not contain the chemical in question.

Purpose of This Study

The purpose of this study was to establish a reproducible method to analyze the effect of different chemicals on angiogenesis in the chick CAM. This approach could

become a future screening process for how different chemicals would affect angiogenesis in the CAM model. Also, the effect of the presence of an oxygen barrier film patch on CAM angiogenesis was evaluated for future investigations of oxygenation through measurements of PO_2 (partial pressure of oxygen).

The chemicals used for these experiments are referred to as "potential angiogenic factors" throughout this thesis because their effect on angiogenesis was being evaluated. These angiogenic factors could either have a pro-angiogenic, anti-angiogenic, or no angiogenic effect on new vessel growth in the CAM. The angiogenic factors tested were angiotensin II, captopril, losartan, L-NAME, nitric oxide (through use of the NO donor SNAP), acetylcholine, adenosine, and H_2O_2. These particular chemicals were chosen for testing because of their known involvement in vasomotor responses and their potential linkage to blood flow responses following reductions in oxygen supply to oxygen demand.

The *ex ovo* chick embryo model system was used because of its simplicity and extensive vascular network. The effect of the angiogenic factors could easily be assessed by observing the blood vessels microscopically. The quantitative assessment of vascularity was based on measurements of the number of vessel segments, number of vessel bifurcations, total vessel length, and complexity of the vascular network as reliable indicators of the degree of angiogenesis.

MATERIALS AND METHODS

Chick Chorio-Allantoic Membrane (CAM) Preparation

Collection and pre-incubation of eggs

Rhode Island Red fertilized hatching eggs were obtained from a local hatchery (Lynn Matz, Mechanicsville, VA). The eggs and the carton that contained them were placed in an insulated box for safe transport to the laboratory. Once they arrived at the laboratory, the eggs were cleaned by wiping them with 70% isopropyl alcohol, removing any debris. The cleaned eggs were then positioned vertically with the larger end up in a "pre-incubator" (Pro Series Circulated Air Incubator, Model 4200, Farm Innovators, Plymouth, IN, see Figure 4). At this point, the eggs were considered to be at Day 0 of embryonic development. Temperature in the "pre-incubator" was maintained at 37 ± 1^{o}C with humidity at approximately 60% for ideal incubation conditions (according to the Farm Innovators manual). A water reservoir in the "pre-incubator" was filled with distilled water to provide a humid environment; its level was checked and maintained daily.

Ex ovo plating procedure for eggs

After three days (Day 3) in the "pre-incubator," all of the eggs were removed one by one and placed in a clear, clean egg tray, keeping them in the same vertical position.

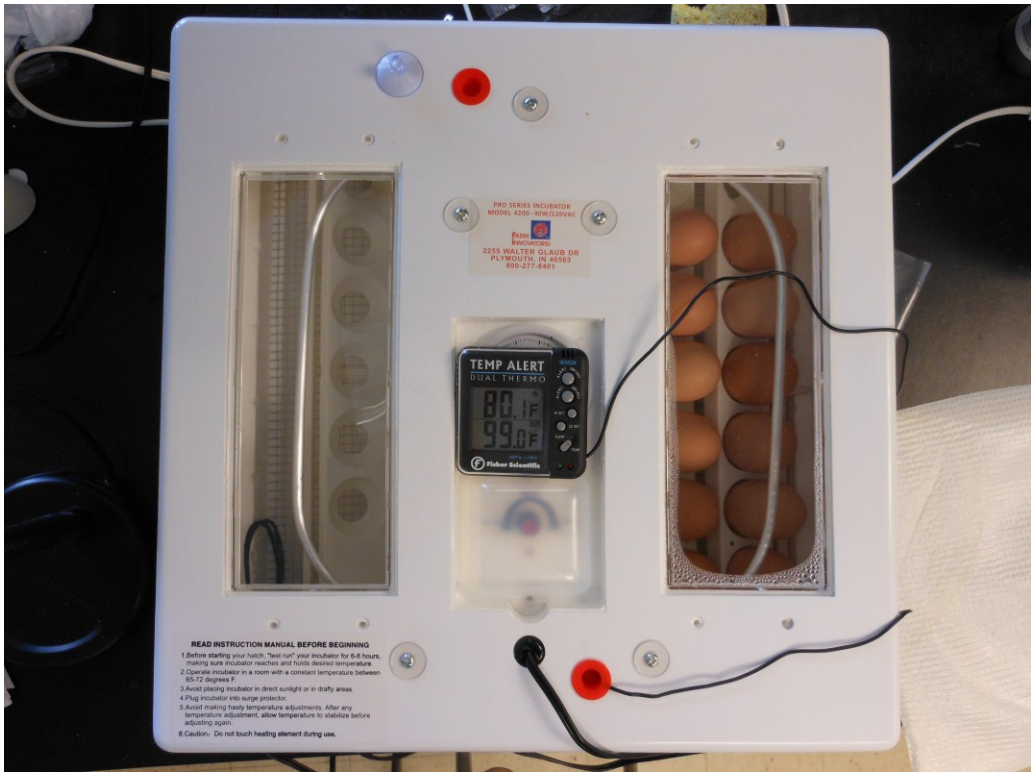

Figure 4. "Pre-incubator." Top view of "pre-incubator" containing one dozen eggs.

The eggs were then individually sterilized with Betadine (providone-iodine 10%, Purdue Frederick, Inc., Stamford, CT), applied liberally to the shell with a cotton ball, and placed back into the "pre-incubator." To avoid potential contamination of the contents of the eggs during the plating procedure, gloves and face masks were worn. Four eggs at a time were removed from the "pre-incubator" for manipulation in the plating procedure. An approximately six inch square piece of plastic film (Kirkland, stretch-tite plastic food wrap, Polyvinyl Films, Inc., Sutton, MA) was placed on a plastic ring (four inches in diameter and one inch high) and a hammock-shaped well was made to hold the contents of the egg. Any of the film that was previously exposed to air was discarded. An egg was cracked by holding its long axis horizontally and bringing it down firmly onto a razor blade which was stabilized by securing it to a countertop. A crack, approximately one centimeter in length and located about two-thirds the distance from the top/larger end, was needed for the liquid (white of the egg) to start leaking out. If no liquid appeared, the egg was rotated and cracked again; the liquid helped coat the broken shell, minimizing the risk of breaking the yolk as it flowed from the shell. The egg was then held approximately two inches over the plastic film hammock so that there was only a short distance the contents had to fall as it flowed from the shell. A thumb was then positioned on each side of the crack and the sides of the egg were slowly forced open. Just before the whole egg shell was about to collapse, the bottom of the shell was quickly forced apart to keep the yolk from breaking on the sharp edges of the broken shell. The contents of the egg were allowed to spill into the plastic film hammock. A "hammock" shape was used because it provided a flexible cushion for the embryo, thereby reducing the potential for physical harm to the embryo as

it was removed from the shell. After all of the contents of the egg were removed, the shell was discarded. At this stage, the embryo was observed to confirm its viability. Normally, a heartbeat was noticeable in the small embryo when the chick was alive. If there was no heartbeat, no visible embryo, or the yolk had spilled during the plating procedure, the embryo was discarded by placing it into a plastic bag and then into a freezer. If the embryo was alive, it was carefully manipulated until the embryo remained centered on top of the yolk.

Once the embryo had been successfully transferred into the plastic film hammock, the film was held against the plastic ring and the whole preparation was picked up and placed into a petri dish which had been modified to accommodate the contents of a fertilized egg. This modified petri dish (see Figure 5) had a 30 mm diameter hole drilled through the bottom of the dish and a small plastic cup (30 mm diameter x 25 mm height) was placed into this hole. The small cup was fastened to the hole in the petri dish with a hot glue gun and the edges were smoothed using #320 sandpaper. Six small holes (2.0 mm in diameter, equally spaced around the perimeter) were punched through its bottom. These holes were used to provide access to the film-encased egg contents when vacuum was applied to the cup to coax the yolk into the small cup. A few drops of K-Y lubricant (McNEIL-PPC, Inc, Johnson & Johnson, New Jersey) were applied to the petri dish and the cup and spread over their surfaces with a cotton swab to facilitate movement of the plastic film-encased yolk into the plastic cup when the vacuum was applied. A second petri dish had a matching hole drilled through it so that it could act as a stand to elevate the assembly on the countertop or other solid surface used in the various procedures.

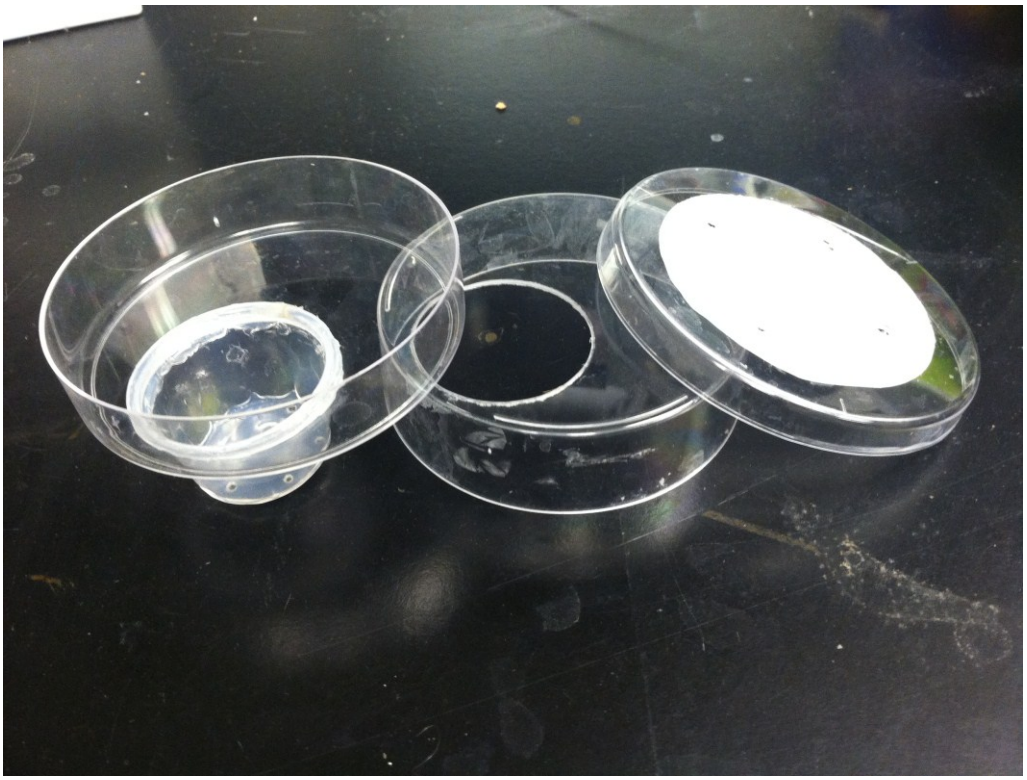

Figure 5. Petri Dish. Modified petri dish with stand and filter paper covered lid.

Also, a petri dish lid was modified by drilling five holes (about 5 mm in diameter) in it and by gluing a piece of filter paper to the inside of the lid (these were ventilation holes for the embryo).

After the embryo was placed in the petri dish, the edges of the plastic film were detached from the plastic ring and the plastic ring was removed. The edges of the plastic film were held and vacuum was applied to the bottom of the small cup. This forced the yolk along with the plastic film into the small cup. The plastic film hammock was manipulated so that the embryo remained on top of the yolk as the yolk was coaxed into the cup. Then 1.2 ml of a penicillin-streptomycin solution (penicillin = 6.67 mg/ml, streptomycin = 5 mg/ml) was applied to the white of the egg to prevent bacterial growth during further incubation. This solution was reapplied to the embryos every 3.5 days. The lid of the petri dish was placed on top of the petri dish and this assembly was then placed into a sterilized incubator (Model 12-140, Quincy Lab, Inc., Illinois, see Figure 6). The incubator was sterilized with soap and water and 70% isopropyl alcohol before introduction of a new batch of plated eggs. The incubator had been modified by placement of a fan for ventilation and a water reservoir to maintain humidity. Also, a motor with a controller was used to gently rock the platform intermittently so that the eggs would experience motion periodically. The motor was set to operate for a period of one minute every hour and a half. Temperature in the incubator was maintained at 37 ± 1 ^{o}C with humidity at approximately 60% for ideal incubation conditions (Dohle 2009). The temperature, humidity, and condensation level within the incubator were recorded each

Figure 6. Incubator

day. The water level of the water reservoir was checked daily and resupplied as needed with distilled water.

Experimental Protocol

Preparation of perforated film patches

Starting on Day 7 when the CAM first becomes visible, patches of gas barrier Krehalon film (Krehalon CB-100, polyvinylidene chloride, 12 μm thick, O_2 transmission rate = 2.19 $cm^3 \cdot ml/100\ in^2 \cdot day \cdot atm$; Krehalon UK, Ltd., United Kingdom) were placed on the developing CAM. The Krehalon patches were made by first placing Krehalon film between two pieces of thin filter paper. The paper, along with the film, was then cut into squares 15 mm on each side.

Five holes were placed in each film patch according to the pattern depicted in Figure 7. The holes were made using blunted hypodermic needles. The needle was dipped in 70% isopropyl alcohol prior to making the holes. The film/paper sandwich was placed on a piece of balsa wood and the needle was twisted through the paper and the film until it reached the wood to make circular holes. Residual pieces of paper and film were extruded from the inside of the needle using a metal rod which fit inside the needle. Table 1 shows the gauge and outside diameter of the needles that were used to make holes in the film.

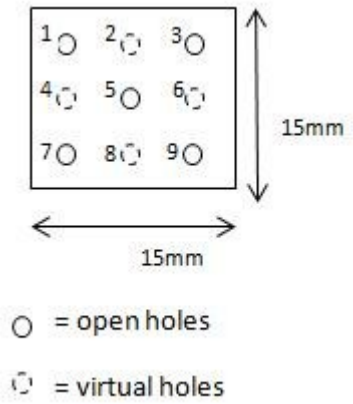

Figure 7. Diagram of Hole Placement in Film Patches.

Table 1. Needle Gauges and Diameter.

Needle size (gauge)		Outside diameter (mm)
25		0.5
20		1.0
14		2.0

Placement of perforated film patches

Film patches were placed on the CAM on Day 7, the day after the CAM first became visible. The patches were dipped in 70% isopropyl alcohol and allowed to air dry before application to the CAM. Fine-tipped Sharpie markers were used to place dots of different colors onto the dried film to facilitate identification of the transparent film patch on the CAM and to visualize the holes under a stereomicroscope. A black dot was placed on the upper left-hand corner, blue on upper right-hand corner, green on lower left-hand corner, pink on lower right-hand corner, and an orange dot on top of the hole in the center of the film patch in order to uniquely identify the holes once the film patch was placed on the CAM. Using forceps, three film patches were then placed on the CAM of one of the embryos. The exact orientation of the film patches and the distance from the center of the embryo were recorded on a data collection sheet (see Appendix A).

Preparation of angiogenic factors

The response of vessel growth to several different angiogenic factors was tested on the CAM preparation. These chemicals were prepared in phosphate buffered saline solution (PBS) at the concentrations listed in Table 2.

Preparation of alginate beads for delivery of angiogenic factors

A 3% solution of alginate (alginic acid sodium salt from brown algase, Biochemika, Sigma Aldrich, Norway) in PBS was made. Several drops of this highly viscous solution were then placed onto a microscope slide cover slip using a plastic

Table 2. List of Angiogenic Factors.

Agent	Formula	Formal Name	Conc.
Adenosine	$C_{10}H_{13}N_5O_4$		1 mM
Angiotensin II	$C_{50}H_{71}N_{13}O_{12}$		10 μM
Captopril	$C_9H_{15}NO_3S$	(*2S*)-1-[(*2S*)-2-methyl-3-sulfanylpropanoyl] pyrrolidine-2-carboxylic acid	0.2 mg/mL
Losartan	$C_{22}H_{23}ClN_6O$	(2-butyl-4-chloro-1-{[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl}-1*H*-imidazol-5-yl)methanol	0.1 mg/ml
Acetylcholine	$C_7NH_{16}O_2^+$		1 mM
SNAP (NO donor)	$C_7H_{12}N_2O_4S$	S-Nitroso-N-acetyl-DL-penicillamine	0.5 mM
L-NAME	$C_7H_{15}N_5O_4 \cdot HCl$	N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride	1 mM
H_2O_2	H_2O_2		1 mM

List of angiogenic factors, their formulas, formal name, and concentration in which alginate beads were incubated (concentrations were determined from previous applications of these chemicals).

pipette tip. Attempts were made to obtain drops of a similar size (~2 mm diameter) and shape. A solution of 10% $CaCl_2$ was then sprayed onto the beads to promote polymerization of the alginate. After about 10 minutes, the cover slips containing the beads were placed in distilled water for 20-30 minutes to remove any residual $CaCl_2$. The cover slips and beads were then placed in small weigh boats and 0.5 ml of the angiogenic factor was placed onto the cover slip, completely covering the beads. For the sham bead, 0.5 ml of PBS was used to cover the bead. The alginate beads were allowed to soak in the angiogenic factor or PBS for one hour. The coverslip containing the beads was removed from the weigh boat and the beads were then carefully placed onto the holes of the perforated film patches, one bead per hole. Three alginate beads with the same angiogenic factor were placed on each film patch at holes 1, 3, and 7 (see Figures 7 and 8). The sham bead was placed on hole 9. The middle hole of the film patch, hole 5, did not contain a bead, so that the tissue of the CAM below this hole was exposed to the ambient atmosphere of the incubator.

Collection of Images from CAM through Perforated Film Patches

Once the film patch and angiogenic factor had been on the CAM for 24 hours (Day 8), images were collected (images were again collected after 48 hours (Day 9)). The embryo was removed from the incubator and placed in an insulated "chicken chamber" for microscopic observation. The chamber was made from a styrofoam box with a clear plastic lid. A heating pad and fan inside the chamber maintained similar

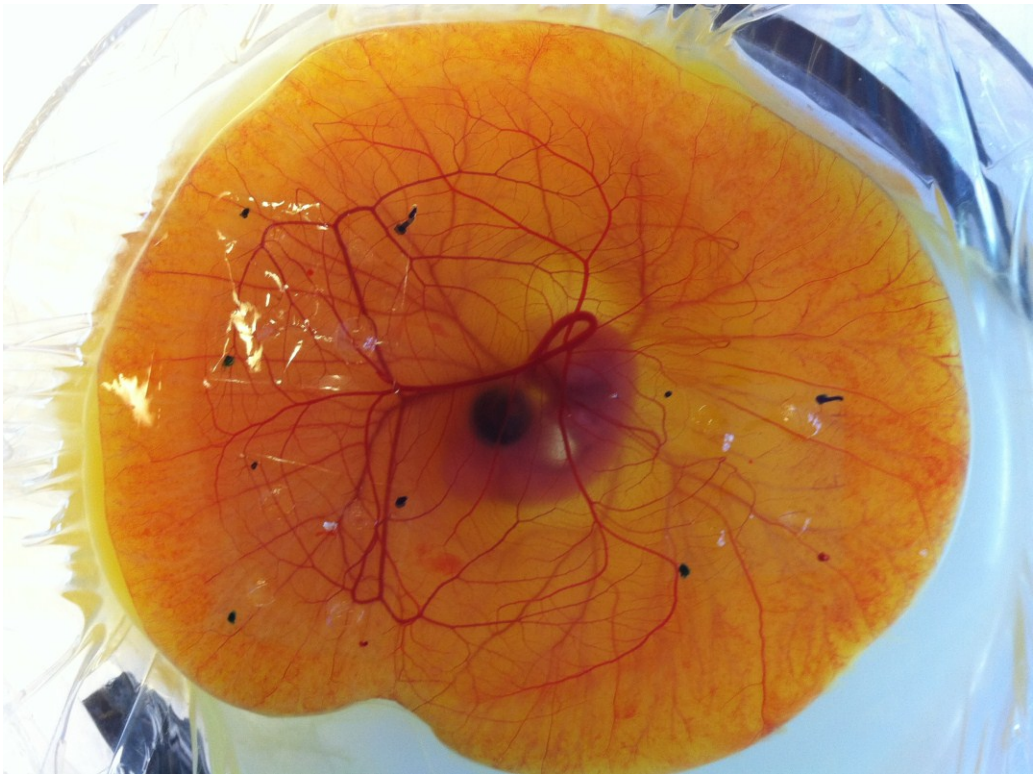

Figure 8. CAM Preparation. An example of perforated film patches with beads placed on a CAM preparation.

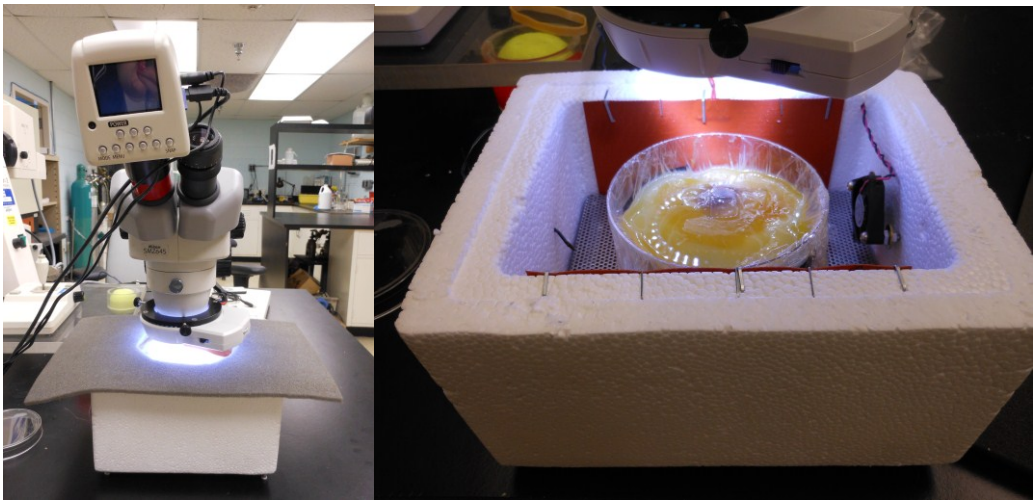

Figure 9. Image Collection. The stereomicroscope setup (left panel) and the "chicken chamber" (right panel) used to collect images of the CAM preparation.

environmental conditions as in the incubator (see Figure 9). The stereomicroscope (Nikon, SMZ645, 0.5X to 5X zoom range) was outfitted with a digital camera (Philips, model 3Z2000825, Philips Co.) in one of the eyepiece ports. The image from the camera was displayed on a laptop computer using Microcap software (version 2.0, ESPA Systems Co, Ltd., 2006). A perforated film patch from which images were to be collected was located on the CAM preparation and the desired hole in the patch was found using the location and specific colors of the dots that had been placed on the film with the Sharpie markers. The focused image was collected at 1X magnification. After image collection, approximately 10 µl of the angiogenic factor (distilled water for the sham bead) was applied to the bead in order to replenish the agent previously supplied by the bead to the region in the CAM under the hole.

Image Analysis of Vascular Networks

Images at 1X magnification were analyzed using the ImageJ 1.46m software platform (Rasband 1997-2011) from the NIH website (National Institutes of Health—http://imagej.nih.gov/ij, version 1.46m, Java 1.6.0_20 (64-bit)). This software allowed manipulation of the image in order to create a binary image which can be processed by the Fractal Analysis plugin of ImageJ (Karperien 1999-2007) (http://rsb.info.nih.gov/ij/plugins/fraclac/FLHelp/Introduction.htm, NIH, FracLac_2.5i.3).

A 0.95 mm diameter circle was created over the hole or virtual hole in the film. The vessels present in that circle could then be outlined in black using a brush tool (see Figure 10). The number of vessel segments and bifurcations were counted and recorded. From there, the Fractal Analysis plugin was used to calculate the number of pixels in the foreground of the image (the black outline of the vessels present) by a method known as "box counting." This method divided the region of interest into a grid of 12 square regions and then separated data in the foreground of the square from the background data. The number of pixels in the foreground is a measurement of the total length of the vasculature. This can be converted into μm by obtaining an image of a calibrated stage micrometer, drawing a 1.0 mm long black line, finding the number of foreground pixels along the 1.0 mm line, and converting this value to μm.

The complexity of the vascular network (fractal dimension, D_f) was also determined using the plugin. Equation 1 shows the equation for D_f, the fractal dimension of an image:

$$D_f = \log N_\varepsilon / \log \varepsilon \qquad \text{[Equation 1]}$$

where N_ε = the number of new parts, ε = the scale (Karperien 1999-2007). This equation compares the number of vessels per square of the grid and assigns it a number $1 \leq D_f \leq 2$. In this way, the complexity of the vascular network (i.e., how much space the vascular network occupies) is found. The more complex the network of vessels, the higher the D_f value (closer to 2) and the less complex the network, the lower the D_f value (closer to 1). See Appendix B for the detailed ImageJ and Fractal Analysis procedure.

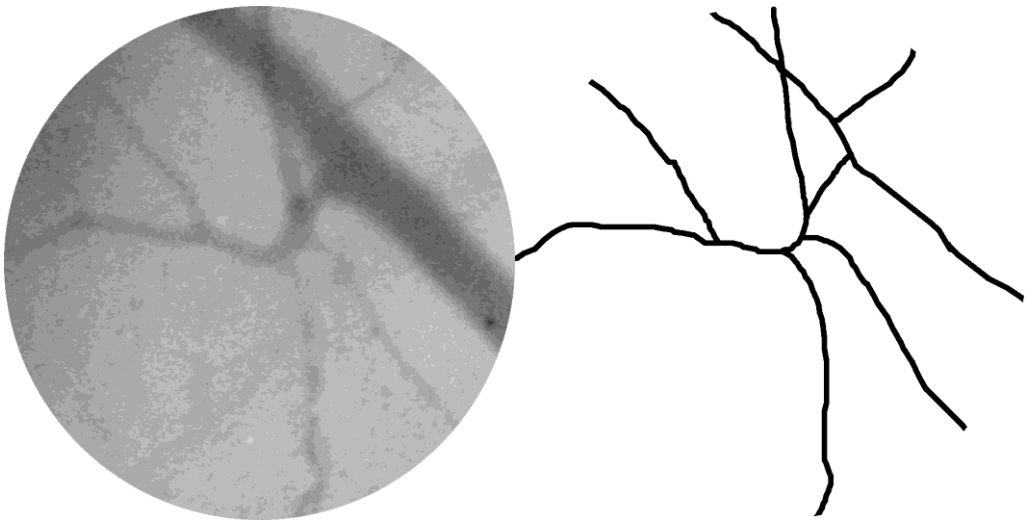

Figure 10. Image Analysis Using ImageJ. ImageJ was used to trace the outline of the vasculature (right panel) from the image collected from the CAM (left panel).

Statistical Methods

Comparisons between the angiogenic factors and the controls were analyzed using the two sample analysis (two tailed) Student's t-test. Differences were considered significant at $p < 0.05$. The degree of pro-angiogenesis or anti-angiogensis between the factors was not calculated, since the goal of this project was limited to determine whether specific chemicals had pro-angiogenic, anti-angiogenic, or no angiogenic effects on the CAM. Values presented in the text, tables, and graphs are the mean ± standard error.

RESULTS

Determination of Optimum Hole Size for Perforated Film Patch

In order to determine which size hole would give the best results in terms of vessel growth, three Krehalon film patches were placed on a seven-day old CAM preparation. Each film contained five holes of the same diameter (see Fig. 9 in the Materials and Methods section). The diameters of the holes were 0.5 mm, 1.0 mm, and 2.0 mm, respectively. Twenty-four hours after placement of the film patches, images of the tissue within each of three of the holes (holes 5, 7 and 9 in the square patch) were collected and analyzed using ImageJ (aliginate beads were placed on the other two holes, 1 and 3, and had no influence on the data collected). The number of vessel segments, number of vessel bifurcations, total vessel length, and the complexity of the vascular network (D_f), along with the standard error for each, are shown in Table 3. The mean of the number of vessel segments for the 0.5, 1.0, and 2.0 mm diameter holes was 17.0 ± 1.0, 25.3 ± 1.8, and 13.7 ± 2.2, respectively. The mean of the number of bifurcations was 7.0 ± 1.0, 11.7 ± 0.9, and 5.3 ± 1.3. Mean total vessel length was 699.9 ± 67.2, 662.3 ± 25.6, and 722.4 ± 84.2 μm and the mean complexity of the vessels was 1.313 ± 0.012, 1.383 ± 0.013, and 1.302 ± 0.030 for the 0.5, 1.0, and 2.0 mm diameter holes. These results are displayed in Figure 11. It can clearly be seen that the 1.0 mm diameter holes gave the most vessel segments and bifurcations and the most complex vascular network.

Table 3. Measure of Vascularity for Different Diameter Holes.

Number of Segments

Hole Diameter (mm)	Hole 5	Hole 7	Hole 9	Mean of Holes 5,7,9	Standard Deviation
0.5	15	18	18	17.0 ± 1.0	1.7
1.0	26	22	28	25.3 ± 1.8	3.1
2.0	18	11	12	13.7 ± 2.2	3.8

Number of Bifurcations

Hole Diameter (mm)	Hole 5	Hole 7	Hole 9	Mean of Holes 5,7,9	Standard Deviation
0.5	5	8	8	7.0 ± 1.0	1.7
1.0	12	10	13	11.7 ± 0.9	1.5
2.0	8	4	4	5.3 ± 1.3	2.3

Vessel Length (μm)

Hole Diameter (mm)	Hole 5	Hole 7	Hole 9	Mean of Holes 5,7,9	Standard Deviation
0.5	824.1	593.2	682.5	699.9 ± 67.2	116.4
1.0	658.1	620.3	708.5	662.3 ± 25.6	44.3
2.0	786.8	555.4	825.1	722.4 ± 84.3	145.9

Complexity (Df)

Hole Diameter (mm)	Hole 5	Hole 7	Hole 9	Mean of Holes 5,7,9	Standard Deviation
0.5 mm	1.336	1.306	1.296	1.313 ± 0.012	0.021
1.0 mm	1.400	1.390	1.359	1.383 ± 0.013	0.022
2.0 mm	1.320	1.242	1.343	1.302 ± 0.030	0.053

A comparison of the number of segments, number of bifurcations, vessel length, and complexity of three different sized holes (0.5, 1.0, and 2.0 mm diameter) placed in the film patch.

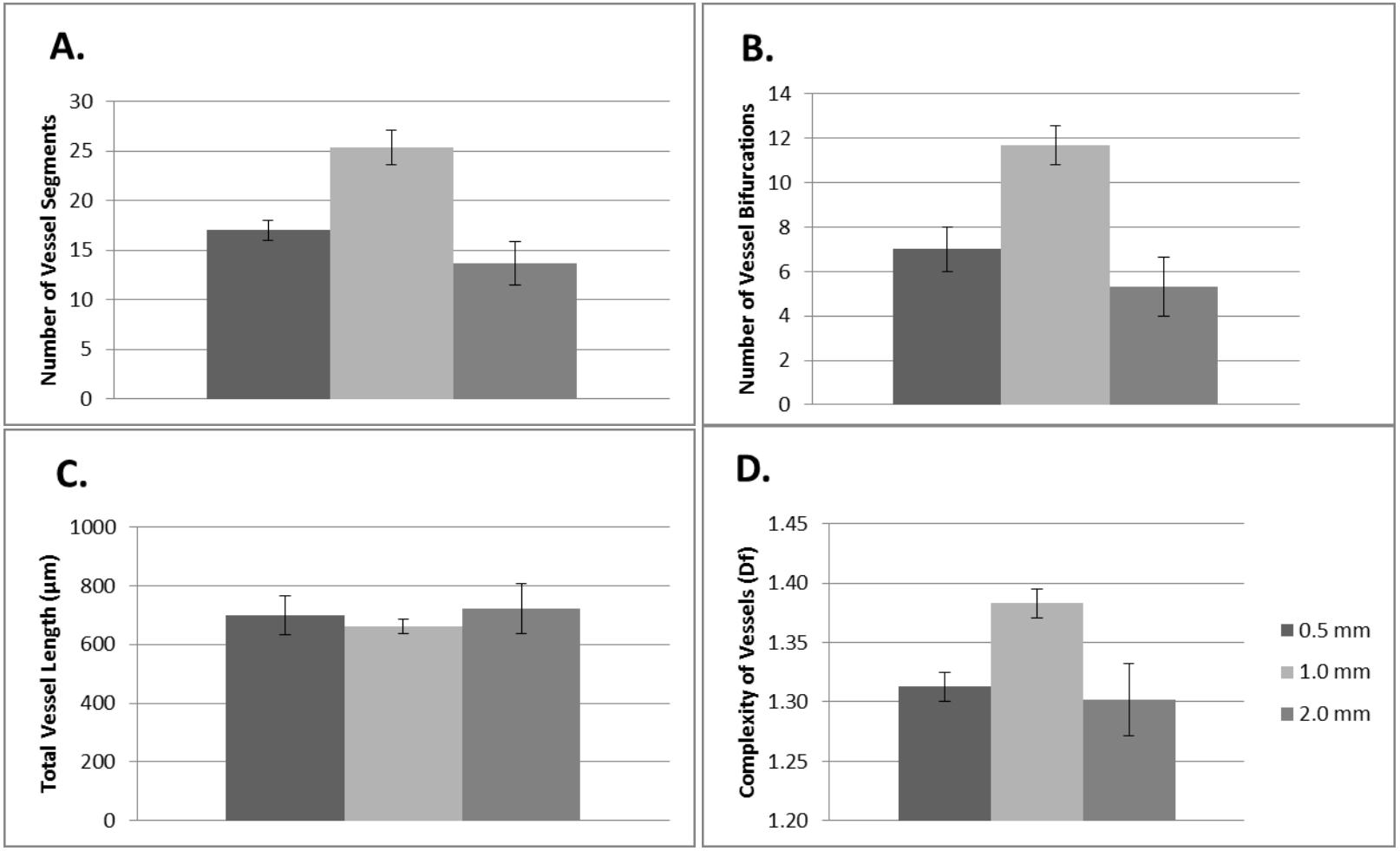

Figure 11. Vascularity as a Function of Hole Diameter. A visual comparison of the (A) number of segments, (B) number of bifurcations, (C) total vessel length, and (D) complexity of three different sized holes (0.5, 1.0, and 2.0 mm diameter) placed in the film patches. The legend is shown in Panel D.

So this size hole was used for the rest of the experiments, since it should provide the most extensive vessel growth.

Tests of Chemical Angiogenic Factors

After determining that the 1.0 mm diameter holes would give maximal results in the parameters to be tested, film patches with five 1.0 mm diameter holes were placed on seven-day old CAM preparations. Aliginate beads which had been incubated in a solution of angiogenic factor were placed on holes 1, 3, and 7 of the film patches and a sham aliginate bead (incubated only in PBS) was placed on hole 9 (see Figure 9 in the Materials and Methods section for hole numbering). The different angiogenic factors tested were Angiotensin II, Captopril, Losartan, L-NAME, H_2O_2, Acetylcholine, Adenosine, and Nitric Oxide (using the NO donor SNAP). Images of the holes were taken on day 8 and day 9 of incubation. These images were analyzed using the four parameters: number of vessel segments, number of vessel bifurcations, total vessel length, and complexity of the vascular network (D_f). The mean value and standard error were calculated by combining data from the three holes containing the alginate beads incubated in the angiogenic factor for both days 8 and 9. For the sham bead, the mean value and standard error were also calculated by combining data from days 8 and 9. Results for each of the factors tested are listed below and are shown in Tables 4-7 and Figures 12-15.

Number of Vessel Segments

The number of vessel segments for the agents tested ranged from 11.3 to 37.0. The angiogenic factors which produced statistical significance compared to the sham beads were Losartan, SNAP, Adenosine and H_2O_2. The mean values of number of vessel segments for the angiogenic beads and sham beads for each angiogenic factor are shown in Table 4 and Figure 12.

Number of Vessel Bifurcations

Table 5 and Figure 13 show the mean values for the number of vessel bifurcations for each angiogenic factor. The range of the number of vessel bifurcations was 4.5-17.8. Losartan and Adenosine exhibited significant differences compared to the sham beads.

Total Length of Vessels

The range of values of the total length of vessels for alginate beads incubated in different angiogenic factors and the sham beads were 569.7-987.3 μm. Table 6 and Figure 14 show the results for this variable. Significant differences compared to the sham beads were found for Angiotensin II, Losartan, SNAP and Adenosine.

Complexity of Vascular Networks

For the complexity of the vascular network, Table 7 and Figure 15 show the mean values for each agent tested. The range for this parameter was 1.245-1.437. Losartan, SNAP and Adenosine gave significant differences compared to the sham beads.

Table 4. Number of Vessel Segments for Each Angiogenic Factor (Days 8 and 9).

Number of Segments—Days 8 and 9

Angiogenic Factor	Mean results of Days 8 and 9
Angiotensin II	16.3 ± 4.2 (6)
Sham	13.0 ± 0.5 (2)
Captopril	18.0 ± 2.3 (6)
Sham	17.5 ± 3.5 (2)
Losartan	11.3 ± 1.1 (6)*
Sham	23.5 ± 5.5 (2)
L-NAME	13.7 ± 1.5 (6)
Sham	12.5 ± 4.5 (2)
SNAP	37.0 ± 6.6 (6)*
Sham	23.0 ± 12.0 (2)
Acetylcholine	17.5 ± 4.2 (6)
Sham	22.5 ± 0.5 (2)
Adenosine	20.8 ± 1.9 (6)*
Sham	31.0 ± 5.0 (2)
H_2O_2	18.0 ± 2.7 (6)*
Sham	11.5 ± 4.5 (2)

Mean number of segments for each angiogenic factor and its corresponding sham bead from Days 8 and 9. The results are presented as mean ± standard error. The number in parentheses indicates how many different beads were included in the mean values. * indicates significance according to Student's t-test between the angiogenic factor and its sham bead.

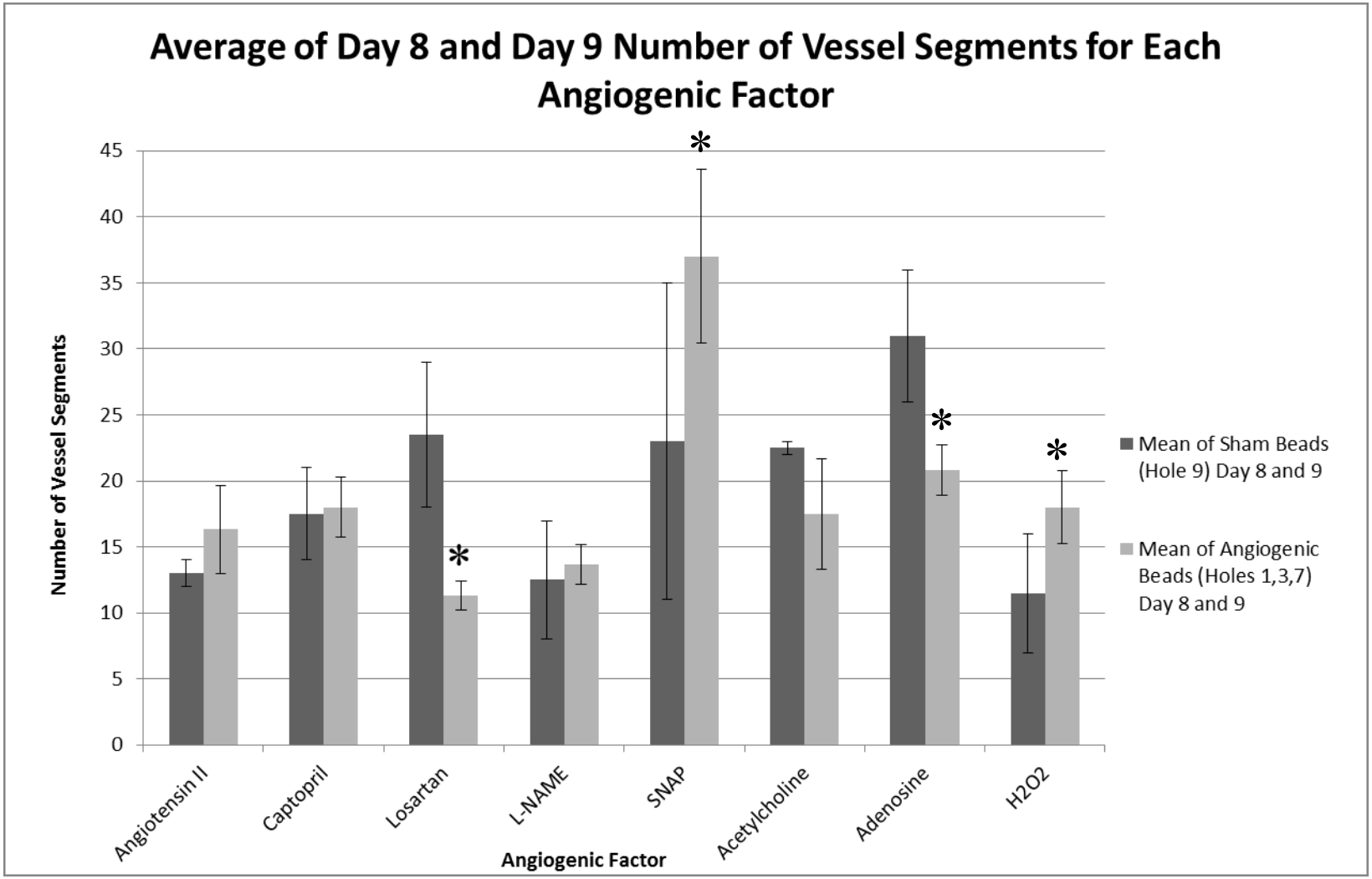

Figure 12. Graph of the number of vessel segments for the beads containing the angiogenic factor and the sham beads (Day 8 and 9). * indicates significance according to Student's t-test between the angiogenic factor and its sham bead.

Table 5. Number of Vessel Bifurcations for Each Angiogenic Factor (Days 8 and 9).

Angiogenic Factor	Mean results of Days 8 and 9
Angiotensin II	6.8 ± 1.3 (6)
Sham	5.0 ± 1.0 (2)
Captopril	8.0 ± 1.2 (6)
Sham	7.0 ± 3.0 (2)
Losartan	4.5 ± 0.7 (6)*
Sham	9.5 ± 2.5 (2)
L-NAME	5.5 ± 0.8 (6)
Sham	5.5 ± 2.5 (2)
SNAP	17.8 ± 3.6 (6)
Sham	11.0 ± 6.0 (2)
Acetylcholine	7.2 ± 2.4 (6)
Sham	9.5 ± 0.5 (2)
Adenosine	9.0 ± 1.0 (6)*
Sham	14.5 ± 2.5 (2)
H_2O_2	7.8 ± 1.5 (6)
Sham	5.0 ± 3.0 (2)

Mean number of bifurcations for each angiogenic factor and its corresponding sham bead from Days 8 and 9. The results are presented as mean ± standard error. The number in parentheses indicates how many different beads were included in the mean values. * indicates significance according to Student's t-test between the angiogenic factor and its sham bead.

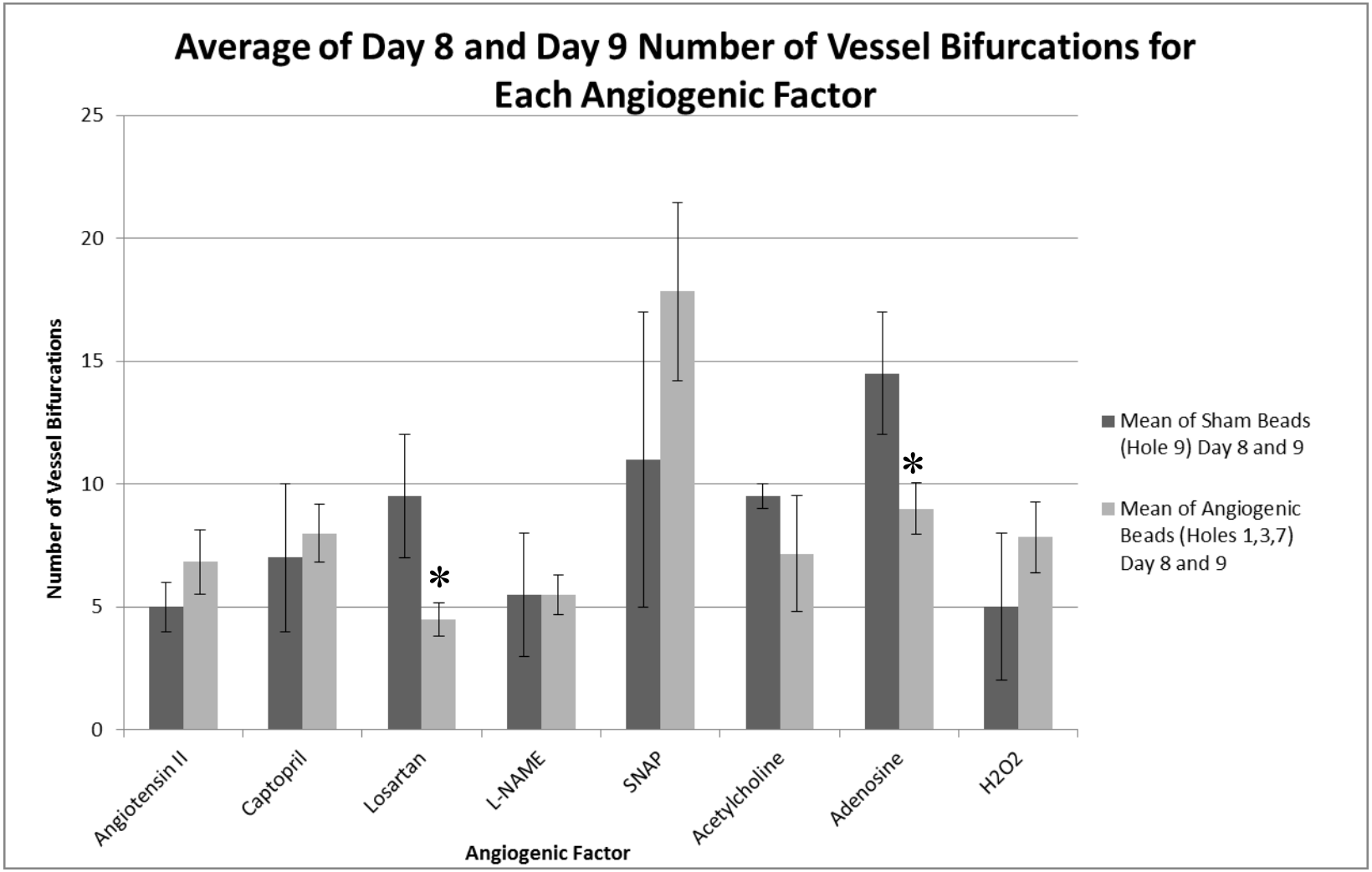

Figure 13. Graph of the number of vessel bifurcations for the beads containing the angiogenic factor and the sham beads (Day 8 and 9). * indicates significance according to Student's t-test between the angiogenic factor and its sham bead.

Table 6. Total Length of Vessels (μm) for Each Angiogenic Factor (Days 8 and 9).

Angiogenic Factor	Mean results of Days 8 and 9 in μm
Angiotensin II	678.8 ± 102.5 (6)*
Sham	765.9 ± 44.2 (2)
Captopril	807.8 ± 71.9 (6)
Sham	801.6 ± 209.7 (2)
Losartan	569.7 ± 37.8 (6)*
Sham	935.1 ± 144.1 (2)
L-NAME	630.4 ± 39.4 (6)
Sham	578.2 ± 107.3 (2)
SNAP	987.3 ± 107.9 (6)*
Sham	605.3 ± 52.2 (2)
Acetylcholine	814.3 ± 104.5 (6)
Sham	815.3 ± 52.6 (2)
Adenosine	818.1 ± 36.2 (6)*
Sham	892.9 ± 39.6 (2)
H_2O_2	711.4 ± 56.2 (6)
Sham	616.2 ± 71.4 (2)

Mean total vessel length (μm) for each angiogenic factor and its corresponding sham bead from Days 8 and 9. The results are presented as mean ± standard error. The number in parentheses indicates how many different beads were included in the mean values. * indicates significance according to Student's t-test between the angiogenic factor and its sham bead.

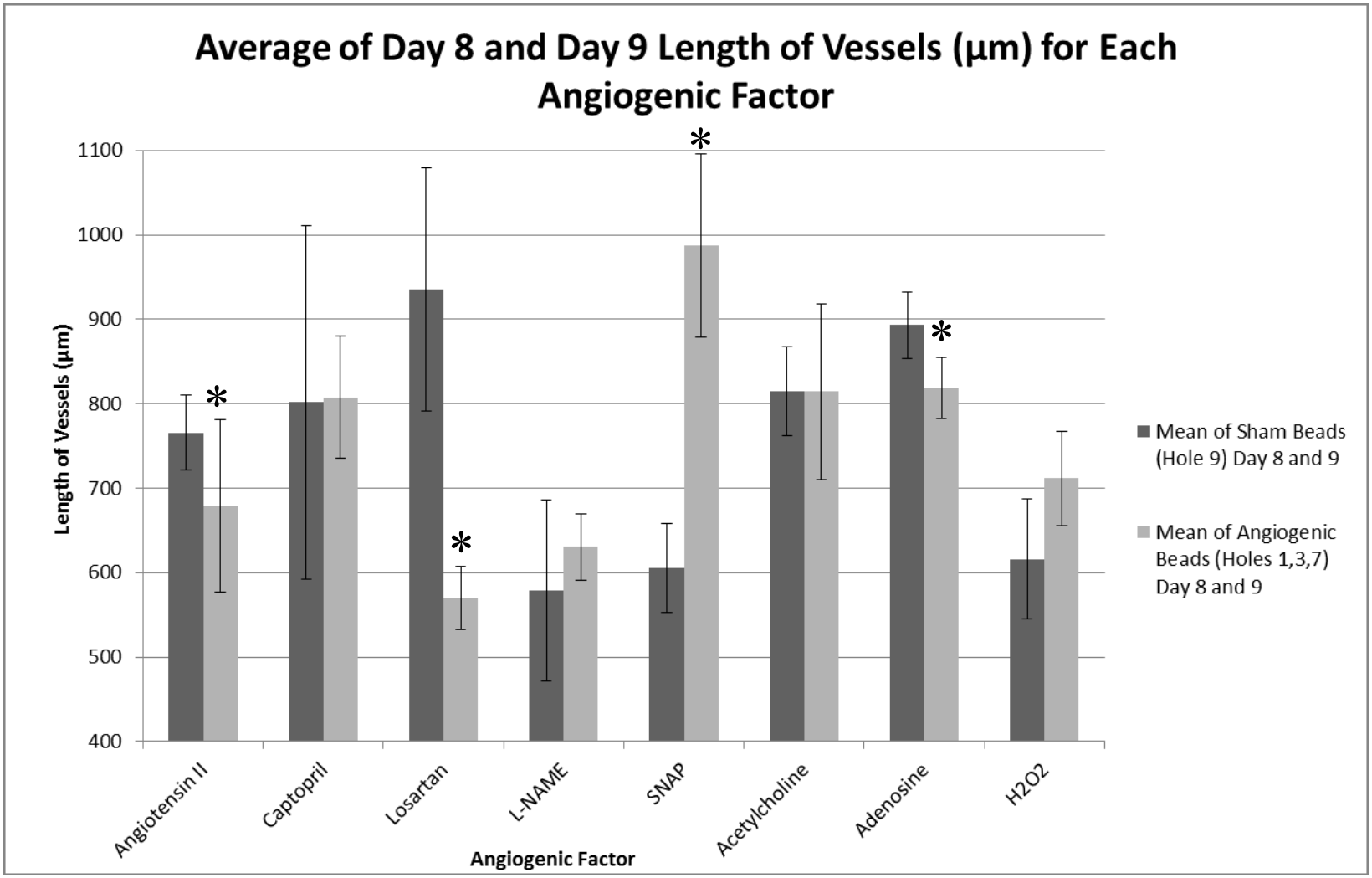

Figure 14. Graph of the length of vessels for the beads containing the angiogenic factor and the sham beads (Day 8 and 9). * indicates significance according to Student's t-test between the angiogenic factor and its sham bead.

Table 7. Complexity of Vessels (D_f) for Each Angiogenic Factor (Days 8 and 9).

Angiogenic Factor	Mean results of Days 8 and 9
Angiotensin II	1.344 ± 0.040 (6)
Sham	1.354 ± 0.008 (2)
Captopril	1.344 ± 0.021 (6)
Sham	1.340 ± 0.071 (2)
Losartan	1.245 ± 0.012 (6)*
Sham	1.364 ± 0.047 (2)
L-NAME	1.286 ± 0.024 (6)
Sham	1.282 ± 0.024 (2)
SNAP	1.437 ± 0.043 (6)*
Sham	1.331 ± 0.096 (2)
Acetylcholine	1.365 ±0.034 (6)
Sham	1.324 ± 0.007 (2)
Adenosine	1.350 ± 0.018 (6)*
Sham	1.393 ± 0.004 (2)
H_2O_2	1.313 ± 0.033 (6)
Sham	1.281 ± 0.051 (2)

Mean complexity of the vessels (D_f) for each angiogenic factor and its corresponding sham bead from Days 8 and 9. The results are presented as mean ± standard error. The number in parentheses indicates how many different beads were included in the mean values. * indicates significance according to Student's t-test between the angiogenic factor and its sham bead.

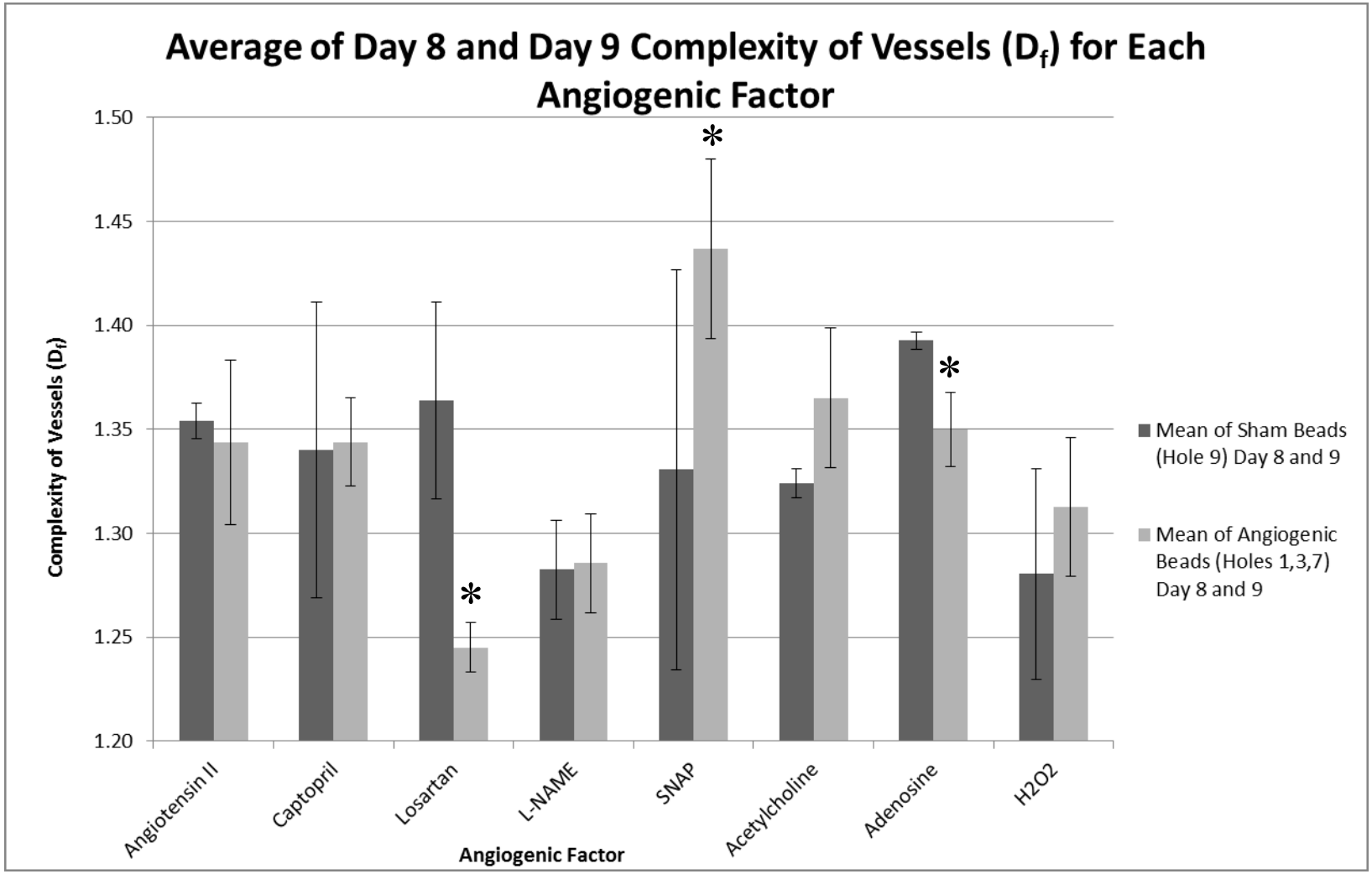

Figure 15. Graph of the complexity of the vessels for the beads containing the angiogenic factor and the sham beads (Day 8 and 9). * indicates significance according to a Student's t-test between the angiogenic factor and its sham bead.

Results for "Virtual" Holes

Each film patch placed on the CAM also contained a hole in the middle that was left open to atmospheric oxygen. No alginate bead was placed on top of it. This hole can be compared to "virtual" holes in the film patch. The virtual holes refer to areas of the CAM that are covered by the film patch and thus very little oxygen can diffuse through the film patch into this area (PO_2 under film = 5-10 mmHg, Connery, personal communication). The virtual holes are equidistant from the center of the holes which contain the alginate beads (see Figure 9 in the Materials and Methods section for location of the virtual holes). Each film patch contained four virtual holes (holes 2, 4, 6, and 8) and one control hole (hole 5). The data recorded from these holes were averaged over two days (Days 8 and 9) and were grouped according to which angiogenic factor was present on that specific film patch. The results for each of these virtual holes (grouped by angiogenic factor) are given below and are shown in Tables 8-11 and Figures 16-19.

Number of Vessel Segments

The number of vessel segments under "virtual" holes for the various angiogenic factors ranged from 14.0 to 42.5. There was a significant difference between number of vessel segments under "virtual" holes compared with the open holes for Angiotensin II, Losartan and H_2O_2. The mean values of number of vessel segments for the virtual holes and control holes for film patches containing specific angiogenic factors are given in Table 8 and Figure 16.

Number of Vessel Bifurcations

Table 9 and Figure 17 show the mean values for the number of vessel bifurcations for each virtual hole and control hole. The range of the number of vessel bifurcations was 5.5-21.5. Angiotensin II, Losartan, SNAP and H_2O_2 gave significant values.

Total Length of Vessels

The range of values for for total length of vessels for the virtual holes and control holes were 621.1-1093.0 µm. Table 10 and Figure 18 show the mean values. Significant differences were found for Losartan and Adenosine.

Complexity of Vascular Networks

For the complexity of the vascular network, Table 11 and Figure 19 show the mean values. The range for this parameter was 1.282-1.446. Angiotensin II, Losartan, L-NAME and Adenosine gave significant results.

Table 8. Number of Vessel Segments for Each Virtual Hole (Days 8 and 9).

Angiogenic Factor	Mean results of Days 8 and 9
Angiotensin II	18.6 ± 2.0 (8)*
Control	14.0 ± 2.0 (2)
Captopril	20.9 ± 2.0 (8)
Control	22.5 ± 1.5 (2)
Losartan	27.1 ± 3.7 (8)*
Control	17.0 ± 8.0 (2)
L-NAME	14.4 ± 2.0 (8)
Control	16.0 ± 1.0 (2)
SNAP	34.0 ± 5.9 (8)
Control	42.5 ± 4.5 (2)
Acetylcholine	19.8 ± 2.7 (8)
Control	17.0 ± 3.0 (2)
Adenosine	30.5 ± 3.2 (8)
Control	31.5 ± 9.5 (2)
H_2O_2	23.6 ±1.4 (8)*
Control	18.5 ± 1.5 (2)

Mean number of vessel segments for each virtual hole for a film patch containing the specific angiogenic factor and its corresponding control hole from Days 8 and 9. The results are presented as mean ± standard error. The number in parentheses indicates how many different holes were included in the mean values. * indicates significance according to Student's t-test between the means for the virtual holes and their control hole.

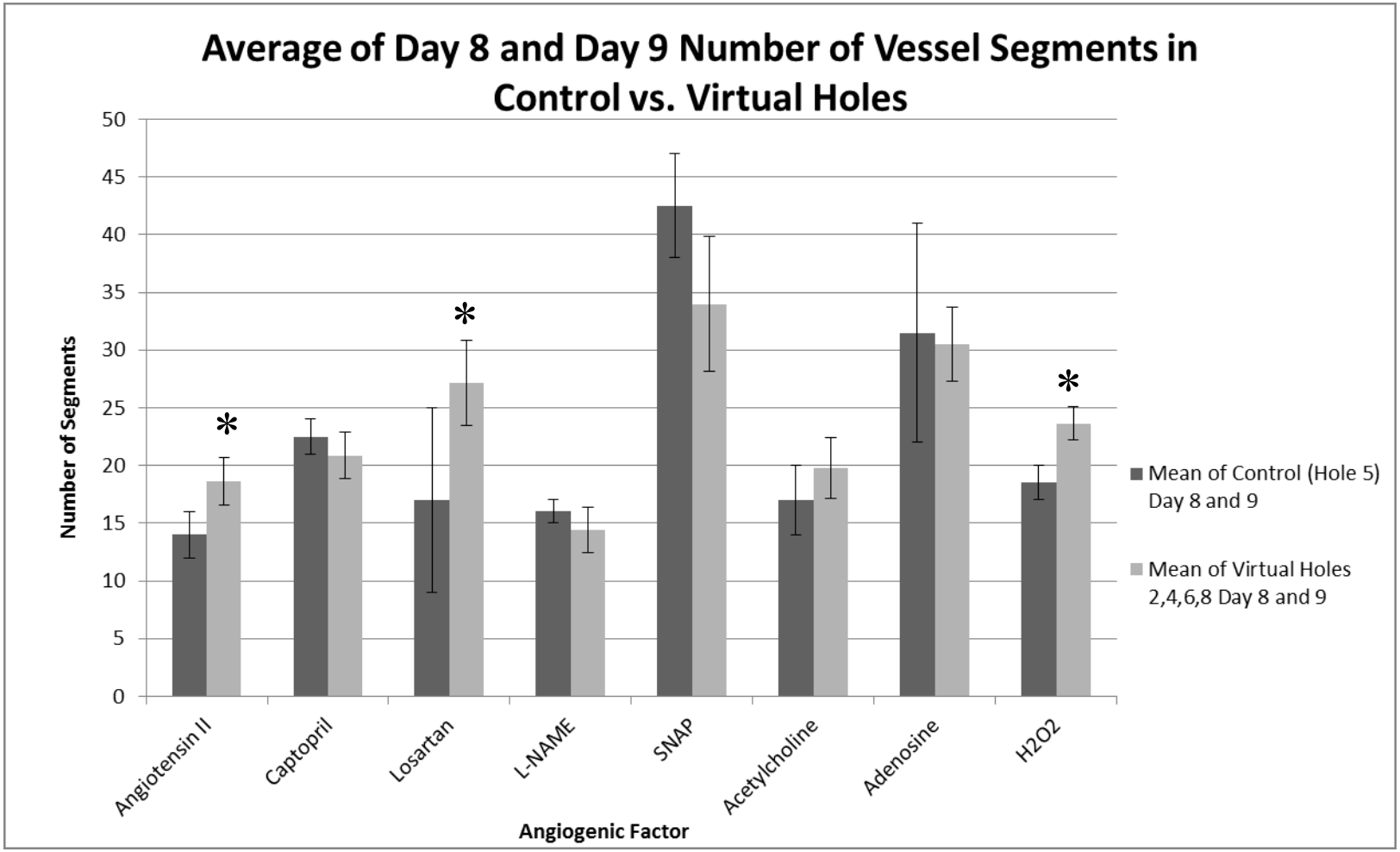

Figure 16. Graph of the number of vessel segments for the virtual holes of each film containing a specific angiogenic factor and the control holes (Day 8 and 9). * indicates significance according to Student's t-test between the virtual holes and their control holes.

Table 9. Number of Vessel Bifurcations for Each Virtual Hole (Days 8 and 9).

Angiogenic Factor	Mean results of Days 8 and 9
Angiotensin II	7.9 ± 1.1 (8)*
Control	5.5 ± 0.5 (2)
Captopril	9.5 ± 1.1 (8)
Control	10.0 ± 1.0 (2)
Losartan	11.8 ± 1.7 (8) *
Control	7.0 ± 4.0 (2)
L-NAME	6.0 ± 1.2 (8)
Control	7.5 ± 0.5 (2)
SNAP	15.9 ± 3.0 (8) *
Control	21.5 ± 3.5 (2)
Acetylcholine	8.4 ± 1.2 (8)
Control	7.5 ± 1.5 (2)
Adenosine	13.5 ± 1.3 (8)
Control	13.5 ± 3.5 (2)
H_2O_2	10.4 ± 0.6 (8) *
Control	8.0 ± 1.0 (2)

Mean number of vessel bifurcations for each virtual hole for a film patch containing the specific angiogenic factor and its corresponding control hole from Days 8 and 9. The results are presented as mean ± standard error. The number in parentheses indicates how many different holes were included in the mean values. * indicates significance according to Student's t-test between the means of the virtual holes and their control hole.

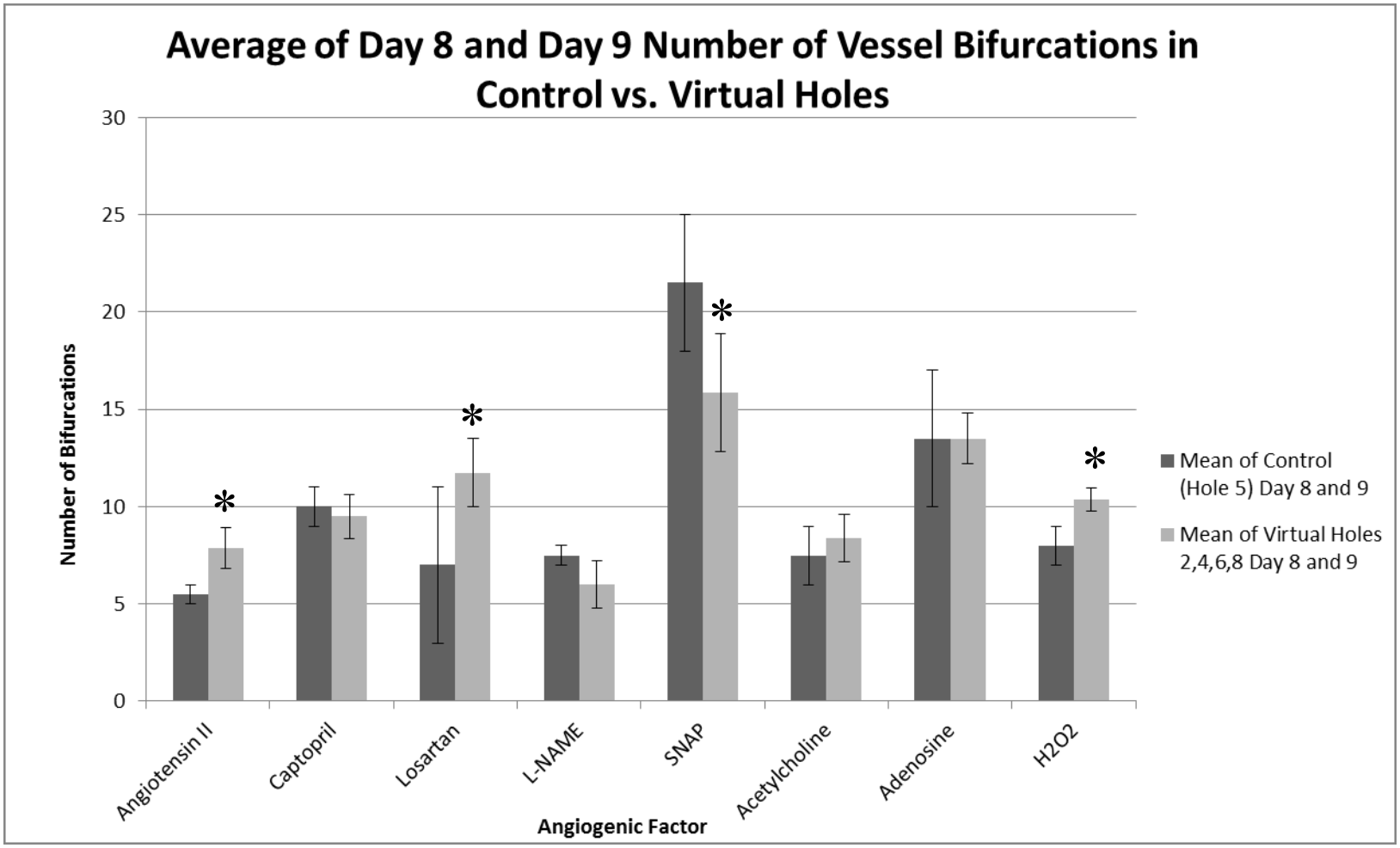

Figure 17. Graph of the number of vessel bifurcations for the virtual holes of each film containing a specific angiogenic factor and the control holes (Day 8 and 9). * indicates significance according to Student's t-test between the virtual holes and their control holes.

Table 10. Total Length of Vessels (μm) for Each Virtual Hole (Days 8 and 9).

Angiogenic Factor	Mean results of Days 8 and 9 in μm
Angiotensin II	780.2 ± 35.9 (8)
Control	754.7 ± 71.3 (2)
Captopril	852.2 ± 67.0 (8)
Control	787.6 ± 19.2 (2)
Losartan	1000.8 ± 99.5 (8)*
Control	621.1 ± 174.2 (2)
L-NAME	677.6 ± 40.7 (8)
Control	675.5 ± 62.8 (2)
SNAP	1093.0 ± 113.6 (8)
Control	987.9 ± 88.5 (2)
Acetylcholine	734.5 ± 59.7 (8)
Control	733.1 ± 87.8 (2)
Adenosine	1053.6 ± 59.9 (8)*
Control	903.1 ± 93.3 (2)
H_2O_2	879.7 ± 44.3 (8)
Control	801.9 ± 108.9 (2)

Mean total vessel length (μm) for each virtual hole for a film patch containing the specific angiogenic factor and its corresponding control hole from Days 8 and 9. The results are presented as mean ± standard error. The number in parentheses indicates how many different holes were included in the mean values. * indicates significance according to Student's t-test between the means of the virtual holes and their control hole.

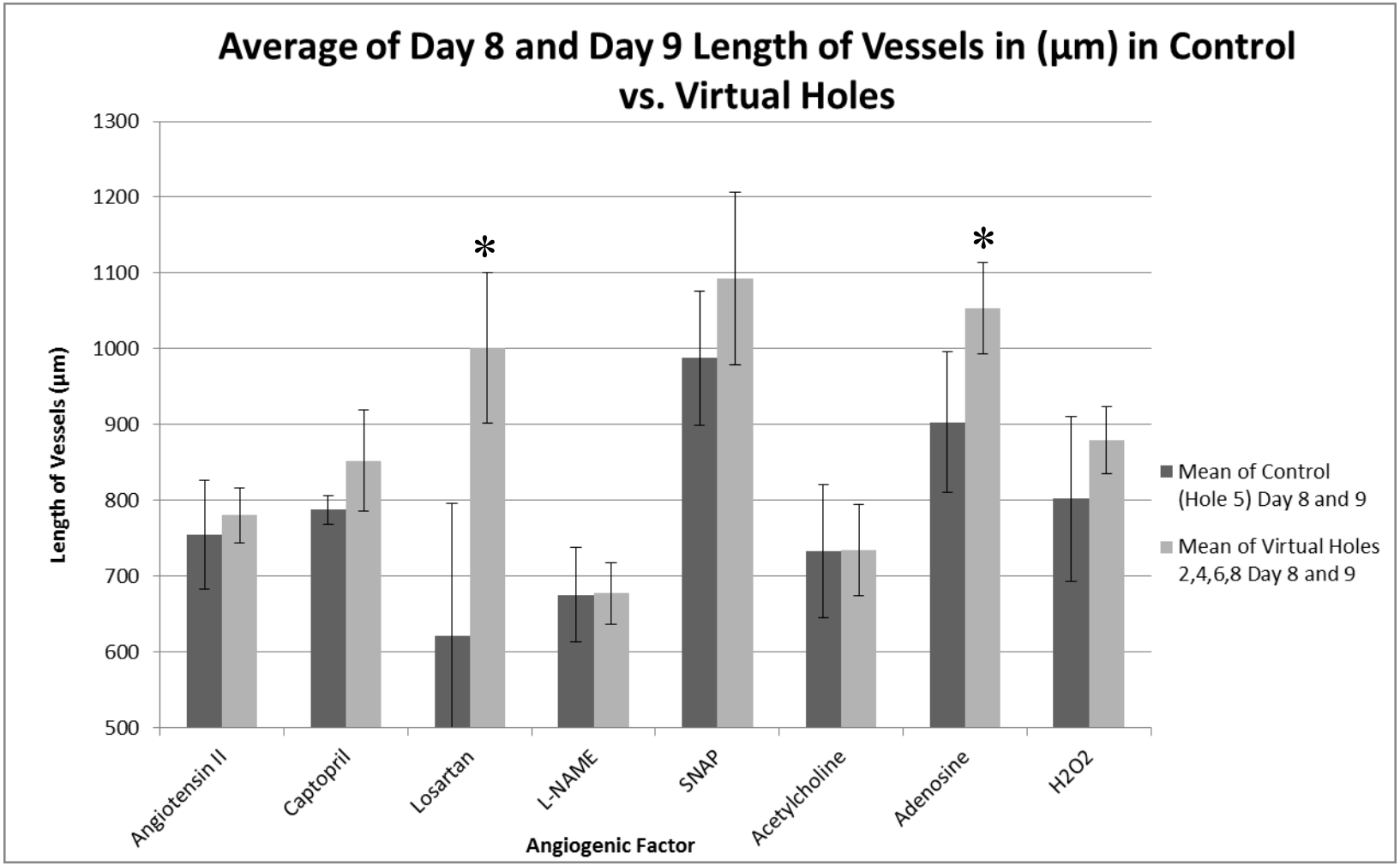

Figure 18. Graph of the length of the vessels for the virtual holes of each film containing a specific angiogenic factor and the control holes (Day 8 and 9). * indicates significance according to Student's t-test between the virtual holes and their control hole.

Table 11. Complexity of Vessels (D_f) for Each Virtual Hole (Days 8 and 9).

Angiogenic Factor	Mean results of Days 8 and 9
Angiotensin II	1.364 ± 0.017 (8)*
Control	1.315 ± 0.035 (2)
Captopril	1.340 ± 0.021 (8)
Control	1.346 ± 0.039 (2)
Losartan	1.401 ± 0.033 (8)*
Control	1.325 ± 0.101 (2)
L-NAME	1.282 ± 0.027 (8)*
Control	1.338 ± 0.031 (2)
SNAP	1.434 ± 0.032 (8)
Control	1.446 ± 0.004 (2)
Acetylcholine	1.319 ± 0.034 (8)
Control	1.344 ± 0.046 (2)
Adenosine	1.418 ± 0.019 (8)*
Control	1.379 ± 0.044 (2)
H_2O_2	1.359 ± 0.015 (8)
Control	1.359 ± 0.040 (2)

Mean complexity of the vessels (D_f) for each virtual hole for a film patch containing the specific angiogenic factor and its corresponding control hole from Days 8 and 9. The results are presented as mean ± standard error. The number in parentheses indicates how many different holes were included in the mean values. * indicates significance according to Student's t-test between the means of the virtual holes and their control hole.

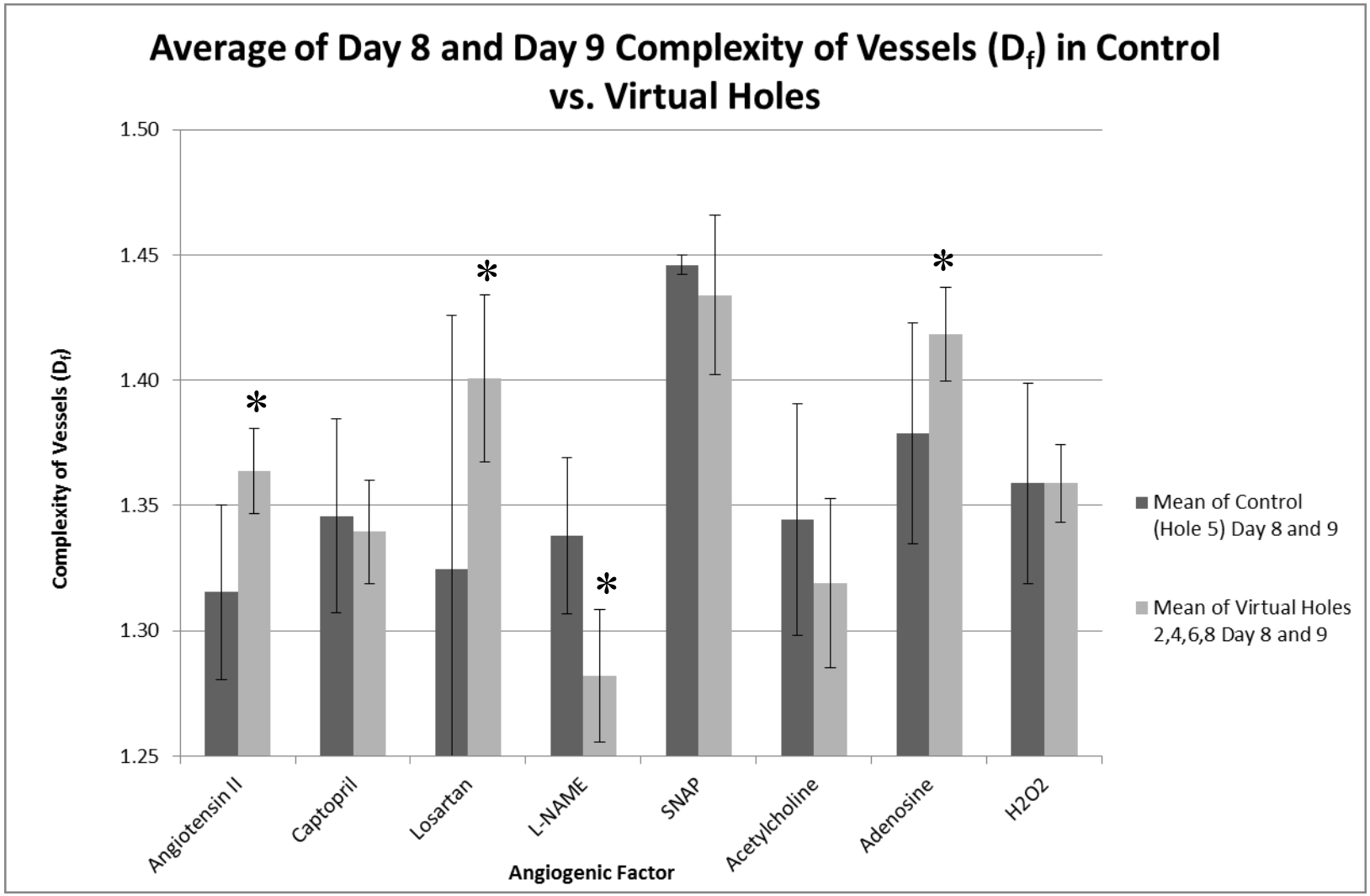

Figure 19. Graph of the complexity of the vessels (D_f) for the virtual holes of each film containing a specific angiogenic factor and the control holes (Day 8 and 9). * indicates significance according to Student's t-test between the virtual holes and their control holes.

DISCUSSION

Summary of the Results

For the angiogenic beads, significant results for at least one of the four parameters (number of vessel segments, number of vessel bifurcations, total vessel length, and complexity of vascular network) were found for Angiotensin II, Losartan, SNAP, Adenosine, and H_2O_2. SNAP and H_2O_2 gave pro-angiogenic results while Angiotensin II, Losartan, and Adenosine were anti-angiogenic. Analysis of the virtual holes gave significant results for Angiotensin II, Losartan, SNAP, Adenosine and H_2O_2. The virtual holes containing Angiotensin II, Losartan, Adenosine, and H_2O_2 gave pro-angiogenic results while SNAP and L-NAME virtual holes were anti-angiogenic. A more detailed description of the findings can be found below.

Summary of the Study

Square oxygen barrier film patches with five 1 mm holes punched in them were positioned on seven day old chick embryos. Polymerized alginate beads incubated in specific angiogenic factors were placed on three of the holes, while a sham bead incubated in PBS was placed on one of the holes. The center hole was left empty to be

considered a control hole. Only one angiogenic factor was associated with a given film patch and each embryo contained three film patches.

After 24 and 48 hours of exposure, images were collected for each of the holes containing the beads, the control hole, and also "virtual holes" or areas covered by the film and, thus, not exposed to the atmospheric oxygen. These images were processed and analyzed using image analysis software. The parameters collected included the number of vessel segments, the number of bifurcations, the total length of the vessels, and the complexity of the vasculature (measured as the fractal dimension). Comparisons between the angiogenic beads and the sham beads could be made. Also, the results from the control holes and the virtual holes could be evaluated. Significance between these values was determined using Student's t-test.

If the results for the comparison between the angiogenic beads and the sham beads were significant, then it was determined if these results were pro-angiogenic, anti-angiogenic, or not angiogenic by noting whether the angiogenic factor gave a response for the parameter evaluated which was larger or smaller than the response for the sham treatment. Table 12 shows this analysis. The angiogenic factors were determined to be pro-angiogenic for a given parameter if the angiogenic beads produced a larger value for that parameter than the sham beads did. If the beads containing an angiogenic factor produced a smaller value for any of the parameters than the sham beads, then the angiogenic factor was determined to be anti-angiogenic based on that parameter. If there was no significant difference between the treated and untreated beads, then the

Table 12. Angiogenesis Analysis for Beads Incubated with Angiogenic Factors.

Angiogenic Factor	Number of Segments	Number of Bifurcations	Length of Vessels	Complexity of Vessels
Angiotensin II	0	0	-	0
Captopril	0	0	0	0
Losartan	-	-	-	-
L-NAME	0	0	0	0
SNAP	+	0	+	+
Acetylcholine	0	0	0	0
Adenosine	-	-	-	-
H_2O_2	+	0	0	0

For each parameter -- the number of vessel segments, the number of vessel bifurcations, the total length of the vessels, and the complexity of the vascular network -- the angiogenic factors were determined to be either pro-angiogenic (+), anti-angiogenic (-), or not angiogenic (0).

angiogenic factor was classified as "not angiogenic." The same procedure was carried out for the control holes compared to the virtual holes (see Table 13).

Analysis of Each of the Angiogenic Factors

Angiotensin II

Angiotensin II is known to increase blood pressure by activating its receptor AT_1, a G-protein coupled receptor (GPCR). Activation of the AT_1 receptor leads to a signaling cascade where one of the outcomes is vasoconstriction. Angiotensin II has been tested on CAM preparations and was found to be pro-angiogenic (Ferdinand et al. 1991) in doses of 67 ng and 670 ng. From the results with the beads treated with potential angiogenic factors, Angiotensin II appeared not to be pro-angiogenic; for the vessel length parameter Angiotensin II gave an anti-angiogenic response. These different results may have occurred because the concentration of Angiotensin II used in the present study (10 μM) was too low even though the concentration was much lower in the Ferdinand et al. study. The concentration should be increased in subsequent tests.

Captopril

Captopril is an angiotensin converting enzyme (ACE) inhibitor used to treat hypertension and some cases of congestive heart failure. Because Captopril inhibits the process that converts Angiotensin I to Angiotensin II, it should have the opposite effects of Angiotensin II on angiogenesis. Research has shown Captopril to be a known

angiogenesis inhibitor (Qiu et al. 2000) at a dose of 12.5 mg. In the present study, Captopril did not show an angiogenic response. As with Angiotensin II, the concentration of Captopril used (0.2 mg/mL) may not have been high enough to elicit a response.

Losartan

Losartan is an Angiotensin II receptor antagonist. Like Captopril, Losartan is primarily used to treat hypertension. It binds to the AT_1 receptor thereby preventing the action of Angiotensin II. Thus it should have the opposite effects on angiogenesis as Angiotensin II and the same response as Captopril. Losartan has been found to inhibit angiogenesis (Lip 2002). This was the case for the beads treated with Losartan. All of the parameters gave an anti-angiogenic response.

L-NAME

L-NAME is a non-specific nitric oxide synthase (NOS) inhibitor. When blood flow is increased in blood vessels, shear stress is transmitted to the endothelial cells. This force activates eNOS or endothelial nitric oxide synthase located in endothelial cells. eNOS then produces nitric oxide which causes dilation of the blood vessels, leading to a decrease in the shear stress on the endothelial cells. L-NAME inhibits eNOS from producing nitric oxide and thus inhibits vasodilation activated by shear stress. L-NAME is anti-angiogenic (Ziche et al. 1994) in concentrations of 0.5 g/L. In the tests involving

alginate beads incubated with L-NAME, no angiogenic response was observed. Again, this could have been because the concentration used was too low (1 mM)

SNAP

SNAP is a nitric oxide (NO) donor. Nitric oxide causes vasodilation by activating soluble guanylate cyclase which increases the amount of cGMP (cyclic guanosine monophosphate) in the smooth muscle cell. cGMP inhibits calcium entry into the cell and activates a cGMP-dependent kinase. This kinase acts on myosin light chain phosphatase which in turn dephosphorylates myosin light chains leading to smooth muscle relaxation. SNAP has been reported to be pro-angiogenic (Ziche et al. 1994). Results from the present study indicate that SNAP is pro-angiogenic except for the number of vessel bifurcations.

Acetylcholine

Acetylcholine binds to muscarinic receptors on endothelial cells and these cells can then contribute to angiogenesis by activation of the MAP kinase (ERK) signaling pathway. Acetylcholine should stimulate angiogenesis (Arias et al. 2009). According to the results of the present study, Acetylcholine is not angiogenic. Higher concentrations of Acetylcholine should be examined to see if this agent is pro-angiogenic.

Adenosine

Adenosine (0.2-3.0 mg contained in Elvax polymer pellets) is known to stimulate angiogenesis in the CAM preparation under global hypoxic conditions (Dusseau et al. 1986). The exact mechanism that causes this effect is not completely understood. The results for the alginate beads incubated with Adenosine indicate that it is anti-angiogenic for all four of the vascularity parameters. These findings are the opposite of what was expected.

H_2O_2

H_2O_2 activates a transcription factor (ETS-1) which turns on angiogenic genes (Fong 2008). Previous studies have shown that H_2O_2 does indeed give pro-angiogenic responses (Yasuda et al. 1999) with concentrations of 0.1-10 μM. Results indicate that H_2O_2 is pro-angiogenic in the number of vessel segments but not angiogenic for the other parameters. Again, perhaps a higher concentration than 1 mM H_2O_2 is needed to produce pro-angiogenic effects for all four of the parameters.

Analysis of the Oxygen Barrier Response

For Angiotensin II, Losartan, H_2O_2 and Adenosine, a pro-angiogenic response was found in the virtual holes compared to the control holes (Table 13). An anti-angiogenic response was found for SNAP (number of vessel bifurcations) and L-NAME (complexity). These responses could either be from the difference in oxygen levels

Table 13. Angiogenesis Analysis of Vascularity in the Virtual Holes for the Various Angiogenic Factors.

Angiogenic Factor	Number of Segments	Number of Bifurcations	Length of Vessels	Complexity of Vessels
Angiotensin II	+	+	0	+
Captopril	0	0	0	0
Losartan	+	+	+	+
L-NAME	0	0	0	-
SNAP	0	-	0	0
Acetylcholine	0	0	0	0
Adenosine	0	0	+	+
H_2O_2	+	+	0	0

For each parameter -- the number of vessel segments, the number of vessel bifurcations, the total length of the vessels, and the complexity of the vascular network -- the angiogenic factors were determined to be either pro-angiogenic (+), anti-angiogenic (-), or not angiogenic (0).

between the open and covered ("virtual") holes or because of the beads treated with the associated angiogenic factor placed on that film patch.

The diffusion coefficient and the diffusion time for each of the angiogenic factors were calculated (see Table 14). The diffusion coefficient is proportional to 1/(sqrt(MW)), where MW is the molecular weight of the angiogenic factor. Thus, if one takes the diffusion coefficient for oxygen in an aqueous medium ($D = 1.01 \times 10^{-5}$ cm^2/s) as a reference value, one can estimate the diffusion coefficient for other molecules based on their molecular weight. From this value, the average time to diffuse a distance x can be calculated as $t = x^2/2D$, where x equals the distance of diffusion and D is the diffusion coefficient. Since the holes containing the alginate beads were 5 mm from the control hole, 5 mm was used as the distance of diffusion. These calculations show that all of the angiogenic factors, except H_2O_2 and Acetylcholine, will take about 10 hours to diffuse 5 mm. Thus it is possible that the agents associated with the treated alginate beads could influence the control holes in 48 hours. Also, the angiogenic factors could have an influence on the virtual holes in the 24 hours before images were taken.

In this case, the differences in angiogenesis may be accounted for by the differences in oxygen levels that the holes were exposed to. Many studies have been done on the effects of hypoxia on vessel growth in the CAM. Fong (2008) and Fraisl et al. (2009) discovered a hypoxia-induced angiogenesis mechanism involving HIF-1 (hypoxia inducible transcription factor 1). Under hypoxic conditions, the levels of HIF-1 increase, leading to increased transcription of their target genes which include

Table 14. Diffusion Coefficients and Diffusion Rates for the Angiogenic Factors.

Angiogenic Factor	Formula	Molecular Weight (MW)	Diffusion Coefficient Factor (sqrt(32/MW)	Time (hours) to diffuse 5 mm ($t = x^2/2D$)
Angiotensin II	$C_{50}H_{71}N_{13}O_{12}$	1046.18	0.175	19.9
Captopril	$C_9H_{15}NO_3S$	217.29	0.384	9.0
Losartan	$C_{22}H_{23}ClN_6O$	422.91	0.275	12.6
L-NAME	$C_7H_{15}N_5O_4 \cdot HCl$	269.70	0.344	10.1
SNAP	$C_7H_{12}N_2O_4S$	220.30	0.381	9.1
Nitric Oxide	NO	30.01	1.033	3.3
Acetylcholine	$C_7NH_{16}O_2^+$	146.21	0.468	7.4
Adenosine	$C_{10}H_{13}N_5O_4$	267.24	0.346	10.0
H_2O_2	H_2O_2	34.01	0.970	3.6

erythropoietin and vascular endothelial growth factor (VEGF)-A. In this way, erythropoiesis and angiogenesis is increased. Stick et al. (1991), Dusseau and Hutchins (1989) and Hoper and Jahn (1995) showed that hypoxia stimulates angiogenesis in the CAM while hyperoxia inhibits CAM angiogenesis, both in a dose-dependent manner.

Initial studies in this laboratory have shown that the partial pressure of oxygen drops from room air pressure (~150 mmHg) to 10 mmHg under the Krehalon film within only 300 μm from the edge of the film (Connery, personal communication). Because of the film patches placed on the CAM, the virtual holes are exposed to much less oxygen than the control holes and thus may be considered to be under hypoxic conditions. Thus any increased angiogenesis in the virtual holes compared to the control holes confirms the results shown by previous studies. Conversely the virtual holes which gave an anti-angiogenic response (SNAP for number of bifurcations and L-NAME for complexity) are not consistent with these studies. It is not known why this response was shown, although it is interesting to note that most studies of angiogenesis using the CAM preparation employ global hypoxia rather than the very localized hypoxia of this study.

Comparison of the Control Holes and the Sham Beads

The control holes and the sham beads in this experiment should give similar results when compared to each other if PO_2 and the chemical environment at these holes was similar. This was the case for all of the results except one. The total length of the

vessels for the film patch containing SNAP gave a significant difference when comparing the control holes with those containing sham beads.

Use of the CAM Preparation

A reliable *ex ovo* CAM preparation was essential for the performance of these experiments. As is often the case, a number of unsuccessful early attempts were made in plating the fertilized eggs before a standard method was produced (see Materials and Methods section for the detailed plating procedure). These efforts led to a reliable and reproducible plating procedure with a relatively high rate of success (~80%). Typically, one-third of the embryos would remain viable until day 6 with only one or two embryos (out of about 10 successfully plated) surviving past day 10.

The CAM of the viable embryo became visible around day 6 of incubation. As the embryo further developed, the CAM would surround the yolk and eventually grow out farther than the boundary of the yolk. Figures 20 and 21 show images of CAM development collected at days 4-11 of incubation.

Perforated Film Patches

Size of patches

The film patches used for these experiments were 15 mm squares. This size was chosen because it was large enough to insert several 1.0 mm diameter holes in the film

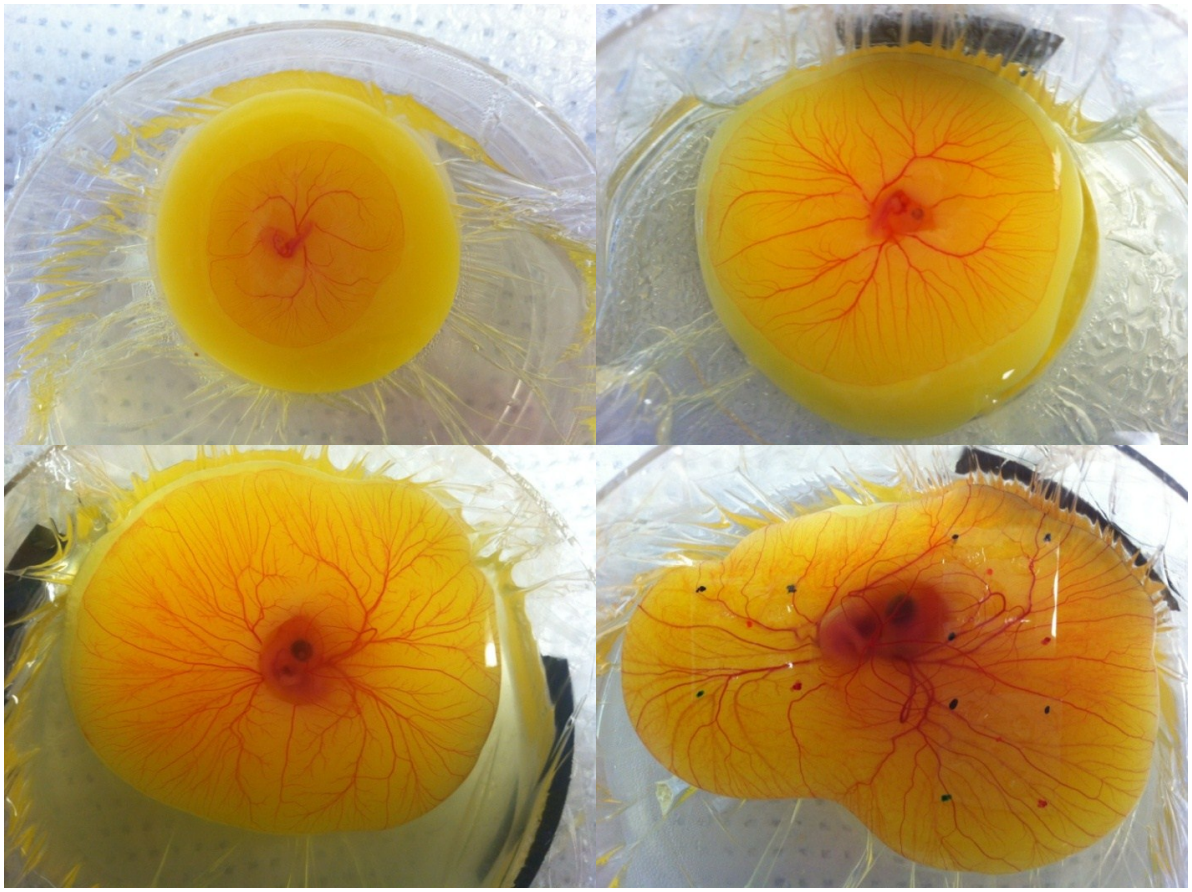

Figure 20. Images of Embryonic Development on Days 4-7 of Incubation. The top left panel shows a 4 day old embryo. The top right panel shows a 5 day old embryo. The bottom left panel is a 6 day old embryo while the bottom right panel is a 7 day old embryo. The CAM is visible on the 6 and 7 day old embryo. It is the slightly darker region surrounding the embryo. An example of the film patches that were placed on the embryos can be seen on the 7 day old embryo (bottom right panel; notice dark dots in the four corners of the film patches).

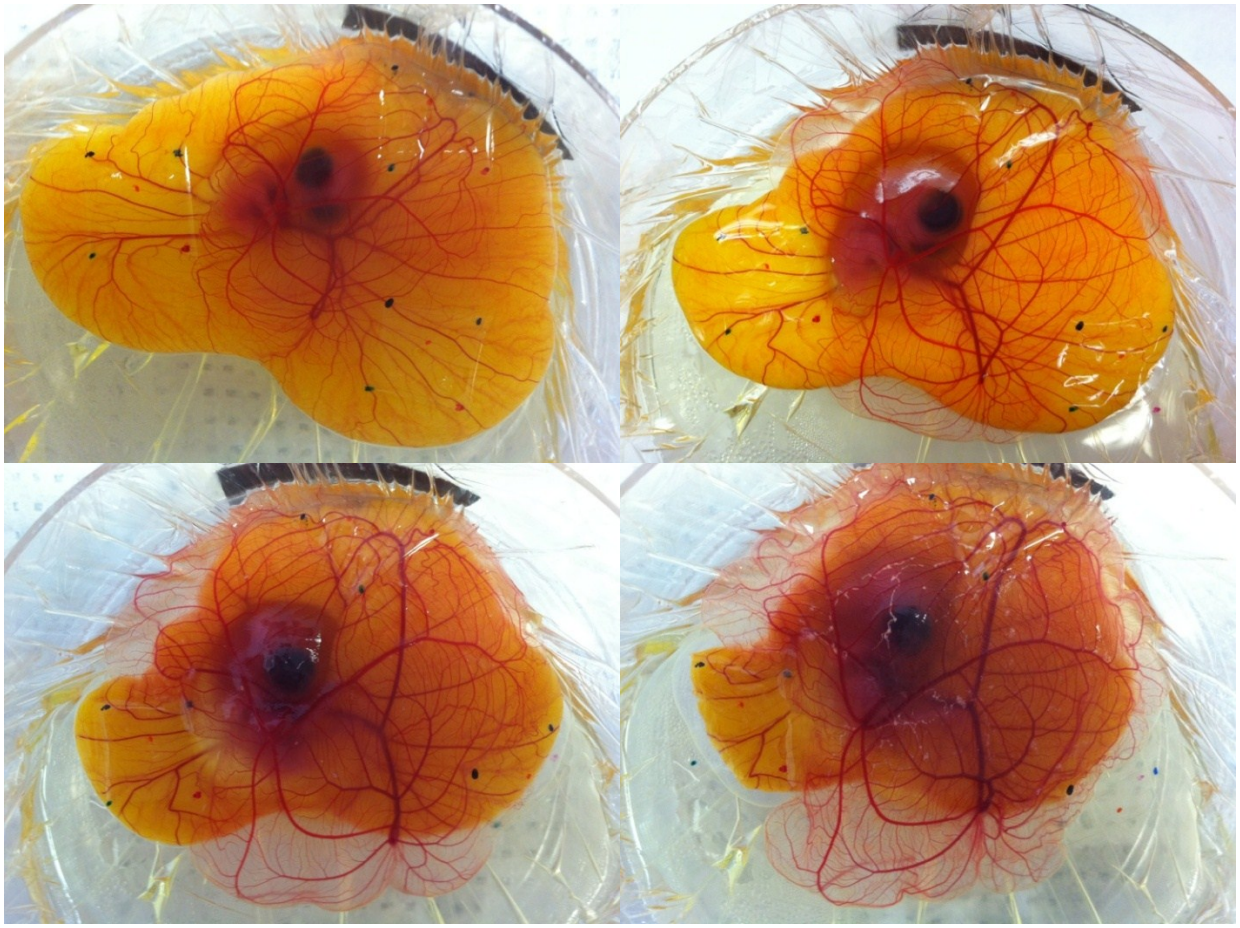

Figure 21. Images of Embryonic Development on Days 8-11 of Incubation. The top left panel shows an 8 day old embryo. The top right panel shows a 9 day old embryo. The bottom left panel shows a 10 day old embryo while the bottom right panel shows an 11 day old embryo. The CAM is visible on all of the images although it is easier to see on the 9-11 day embryos. It is the vascular network that is growing away from the yolk.

without any interactions between the holes. The holes were spaced at least 5 mm apart from each other and 5 mm away from the edge of the film patch. Also, the film patch needed to be small enough so that it did not cover the entire embryo. Covering the entire embryo would kill the embryo because only a small amount of oxygen would be able to diffuse through the film to the embryo.

Hole size

The 1.0 mm diameter hole size was chosen because of the results obtained from the 0.5, 1.0 and 2.0 mm diameter hole size experiments (see Figure 11 and Table 3 in Results). The 1.0 mm diameter holes gave the highest values in the number of segments, number of bifurcations, and complexity of the vascular networks. Other studies have shown that small hole sizes (≤1.0 mm diameter) are ideal for looking at how chemicals affect angiogenesis (Jeong et al. 2011).

Number of patches per CAM

Three film patches were placed on each CAM equally spaced from each other. There were approximately 20-30 mm of space between the centers of the film patches. This was needed to insure that the angiogenic factors on each of the film patches would have no diffusive interactions with each other. Also, only one angiogenic factor was tested per film patch. Attempts were made to cover ≤ 20% of the CAM so that the embryo might still have access to an adequate oxygen supply. It is speculated that covering > 20% may lead to increased mortality of the embryos.

Film composition

The Krehalon film (polyvinylidene chloride) used to make the film patches is used to wrap food. Thus it has very low toxicity. It is 12 μm thick and its oxygen transmission rate is 2.19 $cm^3 \cdot ml/100\ in^2 \cdot day \cdot atm$.

Image Collection and Analysis

Images of the vessels were collected using a stereomicroscope. This was not ideal since it was difficult to determine if the blood vessels were from the CAM or the vitelline membrane. Transillumination image acquisition would have given better images, but the dense yolk prevented this method from being used due to low light transmission. Also by using the stereomicroscope, it was more difficult to obtain a sharp, clear image because there is no fine focus adjustment knob. Blurry images also occurred because the embryo's heart beat caused the whole embryo to move periodically.

The image analysis depended on the quality of images collected from the stereomicroscope. Depending on how clear the images were, the vessels that were visible could be traced by hand with a black brush tool and later analyzed using Frac_Lac software.

Four parameters were used to quantify angiogenesis. They were: the number of vessel segments, the number of vessel bifurcations, the total vessel length, and the complexity of the vascular network. All of these parameters can be used together or separately to show differences in angiogenesis. Based on our limited experience, the best

parameter to use individually would be the total length of the vessels, since it appeared to be the most sensitive parameter tested.

Summary

Because this was the first time chick embryos were used as a model system in this laboratory, many initial pilot studies had to be carried out. Through this study, a systematic method for *ex ovo* plating and incubating chick embryos was established. Also, a unique way to apply angiogenic factors was accomplished by making "beads" of polymerized alginate and incubating the beads with the angiogenic factors. The alginate used is non-toxic and commonly used in the food industry. This method of using alginate beads was adapted from Jeong et al. (2011) where, instead of using alginate beads, a permeable film was manufactured and incubated with the desired chemicals and holes were punched into the film. The effect of oxygen on angiogenesis in the CAM was studied by placing film patches with holes on top of the developing CAM and making comparisons with virtual holes.

From the methods used in these experiments, a potential screening process for determining how chemicals will impact angiogenesis using the CAM model was established for future use.

Future Studies

In the future, many different studies can be done with this CAM preparation. As a followup to these studies, the same angiogenic factors could be re-tested at different concentrations and on many more film patches. Other angiogenic factors such as superoxide anion (using SOTS-1, a superoxide donor), apocynin (an NAD(P)H oxidase inhibitor), or carbon monoxide could be used to test their effects on angiogenesis. The CAM preparation could also be used to study PO_2 profiles near blood vessels.

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LITERATURE CITED

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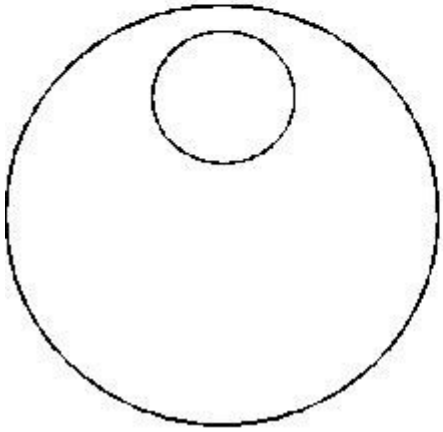
APPENDIX A

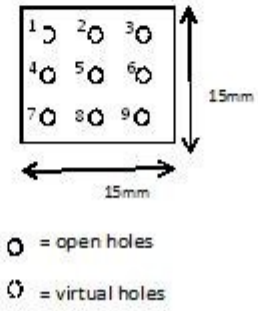
Data Collection Sheet: CAM project—Perforated film placement

Date:___/____/___
Time:_____
Investigator:_____
Egg ID:_____

Date and time of initial incubation (Day 0): ___/____/___ at _____
Date and time of egg plating (Day__): ___/____/___ at _____
Date and time of perforated film placement (Day__): ___/____/___ at _____

Perforated film placement:
**Indicate placement of films, letter of film (A-E), embryo location, boundary of CAM, boundary of vitelline membrane.





Data Collection Sheet: CAM--Image analysis

						Image Collection 1				Image Collection 2			
Patch	Hole	Hole Diameter (mm)	Distance to embryo center (mm)	Chemical applied	Conc.	Date	Time	Saved As	Comments	Date	Time	Saved As	Comments
A	1												
	2												
	3												
	4												
	5												
	6												
	7												
	8												
	9												
B	1												
	2												
	3												
	4												
	5												
	6												
	7												
	8												
	9												
C	1												
	2												
	3												
	4												
	5												
	6												

	7												
	8												
	9												
D	1												
	2												
	3												
	4												
	5												
	6												
	7												
	8												
	9												
E	1												
	2												
	3												
	4												
	5												
	6												
	7												
	8												
	9												

APPENDIX B

Image J procedure
(4/23/12 HH)

Open Image J
Open Saved image (File→open→saved image)
Split color channels (Image→color→split channels)
Keep green channel open. Close blue and red channels.
Use the oval tool to make a circle that is 950 pixels wide and 950 pixels high by adjusting the boxes on yellow outline
Position the circle around the hole in the patch or use on a virtual hole
Crop the circle (Image→crop)
Adjust the brightness of the image (Image→adjust→brightness/contrast)
Move minimum to right until vessels become darker and clearer (maximum should be all the way to the right)
Trace vessels with brush tool (black (color—0,0,0), brush width = 10 pixels). Can go to edit→undo to erase last step
Increase mininium and trace revealed vessels
Get rid of any lines outside the circle (Edit→clear outside)
Double click on the color picker and make sure the color on the bottom left is black (0,0,0) and the color on the bottom right is white (255,255,255) and that the black rectangle is completely showing
Obtain a white background with the black trace shown by reducing the maximum on the brightness/contrast all the way to the left and pressing Apply
Make image binary (Process→binary→make binary)
Save image (File→save as→tiff file→name image)
Count # of bifurcations (marking with brush spots to tract)
Count # of segments
For complexity open FracLac (Plugins→Fractal Analysis→FracLac_)
Select Standard box count
Use default box settings (minimum size = 2 pixels, maximum box size = 45% of ROI, 12 grid positions, show data for each grid). Press ok
If image is still open just select scan image or Roi and the scan will start. If image is not open go to Select files→open image and scan will start (see the progress of the scan on the bottom of the ImageJ tab).

Wait for scan to finish
Close Data window
Look at FracLac_2.5i.3 Box count window
Record data collected (foreground pixels and mean ΛD)
Complexity = mean ΛD

VITA

Heather Amanda Hammond was born on September 10, 1986 in Wellsboro, Pennsylvania. She is a US citizen. After graduating from Wellsboro High School in 2005, she attended the University of Pittsburgh in Pittsburgh, Pennsylvania graduating in April 2009 with a Bachelor of Science in both Biological Science and Chemistry and a minor in Physics. In 2010, she enrolled in the Post-Baccalaureate Pre-Medical Basic Health Sciences certificate program through the School of Medicine at Virginia Commonwealth University in Richmond, Virginia where she remained to complete a Master of Science degree in the Department of Physiology and Biophysics.