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# The influence of blood flow on angiogenesis in the 3-day chick embryo

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# THE INFLUENCE OF BLOOD FLOW ON ANGIOGENESIS IN THE 3 DAY CHICK EMBRYO

by

Brett Mark Clarke

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ABSTRACT

**The Influence of Blood Flow on Angiogenesis in the 3 Day Chick Embryo**

by

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Dr. Carl L. Reiber, Examination Committee Chair
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I hypothesized that experimental elimination of blood flow via ligation of the conotruncus arteriosus would cause a cessation of blood vessel development and even vessel regression. India ink, injected to highlight the vasculature, had no effect on blood flow. Embryos were divided into ligated, sham, and control groups. After ligature placement and 6 hr. reincubation, KCl, added topically induced cardiac arrest. The diameters of the anterior cardinal vein, anterior vitelline vein, vitelline artery, and vitelline vein were not statistically different from sham and control values. Eye diameter increased equally in the presence and absence of blood flow. These results demonstrate that blood flow had no influence on vessel growth in diameter during a 6 hr. period.

Blood flow did appear to offer a stabilizing influence against vessel degradation. A critical window of 6 hr. may exist during which the absence of blood flow has no detrimental effects on embryonic vasculature.

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Brett Mark Clarke

# CHAPTER 1

# INTRODUCTION

## An Alternative Role for the Embryonic Heart

The embryonic heart is recognized to be the first organ to function in an embryo and is the origin of hemodynamic factors such as blood pressure and blood flow within embryonic vessels. In the chick embryo, the heart developed simultaneously with the intra and extraembryonic vasculature (Romanoff, 1960), though the formation of vascular structures was independent of heart development, as indicated by the formation of capillary networks and some larger vessels prior to or in the absence of heart function (Chapman, 1918; Lillie, 1952). The heart of the chick embryo began to contract at approximately 33 hr. incubation (HH stage 10 (Lille, 1952)), but was not sufficiently strong or its contractions not rhythmic enough to initiate blood flow until approximately 10 hr. after contractions began (HH stage 12) (Romanoff, 1960). By 3 days incubation (HH stage 18), the heart tube has twisted to become a loop that protrudes ventrally, and blood flow was well established (Romanoff, 1960).

It has been commonly assumed that in the embryo the heart beat and blood flow function for the purpose of convective nutrient and endocrine delivery, and waste removal as they do in the adult. Contrary to this belief, blood flow has been shown not to be

significant for nutrient delivery and waste removal in the early embryo. Pelster and Burggren (1996) demonstrated in the Zebra fish (*Danio rerio*) embryo that atmospheric inhibition and chemical ablation of hemoglobin did not alter oxygen consumption. In the day 3 (HH stage 18) chick embryo, Warburton et al. (1996) demonstrated via ligation of the heart outflow tract that oxygen consumption did not change in the absence of blood flow. Burggren and Territo (1995) pointed out, therefore, that the heart beat and blood flow in the early embryo may have an alternative function more related to circulatory development (angiogenesis) than to nutrient delivery and waste removal. Indeed, the heart beat and blood flow may promote various of patterns of angiogenesis.

## Patterns of Angiogenesis

Angiogenesis, in the simplest sense, has been defined as vascular growth and development. During embryonic development, blood vessels were recognized to have three origins: 1) angioblasts, endothelial cell (EC) precursors, aggregated to form blood vessels (vasculogenesis); 2) current vascular ECs mitotically divided (angiotrophic growth); or 3) an integration of the first two processes occurred where dividing vascular ECs encountered and integrated isolated angioblasts (Wilting and Christ, 1996). In adult animals, where angioblasts are no longer prevalent and vessels are already established, other patterns of angiogenesis have been described. These patterns have included sprouting, intussusceptive growth, blood vessel fusion, and intercalated growth (Wilting and Christ, 1996).

Of these patterns of angiogenesis, to date, sprouting has been the most well

described. The steps of sprouting include 1) a dissolution of the EC basal lamina at a particular point, 2) EC proliferation, evagination, and migration into the perivascular area, and 3) further proliferation and migration as drawn toward an angiogenic stimulus. In the process of migration in the perivascular area, ECs deposited extracellular fibronectin and laminin in cooperation with local fibroblasts. Fibronectin and laminin allowed EC integrin attachment and thus facilitated scalar movement toward an angiogenic stimulus. Meanwhile vascular endothelial growth factor (VEGF) from vascular smooth muscle, with the aid of nitric oxide from ECs, provided a vectorial or directional chemotactic stimulus (Kubota et al., 1988; Davis and Camarillo, 1995; Drake et al., 1995; Wilting and Christ, 1996; Noiri et al., 1998; Tomanek and Ratajska, 1997). Cellular processes of apoptosis, vacuolation, and phagocytosis of ECs occurred within EC-dense sprouts to produce a lumen (Wilting and Christ, 1996; Meyer et al., 1997).

Other patterns of angiogenesis have not been as well described as sprouting. Intussusceptive growth involved the septation of larger sinusoidal capillaries transversely through the lumen to produce two smaller diameter vessels (Burri, 1992; Wilting and Christ, 1996). Blood vessel fusion involved the coming together and fusion into one larger vessel of two vessels derived from the bifurcation of a common vessel (Wilting and Christ, 1996). Intercalated growth, the focus of the present study, involved mitotic division to increase vessel diameter and length, similar to angiotrophic growth described above (Wilting and Christ, 1996). These patterns of angiogenesis have been observed during embryonic development, wound healing, reproduction, and various pathogenic states such as cancer.

*Humoral Regulation of Angiogenesis*

The circulatory system of the chick embryo grows rapidly throughout the incubation period, as indicated by an increase in the area of the extraembryonic vasculature from 5 to almost 35 $cm^2$ between day 2 and day 6 of incubation (Romanoff, 1960). The growth rate of the embryo and its vasculature has been attributed to the presence of chemical (humoral) factors. At least three such factors have been tied to the formation of vascular structures in the chick embryo. Eichmann et al. (1997) demonstrated that vascular endothelial growth factor (VEGF) was important for the differentiation of endothelial and hemopoietic cells from the mesoderm and the formation of primitive blood vessels. Transforming growth factor- β (TGF-β) was shown to be important for chick intracardiac valve formation (Brown et al., 1996). Basic fibroblastic growth factor (bFGF) was demonstrated to have a role in the vascularization of the chick chorioallantoic membrane (Ribatti et al., 1995). These (VEGF in Senger et al., 1997; Transforming growth factor-β in Giudice, 1994; Fibroblastic Growth Factors in Staab et al., 1997; Lopez et al., 1997; and Giudice, 1994) and other factors such as platelet activating factor (PAF) (Andrade et al., 1992), endothelins (Giudice, 1994; Pedram et al., 1997), and estrogen (Cullinan-Bove and Koos, 1993), have been reported to induce angiogenesis in other *in vivo* as well as *in vitro* systems. Angiogenesis has, therefore, been described as the result of an imbalance between positive and negative humoral angiogenic regulators (Folkman, 1995).

### *Physical Regulation of Angiogenesis*

Several studies, including Clark (1918) and Champman (1918) have indicated that hemodynamic (physical) factors, which changed with vascular development (Hu and Clark, 1989), also play a role in vascular development and maintenance. Clark (1918) observed in the frog larvae (*Rana*) that those vessels in established capillary networks which received increasing blood flow, continued to develop, while those vessels in the same network, which received little blood flow, regressed and disappeared in time. Chapman (1918) demonstrated via heart ablation in the chick embryo that, while capillary networks and some larger vessels form, vascular structures did not grow and were not maintained in the absence of blood flow. Hence, a combination of both humoral and physical factors appeared to be important for vascular growth; i.e., angiogenesis.

Determination of the isolated influence of hemodynamic variables on angiogenesis in the embryonic model is impossible because of the presence of endogenous growth factors. The adult model, therefore, has often represented a more static system in which to study the influence of blood flow, and has offered insight into the influence of blood flow on angiogenesis. Commonly, studies have examined the influence of blood flow on angiogenesis in systems with increased nutrient demand and increased blood flow, as well as systems with reduced blood flow. Chronic nervous stimulation of adult skeletal muscle increased and maintained VEGF production (Annex et al., 1997), and increased capillary density (Myrhage and Hudlicka, 1978; Hudlicka and Price, 1990). Hudlicka et al. (1992) suggested that the angiogenic response of capillaries to long-term vasodilation which resulted in increased capillary density and increased

capillary to fiber ratios in cardiac and skeletal muscles, was presumably produced by increased erythrocyte velocities and luminal shear stresses.

To regulate blood flow for experimentation, vessel ligation or clamping has been used in adult animals. Vessel ligation after the point of bifurcation caused increased blood flow in collateral vessels (Bloomer et al., 1949). In such instances, collateral vessels then demonstrated intercalated-growth-angiogenesis by increasing in diameter (Bloomer et al., 1949; Charan and Carvalho, 1997). Vessel ligation also caused an accumulation of metabolic wastes in the tissues and introduced localized hypoxia, as evidenced by the reactive hyperemic response demonstrated after the reinstatement of blood flow (Berne et al., 1998). Hence, ligation introduced an altered state in more than just blood flow; this may provide argument against the moderation of blood flow via ligation.

Ischemia and accompanying tissue hypoxia have been demonstrated to induce angiogenesis. In cardiac muscle, ischemia, and resulting tissue hypoxia, induced angiogenesis (Hudlicka et al., 1992). Induced myocardial infarction produced increased capillary density in ischemic regions surrounding the infarcted tissue (Xie et al., 1997). Interestingly, ischemia in the rat *tibialis anterior* (TA) muscle produced no change in capillary-to-fiber ratios (Hudlicka and Price, 1990). Change in the oxidative capacity of the muscle fibers in the ischemic rat TA, however, indicated that hypoxia did not result from the induced ischemia in this instance (Hudlicka and Price, 1990). In an effort to circumvent the confounding effects of hypoxia on flow-induced angiogenesis, Wang and Prewitt (1991) examined cremaster muscle capillary density with uninterrupted blood

flow in a unilaterally orchidectomized rat. They found that reduced functional load inhibited muscle growth and inhibited normal age-increased capillary density simultaneously, when compared to a younger control and the normal contralateral cremaster muscle. This demonstrated that reduced blood flow alone, isolated from the confounding effects of hypoxia, was able to induce vascular remodeling.

Their variable environment requires that ECs must be able to sense and react to changes around them. *In vivo* and *in vitro* studies have shown that endothelial cells respond to vascular hemodynamic stimuli (Ando and Kamiya). In fact, Koller and Kaley (1991) suggested that one major role of the endothelium is to optimize wall shear stress through regulation of vessel diameter. Reviews by Ando and Kamiya (1993) and Papadaki and Eskin (1997) concluded that the mechanism for exactly how ECs recognize and convert a mechanical stimulus to biochemical information is yet unclear. They suggested that mechanostimulus may be transduced via cytoskeletal-extracellular connections such as transmembrane intregins, or maybe via desmosomes and tight junctions. Furthermore, Papadaki and Eskin advanced that mechanostimulus may be transduced through channel-mediated ion influx of $K^+$ and $Ca^{2+}$, for example, or through linkage to G-protein receptors. Evidence exists which could implicate any one or all of these pathways (for review see Ando and Kamiya, 1993; and Papadaki and Eskin, 1997).

Endothelial cells (ECs) experience two kinds of forces– a pulsatile, stretch-force which acts circumferentially, and a shear, frictional force which acts longitudinally in the direction of the blood flow (Papadaki and Eskin, 1997). Thoma (in Clark, 1918) suggested that stretch forces caused vessel thickening as well as vessel longitudinal

growth. Stretch was shown, more recently, to increase transforming growth factor-β mediated myocardial VEGF expression (Annex et al., 1997; Li et al., 1997). Hudlicka et al. (1992) suggested that stretch-induced release of basic fibroblastic growth factor from stored pools in the basal lamina produced stretch-resultant EC proliferation. Shear stresses were also shown to cause EC cytoskeletal actin to align in the direction of flow (Wong et al., 1983; White and Fujiwara, 1986), promote EC proliferation and migration, and increase EC production of histamines which increase vascular permeability (Ando and Kamiya, 1993). Shear stresses also modulated EC production of prostacyclin, an anticoagulant and vasodilator, epidermal growth factor (EDRF) (also known as nitric oxide), possibly endothelin-1 (ET-1), a vasocontrictor and mitogen, tissue plasminogen activator (tPA), a growth factor, and platelet-derived growth factor (PDGF), a smooth muscle cell mitogen and vasocontrictor (Ando and Kamiya, 1993). Shear stress was also demonstrated to down-regulate EC fibronectin production, which is important to EC migration (Ando and Kamiya, 1993; Heubsch et al., 1996; Chon et al., 1997; Grant and Kleinman, 1997). Though it is unclear how ECs mechanotransduce shear and circumferential stresses to biochemical signals, their stimulus-induced production of these numerous humoral factors is evidence that ECs mediate hemodynamic information for numerous angiogenic pathways.

## Role of Hemodynamics in Embryonic Angiogenesis

It has been established that humoral and physical factors can modulate vascular growth. Allusions to the high rate of vascular growth experienced during the first few

days of embryonic development in the chick embryo have also been made. The roles of humoral and physical factors in angiogenesis are often treated as distinctly separate. It has been demonstrated above, however, that an important interplay between both factors does exist. The question thus arises for a model system such as the developing embryo, where humoral and physical stimuli are present in abundance, which stimulus is most important for angiogenesis? Furthermore, demonstration of the value of physical angiogenic stimuli in vessel maintenance or growth might designate an new role for the early embryonic heart beat and blood flow.

The present study attempts to address the importance of hemodynamic angiogenic stimuli in the embryonic model. I hypothesized that even in the presence of humoral angiogenic stimuli such a growth factors, vessel maintenance would require the presence of hemodynamic, physical angiogenic stimuli. Chapman (1918) observed that by 3.5 days (HH stage 19) of no blood flow due to cardiac ablation, vessels which had begun to establish themselves, began to regress. In the present study, the vessels of 3 day chick embryo (HH stage 18), in contrast to those vessels in Chapman (1918), were established for more than 24 hr. and had experienced hemodynamic stresses such as blood pressure and flow during this time prior to experimental elimination of blood flow. Using the 3 day chick embryo as a model, one pattern of angiogenesis, intercalated growth, was quantified. I predicted that through systemic blood flow elimination via the minimally invasive technique of conotruncal ligation, I would quantify the absence of intercalated growth or a regression of intercalated growth as measured by vessel diameter, compared to vessels with normal blood flow.

# CHAPTER 2

# MATERIALS AND METHODS

## Egg Storage and Growth

Medium to large sized white fertile chicken eggs (Leghorn) were obtained from California Golden Egg (Sacramento, CA) and Hyline International (Lakeview, CA). Eggs were either refrigerated in storage at 6-10°C and 80% humidity or immediately placed in an incubator in Hobart rotating trays, blunt end up, at 38°C and 60% humidity. Eggs were allowed to incubate until the embryos had reached Hamburger-Hamilton (HH) (Lillie, 1952) Stage 18 (approximately 3 days). Eggs were not stored longer than 2 weeks prior to incubation because extended storage increases mortality and delays growth (Haque et al., 1996). During procedures below, intact eggs were held in a thermostated chamber which kept the embryo's temperature between 33 and 35°C, as measured by mercuric thermometer. The eggs were windowed above the air cell and embryos were reached by removal of the outer membrane.

## Cardiac Rhythm, Blood Flow, and Ink Injection

Preliminary trials indicated that injection of India ink into each embryo was

necessary to visualize vessels via videomicroscopy for measurement (see Ahemorrhea procedure below). In an effort to understand the influence of ink injection on heart rate and blood flow, HH Stage 18 embryos ( n = 21) were separated into Control (n = 7), Ink-injected (n = 7), and Saline-injected (n = 7) groups, and heart rate and blood flow were measured. The control group received no injection. The ink-injected group received ink injections of adequate dosage (0.186 ± 0.017 μl) to darken the vessels for use with videomicroscopy. The saline-injected group received 0.276 μl injections of 0.75% saline mixed at a volume:volume ratio of 0.5 ml Schilling green food coloring to 15 ml of saline. Ink and Saline were injected through a pulled capillary tube (diameter between 25 and 50 μm) using a nanoliter injector (World Precision Instruments, Inc) mounted on a micromanipulator. All injections occurred over a one minute period.

Flows were measured using a 545C-4 Directional Pulsed Doppler Flowmeter (Bioengineering, Univ. of Iowa) attached to a 20MHz Doppler probe. The probe consisted of a 1 mm piezoelectric crystal threaded through polyethylene tubing which was subsequently threaded through and attached to glass Pasteur pipette for stability (similar to the setup described in Clark and Hu (1982)). The probe was angled 45° to the dorsal aorta and placed caudal to where the posterior cardinal vein , which travels parallel to the dorsal aorta, separates from the dorsal aorta to enter the sinus venosus. The voltage signal emitted from the flowmeter was filtered through a Narco Bio-systems (Houston, TX) 4-channel physiograph, model 780-1810, with a universal biocoupler type 7189 (sensitivity set at 500 mV and the filter set at 3 Hz), and ultimately captured and recorded using a computer data acquisition program (Sable Systems International). Recorded

voltage changes were averaged and converted to flows using the formula: 1kHz shift • 1.25 • $d^2$, where 1kHz shift = 500 mV and d = diameter of the vessel in which flow was measured. Total measurement duration lasted no more than 20 minutes, and all evaluated measurements occurred at the end of the collection period for consistency. The average time collection periods were 10.2 min for the Control group and 17.6 min for the Ink and Saline-injected groups. Heart rate values were derived from the voltage oscillations captured during the above flow measurement.

## Ahemorrhea

Embryos were randomly divided into 3 groups: a control group (n = 7), a sham group (n = 6), and a ligated group (n = 10). The sham group underwent surgical placement of 11-0 silk suture through the heart loop and the suture was left unconstricted throughout the treatment period. The ligated group also underwent surgical placement of 11-0 silk suture through the heart loop. The suture was looped around the conotruncus arteriosus and constricted to eliminate systemic blood flow throughout the experiment. The absence of blood flow was confirmed by visual examination of the extraembryonic vessels.

Prior to any treatment, embryos of all groups received mouth-pipetted injections of India ink to provide contrast to the vasculature. The mouth pipette consisted of a pulled glass capillary tube with a tip diameter of 25 to 50 μm attached polyethylene tubing (Fisher Scientific, Inc). The pulled capillary tube was angled to between 20° and 45° under open flame to facilitate vessel wall penetration. Ink was injected into the

posterior vitelline vein and was allowed to flow until the vasculature was adequately constrasted for videotaping. Injection was completed within 1 min. Small hemorrhage (volume unquantified) occurred at the site of injection immediately following the removal of the pulled capillary tube and was stopped within 4 sec. using fine tipped forceps to pinch and seal the vessel.

After ink injection and treatment, all embryos were reincubated for 6 hr. Because ligature placement required approximately 30 min, the reincubation time of the sham and ligated groups was offset from that of the control group (Figure 1). To stop the heart after the reincubation period, embryos from all groups received a topical application of one drop of 0.75% KCl (14.1± 1.5 μl) from a 1 cc tuberculin syringe, except in the case of one control group embryo and one sham group embryo where two drops of 0.75% KCl (24.8 ± 3.0 μl) were used. Dyed KCl was used to visually determine the spread of the KCl. One drop of KCl placed over the heart spread to all the measurement points (below).

Each embryo was videotaped at 400 X using a Javelin camera mounted to a Leica Wild Mz32 or Zeiss surgical dissecting scope immediately following ink injection, after treatment, after 6 hr. reincubation, and after the heart was stopped. Each respective embryo was videotaped at all points in time with the same dissecting scope. Videotape of each embryo was analyzed using an OPTIMAS imaging and analysis system (Bioscan, Inc., Edmunds, WA). Measurements were taken of the outer-diameter of the eye, the anterior cardinal vein, the anterior vitelline vein, a vitelline artery, and a vitelline vein. To establish consistency, vessel branches were described as primary, secondary, etc..., by

counting visible branches from the embryo distally. All vitelline vessel measurements were taken from a primary artery and a primary vein. Since large variations were seen between embryos of the same treatment group (within-group variations), vessel outer-diameter values were normalized for each embryo as a percent change of initial post-contrast dye injection measurements.

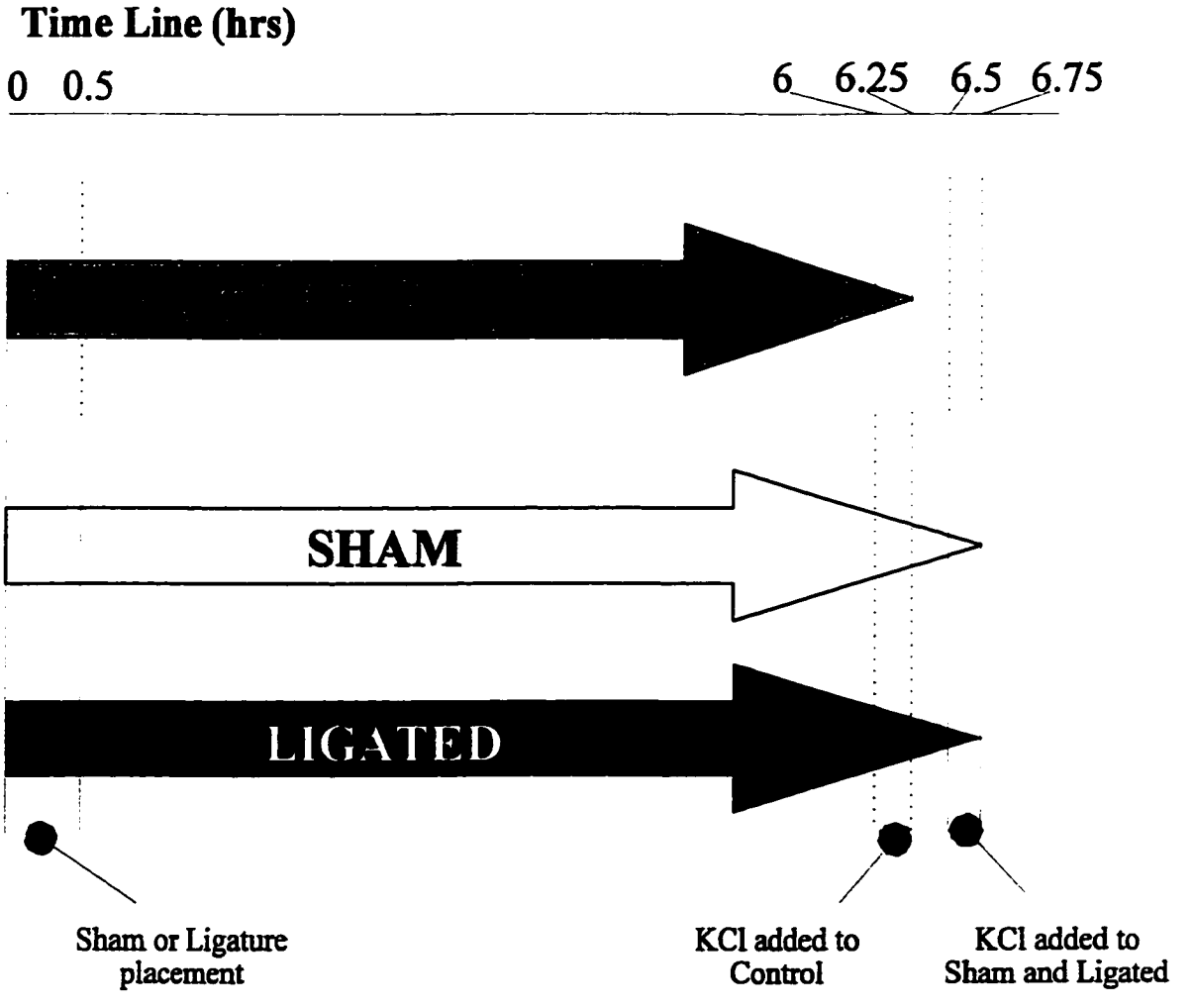


**Figure 1**: A treatment period time line for the control, sham, and ligated groups. Measurements were taken at each the point of time represented by solid lines.

## Quantification of Injection Volume

The nanoliter injector (mentioned previously) delivered a precise, known volume of ink into the blood stream. Mouth-pipetted injections, in contrast, delivered an unknown volume of ink into the blood stream. Also, the volume of ink injected into each embryo depended upon each embryo's respective blood volume and presumably vascular area. To quantify the volumes injected via mouth pipetting into each embryo, two approaches were taken. The first approach involved deriving volume values from changes in the mass of the mouth pipette as a result of injection. HH Stage 18 embryos (n = 5) received mouth-pipetted injections of India ink of adequate dosage to darken the vessels for videomicroscopy as previously described. The mass of the injection apparatus was verified immediately before and after each injection. Changes in mass seen as a result of injection were then averaged and then divided by the average density of the ink to obtain an average injection volume (Table 1).

The second approach involved creating a standard curve to correlate a known injected volume to various morphometric measurements. Using the nanoliter injector mentioned previously, HH Stage 18 embryos (n = 7) received injections of India ink of

**Table 1**: Mean injected volume estimated for injections via mouth pipetting (± SEM)

| Mean Ink Mass Differences (mg) (n = 5) | Mean Ink Density (mg/ml) (n = 6) | Mean Injected Volume (μL) |
|---|---|---|
| 0.56 ± 0.1 | 1064.2 ± 17.3 | 0.53 |

adequate dosage to darken the vessels for videomicroscopy (0.183 ± 0.017 µl) into the posterior vitelline vein. Each embryo was then videotaped. Measurements were then taken of the area of the eye, area of the embryo, and perimeter of the embryo using the above mentioned OPTIMAS computer program. A vascular index of the *area vasculosa* similar to that used in Birchard and Reiber (1993) was also produced. Briefly, a digital image of the *area vasculosa* was captured through the OPTIMAS program and pulled into Corel Presentations 8 (Corel Corporation Limited, Dublin 2, Ireland). There, four rings of 1, 2, 3, and 5 mm diameters were overlaid onto the image, and then the number of vessels which transected each ring were counted (Figure 2). All counting was carried out by the same individual, someone independent of this study, in a single-blind fashion. No distinction was made between arteries or veins.

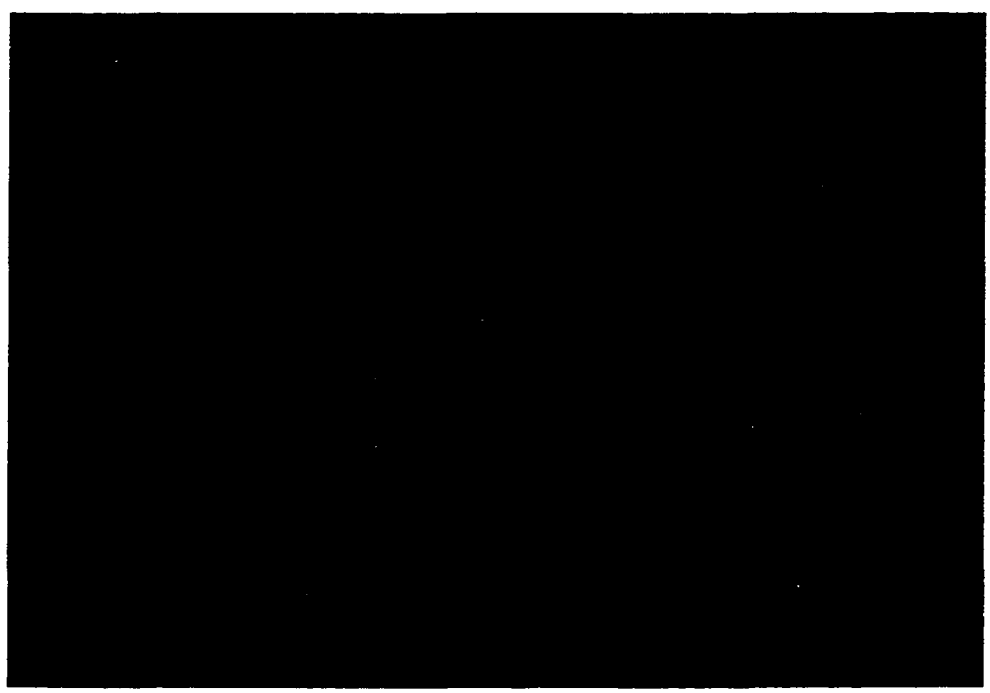

**Figure 2:** The area vasculosa with a circle overlay used to produce a vascular index.

## Statistics

One-way ANOVA was used to compare blood flow and heart rate values between control, saline-injected, and ink-injected groups. One-way ANOVA was also used to compare the mean volumes of ink injected to determine the influence of ink injection on blood flow and to establish standard curve for a morphometric characteristic. An independent t-test was used to compare the larger of these two mean ink volumes to the maximum mean volume approximated by ink mass-density relationships.

Repeated Measures ANOVA was used to compare changes in vessel diameter between control and sham, and sham and ligated groups. F and P-values are reported for ANOVA tests. As a note, within-group (group versus time) and between-group (group versus group) comparisons are reported. Emphasis must be placed on between-group comparisons (group versus group). Regression analysis was used to correlate injected volume to the morphologic variables (area of the eye, area of embryo, perimeter of the embryo, and vacular index) and $r^2$ values were evaluated as a relationship indicator. Herein, values are mean ± SEM. SPSS 8.0 statistical software (SPSS, Inc) was used for all statistical analyses and the critical value for significance was set at $\alpha = 0.05$.

# CHAPTER 3

# RESULTS

## Cardiac Rhythm, Blood Flow, and Ink Injection

No significant difference in flow existed between the control, saline-injected, and ink-injected groups (Figure 3; df 2, 18, $F = 0.359$, $p = 0.703$). Likewise, no significant difference in heart rate was found between the control, saline-injected, and ink-injected groups (Figure 4; df 2, 18, $F = 1.618$, $p = 0.226$).

## Ahemorrhea

In control, sham, and ligated groups, the eye as measured by outer diameter increased by approximately 20% during the 6 hr. reincubation period (Figure 5; control df 1, 5, $F = 41.615$, $p = 0.001$; sham df 1, 5, $F = 114.593$, $p = 0.000$; ligated df 1, 9, $F = 26.995$, $p = 0.001$) and showed no difference in the rate of growth between the three groups (sham v. control, df 1, 10, $F = 1.383$, $p = 0.267$; ligated v. sham, df 1, 14, $F = 2.043$, $p = 0.175$).

The intraembryonic anterior cardinal vein in the control group, grew significantly by more than 35% over the 6 hr. treatment period (Figure 6; df 1, 6, $F = 11.789$, $p =$

0.014). The anterior cardinal vein in the sham group experienced a significant 20% increase over the treatment period (df 1, 5, F = 9.28, p = 0.029). The anterior cardinal vein of the ligated group did not change significantly with time (df 1, 5, F = 4.922, p = 0.077), though a trend of bulging is visible immediately after ligation (Figure 6). Statistical comparisons among groups indicated that the final anterior cardinal vein

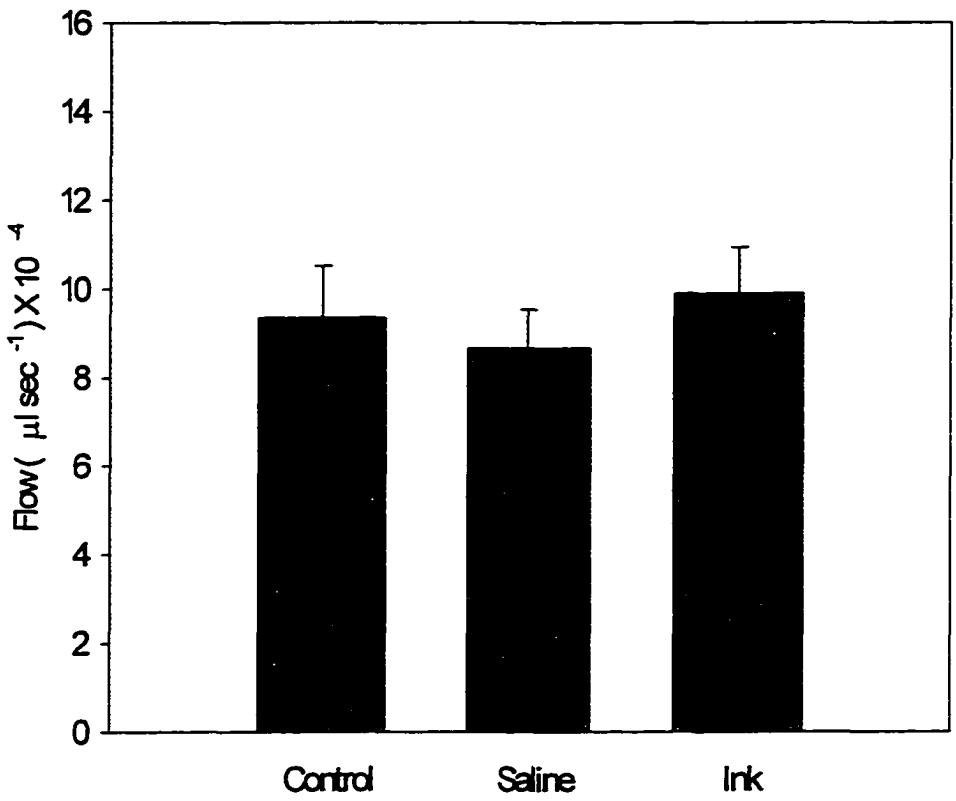


**Figure 3**: Average blood flows during 2 a two min. period in the control (n = 7), saline-injected (n = 7), and ink-injected (n = 7) groups in the chick embryo (HH stage 18) dorsal aorta. (Mean ± SEM)

diameters after the addition of KCl were no different between the sham and the control groups (df 1, 11, F = 1.123, p = 0.312), and the ligated and the sham groups (df 1, 10, F = 0.469, p = 0.509).

The diameter of the extraembryonic anterior vitelline vein did not change significantly during the treatment period in either the control (Figure 7; df 1, 5, F = 1.495,

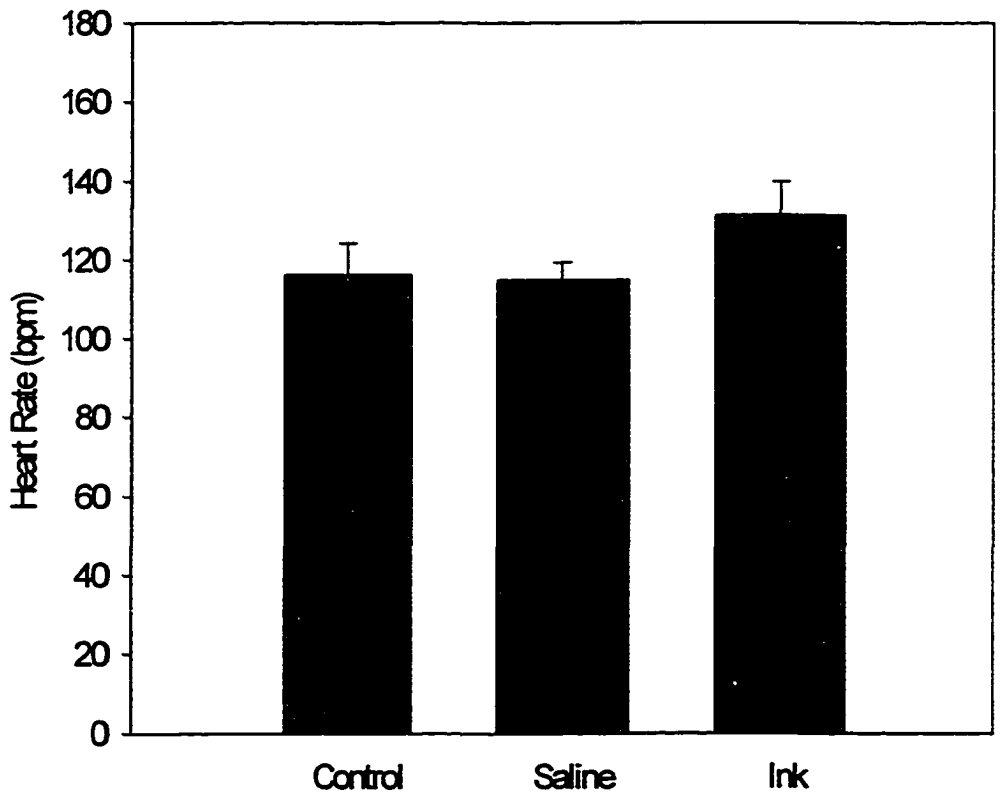


**Figure 4**: The heart rate of the chick embryo (HH stage 18) during flow measurement in the control (n = 7), saline-injected (n = 7), and ink-injected (n = 7) groups. (Mean ± SEM)

p = 0.226), or the sham groups (df 1, 4, F = 6.293, p = 0.066). The anterior vitelline vein of the ligated group decreased significantly in diameter during the treatment period (Figure 7; df 1, 9, F = 13.827, p = 0.005), with its constriction most prevalent after the addition of KCl. Cross-group comparisons demonstrated that at the end of the treatment period after the addition of KCl, no difference existed between the control and sham

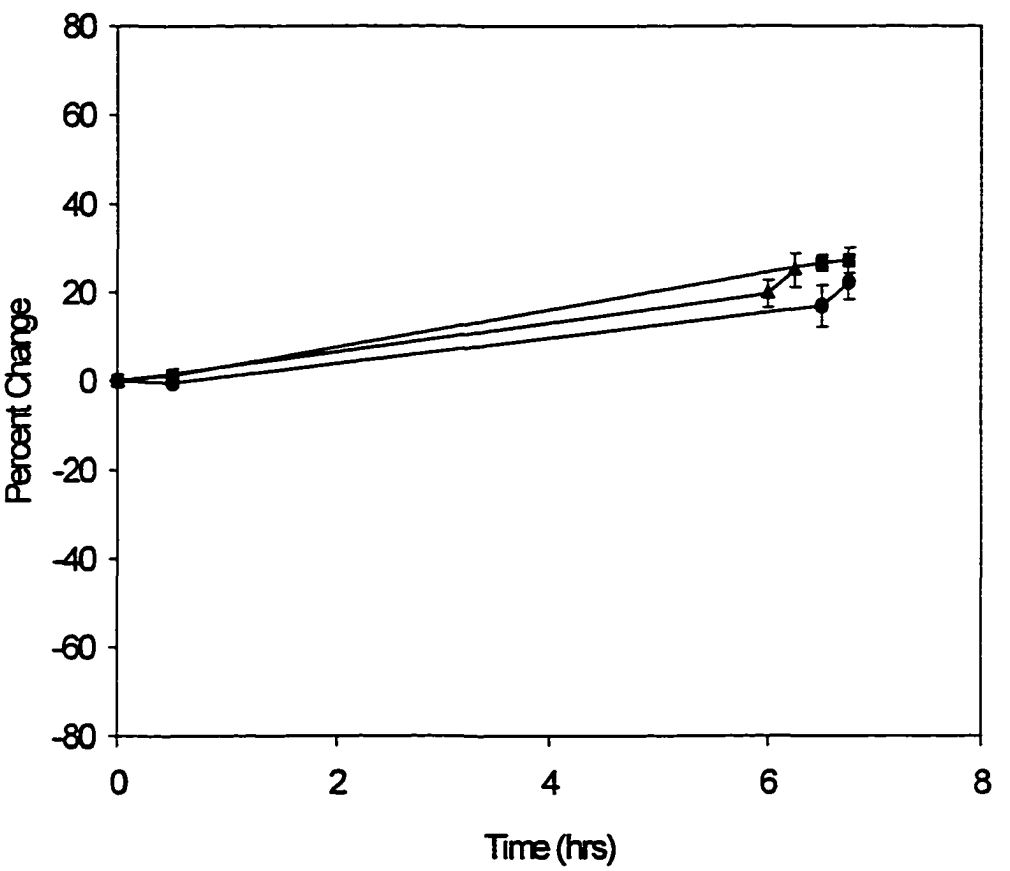


**Figure 5**: Percent change in diameter of the eye of the chick embryo (HH Stage 18) in the control (▲), sham (■), and ligated (●) groups.

groups (df 1, 9, F = 4.797, p = 0.056) and no difference existed between the ligated and sham groups (df 1, 13, F = 0.121, p = 0.734).

The vitelline artery of the control group did not significantly change over the treatment period (Figure 8; df 1, 6, F = 2.891, p = 0.140). The vitelline artery of the sham and ligated groups changed significantly after the treatment period (Figure 8; sham df 1,

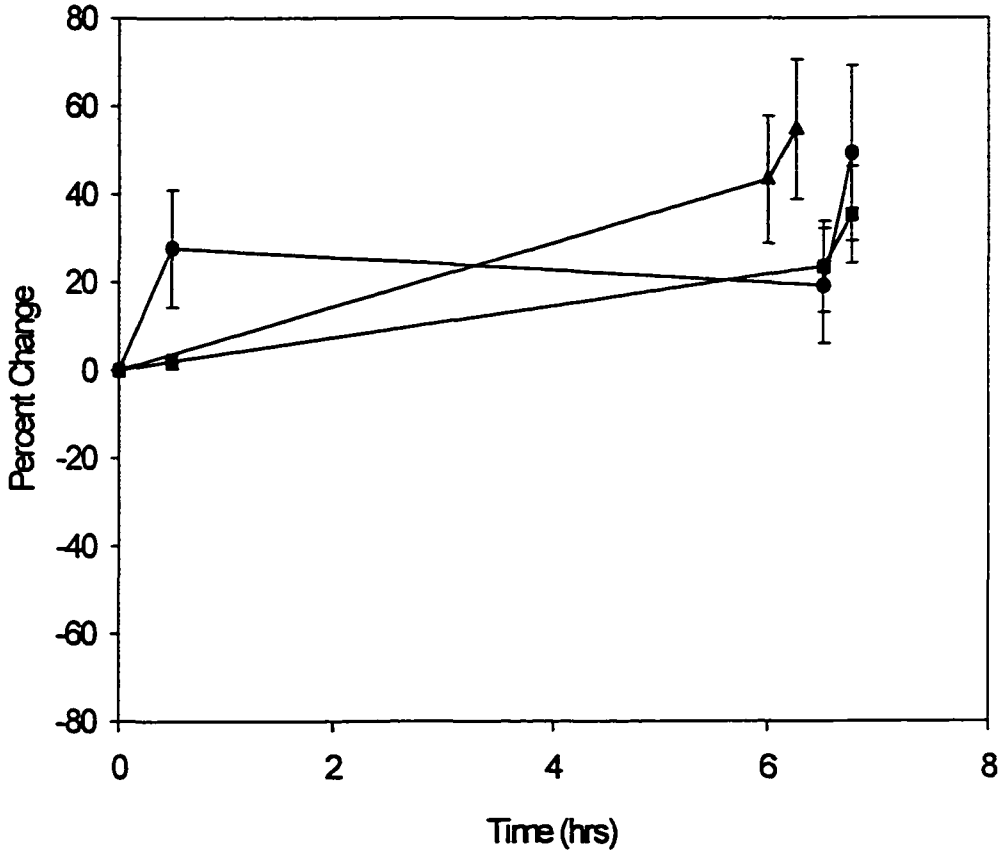


**Figure 6**: Percent changes in diameter of the chick embryo (HH Stage 18) anterior cardinal vein in the control (▲), sham (■), and ligated (●) groups.

5, F = 23.925, p =0.005; ligated df 1, 9, F = 13.827, p = 0.005). The vitelline artery of the sham group appeared to grow in diameter until the addition of KCl with caused a decrease in diameter of nearly 40 %. The vitelline artery of the ligated group decreased in diameter after ligature placement, showed no growth after 6 hr., and showed 10%

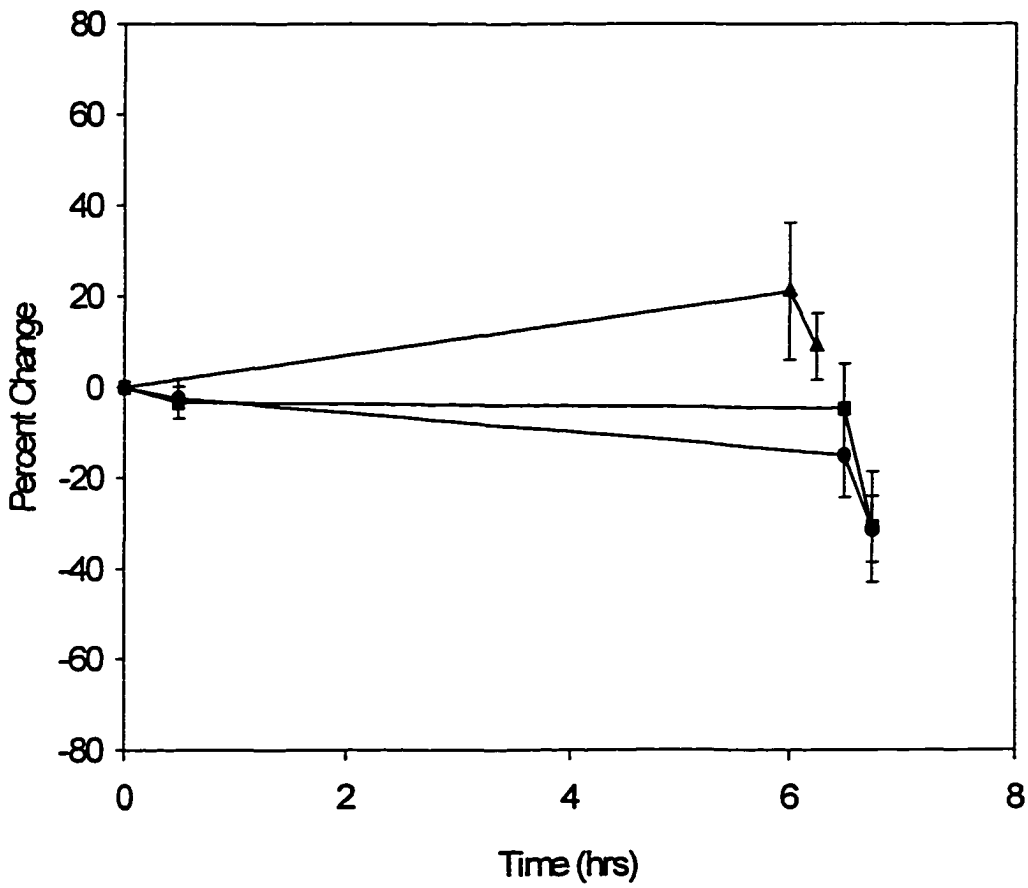


**Figure 7**: Percent changes in diameter of the chick embryo (HH Stage 18) anterior vitelline vein in the control (▲), sham (■), and ligated (●) groups.

decrease in diameter after the addition of KCl. Comparisons of sham and control groups indicated that no significant treatment effect existed between them (Figure 8; df 1, 11, F = 0.104, p = 0.753). Comparisons of the ligated and sham groups indicated a significant treatment effect existed between them after ligature placement (df 1, 14, F = 16.231, p =

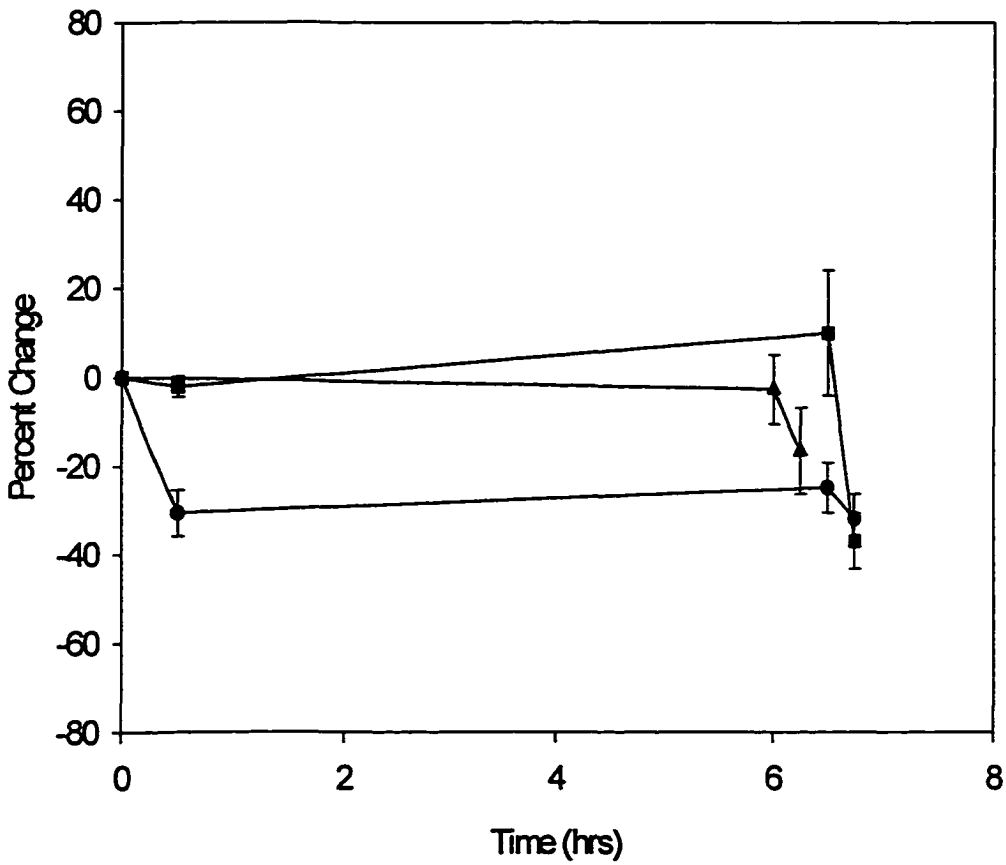


**Figure 8**: Percent changes in diameter of the chick embryo (HH Stage 18) vitelline artery in the control (▲), sham (■), and ligated (●) groups.

0.001) and after the 6 hr. treatment period (df 1, 14, F = 18.305, p = 0.001), while no difference existed after the addition of KCl (df 1, 14, F = 2.213, p = 0.159).

The vitelline vein did not change significantly in the control or ligated groups over the treatment period (Figure 9; control df 1, 6, F = 0.170, p = 0.695; ligated df 1, 8, F = 2.265, p = 0.171). It did decrease significantly in the sham group (df 1, 5, F = 29.437, p = 0.003). The diameter of the vitelline vein in the sham group remained unchanged during

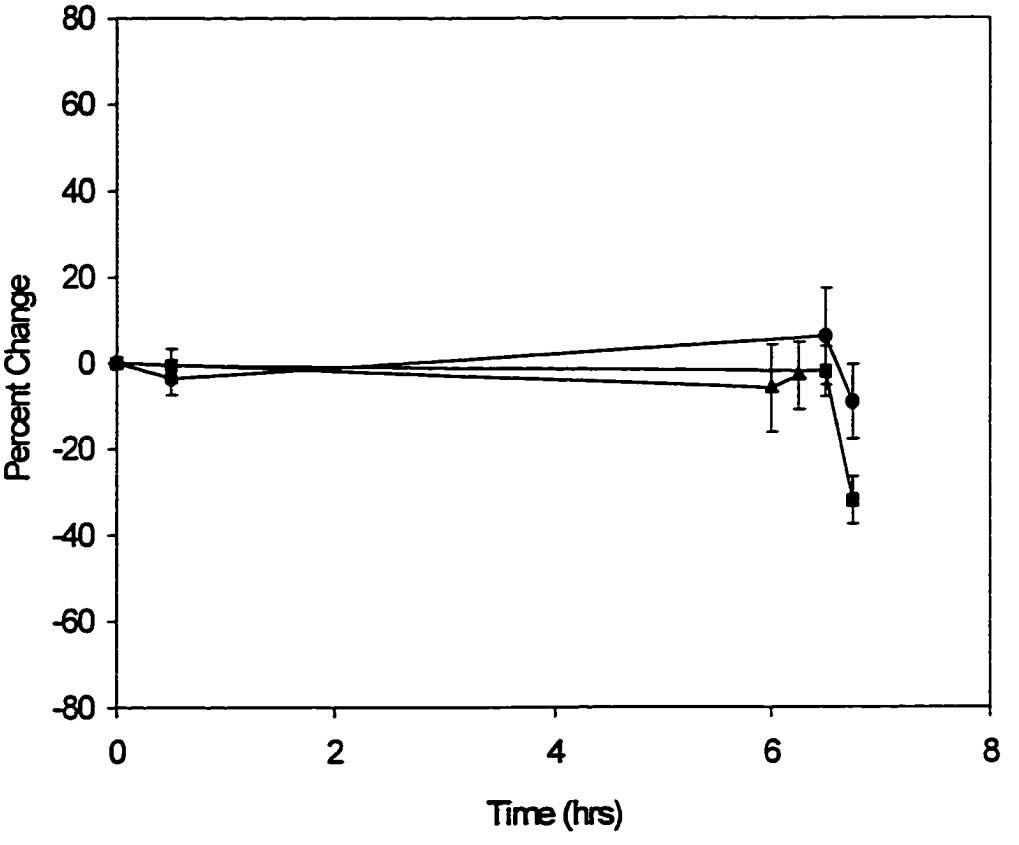


**Figure 9**: Percent changes in diameter of the chick embryo (HH Stage 18) vitelline vein in the control (▲), sham (■), and ligated (●) groups.

the 6 hr. treatment period (Figure 9). After the addition of KCl, the diameter decreased by approximately 35%. When compared against each other, the sham and the control groups were not statistically different after the treatment period (df 1, 11, $F = 2.239$, $p = 0.163$). Similarly, the ligated and sham groups were not significantly different after the treatment period (df 1, 13, $F = 1.047$, $p = 0.325$).

## Quantification of Injection Volume

None of the tested variables proved to have exceptionally strong correlation with injected volume (Table 2). It was, therefore, not possible to create a standard curve to approximate the volume injected by mouth pipetting during the Ahemorrhea experiment. The mean volume of ink injected to determine the effect of ink injection on blood flow ($0.186 \pm 0.017$ µl) and the mean of the volumes injected to correlate injection volume to a

**Table 2**: Statistical correlation results to facilitate determination of injected volume

| Variable | $r^2$ | P value (slope different from zero) |
|---|---|---|
| Area of Embryo (n = 7) | 0.16 | 0.373 |
| Snout-Eye Cephalic Transect (n = 7) | 0.51 | 0.07 |
| Circular Area of Eye (n = 7) | 0.15 | 0.389 |
| Perimeter of Embryo (n = 7) | 0.01 | 0.498 |
| Vascular Index (n = 7) | 0.23 | 0.279 |

morphometric characteristic (0.183 ± 0.017 µl) were not significantly different (df 1, 12, F = 0.02, p = 0.891). The mean ink volume injected by mouth pipette (Table 1) provided an approximate injected volume which was significantly greater (df 6 T = -20.71, p = 0.000) than the largest mean volume injected (0.186 ± 0.017 µl ) using the nanoliter injector to determine the influence of ink injection on blood flow. Two methods suggest that the volume of ink injected during the Ahemorrhea experiment was between 0.183 and 0.186 µl. A third method placed the ink injection volume during the Ahemorrhea experiment at 0.53 µl.

## Further Observations

Ligation produced several interesting and noteworthy effects. Immediately following ligation, and in some instances where the heart continued to beat throughout the treatment period, blood in the venous system began to pulsate forward and reverse with each contraction of the heart. This was seen in the anterior cardinal vein as well as the large trunks of the lateral vitelline veins, but rarely in the anterior vitelline. Furthermore, and in contrast to heart surgical ablation studies as discussed in the Discussion section, blood drained from the capillaries and venous vessels of the *area vasculosa* to pool in the large veins proximal to the embryo. Larger arteries, however, maintained a small volume of intralumenal blood. Except in the case of these arteries, if it were not for the presence of carbon particles from injected India ink which clung to the vessel walls, the extraembryonic vasculature would have become invisible. Also to be noted, a lack of blood flow produce a tremendous "edema" effect within the amnion and

under the surface of the inner vitelline membrane. A similar effect, though much less pronounced occurred in the sham group. Ligation also caused change in the structure of the *area vasculosa*. A lack of blood flow seemed to cause the *area vasculosa* to shrivel and contract in a radial fashion toward the embryo. A lack of blood flow also appeared to cause a loss of structural integrity most notably to the walls of the larger veins. It was indeterminable whether similar changes occurred to capillaries or smaller veins. No loss of structural integrity was visible in vitelline arteries.

# CHAPTER 4

# DISCUSSION

## Blood Flow and Intercalated Vessel Growth

The aim of this research was to address an alternative role for the early embryonic heart beat and blood flow. The early embryo grows in a milieu of stimuli, humoral and hemodynamic, which can work in parallel as well as cooperatively to promote angiogenesis. I hypothesized that even in the presence of these humoral angiogenic stimuli, vessel maintenance would require the presence of hemodynamic, physical stimuli. The approach of this study was to measure changes in vessel growth – intercalated (mitotic) growth – from the perspective of vessel diameter under conditions of experimentally eliminated blood flow. Results from this study indicate that vessel maintenance and intercalated growth in diameter may be independent of blood flow during a 6 hr. period, contrary to my original hypothesis. In the absence of blood flow, the diameter of the intraembryonic anterior cardinal vein remained unchanged and ultimately did not differ from the diameter of control vessels which had normal flow (Figure 6). The diameter of extraembryonic anterior vitelline vein decreased in the absence of flow for 6hr., but ultimately measured no differently than control vessels

which had had normal flow (Figure 7). The diameter of the extraembryonic vitelline artery decreased significantly after the elimination of blood flow, but after 6 hr., its diameter also remained unchanged compared to control vessels which had had blood flow (Figure 8). Finally, the diameter of the extraembryonic vitelline vein did not change over a 6 hr. period in the absence or presence of blood flow (Figure 9). To summarize, in this study blood vessel diameter changed independently of the influence of blood flow. This suggests that even though both humoral and hemodynamic stimuli are present and dictating growth, any growth, lack of growth, or regression at this point in development is solely under the command of humoral angiogenic factors. However, since vessels did not show measurable signs of regression, this also suggests that the presence of a hemodynamic stimulus such as blood flow, while not important for dictating growth, somehow stabilized the vessels against regression.

It is well established that blood flow is an important angiogenic regulator, though it mechanisms for regulation are not entirely clear. (For complete reviews see Hudlicka, et al., 1992; Ando and Kamiya, 1993; Skalak and Price, 1996; Ando and Kamiya, 1996; Papdaki and Eskin, 1997; and Hudlicka, 1998.) In brief, it has been shown that endothelial cells (ECs) experience circumferential, tensional, and shear stresses from vascular pressure and flow changes. Information regarding these forces can be transduced by ECs and ultimately translated to produce many forms of vascular growth (angiogenesis). Blood flow is, therefore, important, if not required, for vascular maintenance and growth. Thoma (in Clark, 1918) indicated, among other things, that 1) increase or decrease in the size of a vessel is dictated by the magnitude of blood flow, and

2) increase or decrease of vessel wall thickness depends on blood pressure. If Thoma (in Clark, 1918) had been referring to immediate changes in vessel diameter, the results in Figure 8 would support his statement. In terms of growth, however, results from this study suggest that in the short-term, trends of dilation (Figure 6) or constriction can occur (Figures 7 and 8), but over a 6 hr. period in the absence of blood pressure and flow, no significant changes in vessel diameter occur. The intraembryonic anterior cardinal vein increased in diameter (growth) independent of blood flow (Figure 6). The extraembryonic anterior vitelline vein decreased in diameter in the presence and absence of blood flow (Figure 7). Interestingly, though, this decrease in diameter is partially linked to the act of microsurgery required to eliminate blood flow, and not entirely tied to the elimination of blood flow itself. In the vitelline artery and vein, apart from the decrease in diameter seen in the vitelline artery due to the pressure drop immediately after ligation of the conotruncus arteriosus, the vitelline artery and vitelline vein diameters remained unchanged after 6 hr. of no blood flow (Figures 8 and 9). These results would not be predicted from those of Thoma (in Clark, 1918).

The results of the present study also appear to contradict the results of several other studies, including Chapman (1918) and Clark (1918). Chapman (1918) demonstrated that ablation of the chick embryo heart before circulation began (HH stage 12) did not prevent capillary network formation and formation of ill-defined, sinusoid-like large vessels in the extraembryonic vasculature. A lack of circulation also did not prevent large vessel formation such as that of the dorsal aorta and cardinal veins intraembryonically. A lack of blood flow for 72 hr. caused large extraembryonic vessels

to begin regression and reversion back to capillary networks. By 8 days with no blood flow, even the capillary networks had begun to regress to formations which resembled primitive blood islands, the first structures seen in circulatory development. Through non-invasive observations, Clark (1918) described how from initial capillary networks those vessels which received progressively greater blood flow thickened and lengthened, while those vessels which received less flow regressed and disappeared. More recently, Männer et al. (1995) observed that a lack of blood flow generally caused structural changes to the vitelline vasculature.

In the present study, while no measurable changes due to a lack of blood flow were observed, unmeasured changes did occur which resemble those reported by Chapman (1918), Clark (1918), and Männer et al. (1995). The absence of flow initiated what appeared to be a dissolution or loss of structural integrity of the vessel walls especially in the anterior and lateral vitelline veins. Dissolution or regression of capillary networks was indeterminable, but unmistakable contraction of the *area vasculosa* similar to that observed by Champan (1918) and Männer et al. (1995) was noted. The apparent disagreement between the results of this study with its stated hypothesis and with the predictions which can be made from the literature, may be resolved or explained by examination of the various techniques applied, by examination of how vessels grow, and by the examination of oxygen availability.

## Critique of Techniques

### *Treatment Period*

One of the possible explanations for why an absence of flow did not induce changes in vessel diameter in the present study is the length of the treatment period. In studies such as Chapman (1918) and Männer et al. (1995), where flow dependent changes were reported, treatment periods ranged from 24 hr. to several days. Clark (1918) made his observations on the tail of the frog larvae (*Rana*) over more than a one month period. Chapman (1918) reported that no change in the vasculature had occurred in the absence of flow after 24 hr., though the embryos were comparably much younger than those of the present study. Other studies which examined vascular changes due to increased flow used short and long duration treatment periods. Hudlicka and Price (1990) used daily stimulation of adult rat skeletal muscle for 2 weeks to demonstrate that increased blood flow was necessary to increase capillary to fiber ratios. Wang and Prewitt (1993) demonstrated in the adult rat cremaster muscle the influence of decreased blood flow on arterial diameter and arteriolar number after a treatment period of 4 weeks. *In vivo* and *in vitro* studies on the structural as well as mitotic effects of shear stress on endothelial cells implemented treatment periods ranging from 24 hr. up to 1 month (Ando and Kamiya, 1993).

In the present study, the treatment period was designated 6 hr. for several reasons. It was observed that the eye grew over the 6 hr. period (Figure 5). Furthermore, Chapman (1918) observed the greatest effect of a lack of blood flow on the extraembryonic

vasculature between 63 hr. (HH stage 17) and 80 hr. (HH stage 19) incubation. Based on these observations, changes in vasculature structure were expected. It is also important to note that the videometric technique being applied in the present study, required the embryo to be as close as possible to the inner membrane of the air. Romanoff (1960) observed (and prelimary observations in this study agreed) that the embryo sank away from the inner membrane of the air cell starting as early as 96 hr. (HH stage 21) incubation. In present study, it was observed that changes occurred in the embryos of the ligated group which caused them to sink within the 6 hr. treatment period, and thus made them difficult to videotape and measure. Hence, while reincubating until approximately 96 hr. (HH stage 21) would have added 24 hr. to the treatment period, it would have been impossible to collect data via videometric analysis at the end of the treatment period.

Though an extended treatment period in the present study might have required more extensive procedures for data collection, different changes might have occurred as the embryo sank from the surface toward the center of the egg. Consideration of trends in the data, however, reveals that only the changes due to the absence of flow in the anterior cardinal vein may have been more dramatic were an increase in the treatment period to have been implemented (Figure 6). The other vessels and the eye indicated trends of either very little change (Figures 8 and 9) or of changes which were almost parallel to control vessels which had flow (Figures 5 and 7).

### *Sample Size*

Small sample size could have contributed to the results of the present study.

Numerous studies in which the chick embryos were used to measure any number of cardiovascular variables incorporated sample sizes ranging from 5 (Hu et al., 1996) to 90 embryos (Yoshigi et al., 1996), though sample sizes of 10 to 30 embryos seemed most common. The present study suffered from a large degree of variation among animals of the same treatment group at different points in time. It is possible that a larger overall sample size would have decreased the variation in each group. Initially, the ligated group sample size was 6 embryos. In an attempt to decrease within-group variation, its sample size was increased to 10 embryos. Rather than decrease the ligated within-group variation as measured by group variance, the addition of 4 embryos increased variance, on average, 50% for the diameter of the eye, 0.5% for the diameter of the anterior cardinal vein, 3% for the anterior vitelline vein, 4% for the vitelline artery, and 19% for the vitelline vein. This suggests that increasing the sample size by only a few embryos in this case, might not have effectively decreased the overall variation in the control and sham groups. As a point of interest, to test a larger sample size, a random number generator confined to actual data ranges was used to produce an artificial sample size, n = 30. Random numbers produced this way for anterior cardinal vein values were then tested (t-test) against actual values at each point in time for the sham and ligated groups. The t-test indicated that no difference existed between the random and actual values in the sham group and only the randomly produced values for after ligature placement (0.5 hr.) differed from actual values for the ligated group. Random number sample variances seemed smaller than the variances for the actual data, and when run comparing the random number samples from the ligated group to those of the sham group in a repeated

measures ANOVA, significant differences were seen between the groups during what would represent the treatment period. Only at the very end of the treatment period did values meet (similar to what is seen with mean values in Figure 6). While analysis of the actual data indicated that no between-group difference existed for the ligated and sham anterior cardinal vein, the fact that both the random number comparison and the actual data analysis arrived at the same conclusion (i.e., when all was done, the diameter of the anterior cardinal vein in ligated and sham groups was the same) – the results even with a small sample size may be accurate. Similar results were achieved in random number comparisons of other vessels.

### *Use of KCl*

To permit ligated (unpressurized) and sham (pressurized) between-group comparisons, it was necessary to induce cardiac arrest in the sham groups to depressurize their systems. Induction of cardiac arrest may have been provided by electrical, athermal, or chemical means. Each of these means could have induced vasoactive changes in vessels proximal to the heart. KCl was chosen because it was easily obtained and administered, and its action on cardiac tissue was understood (Winkler et al., 1940). It has been well established that KCl plays an important role in the establishment of cell membrane potentials. Furthermore, it has been established that fluctuations in membrane potential are closely coupled with the contractile events of the cell.

Depolarization of the cell membrane requires that a certain threshold membrane potential be reached. The addition of KCl at 0.75% (m/v) to the interstitium, effectively

removes the cell membrane potential and prevents it from repolarizing – hence, no further contraction occurs. In the present study, the presence of excess KCl eliminated the cardiac cycle (stopped the heart). Also, Figures 6 - 9 suggest that KCl may have acted as a vasodilator and/or a vasoconstrictor: intraembryonic variables (eye and anterior cardinal vein) tended toward dilation whereas extraembryonic variables (anterior and vitelline veins, and vitelline artery) tended toward constriction (the more common vascular response to KCl (Karoon et al., 1998)). Hence, the apparent trend toward dilation or constriction in the present study may have been linked to location, more than to vessel type (artery versus vein).

More than environment-related, the trends in the present study may be related to the state of the vessel at the time of the KCl application. If, for example, the $Ca^{2+}$ channels (inherently slow) were open at the time of KCl application, they might remain open longer or indefinitely since no movement of $K^+$ would occur to repolarize the membrane and signal their closure. Calcium from intra- and extracellular sources would then be allowed to establish its own electrochemical equilibrium at high cytosolic concentrations, promoting constriction. If, on the other hand, $Ca^{2+}$ channels were to have just closed at time of KCl application, the membrane potential would remain locked, cytosolic $Ca^{2+}$ would be reduced in time, and a dilation effect might occur (from more in depth information, see Sperelakis, 1998). Regardless of the exact cascade of events produced by the addition of KCl, its presence in the present study introduced a confounding variable that clouded what may have been more clearly explainable changes in diameter due to changes in pressure. Because of the presence of KCl, it is unclear how

much change in vessel diameter was a result of a loss of pressure in the system and how much was a KCl effect. It also is unclear whether another means of cardiac arrest would have produced less interference with neighboring vessels.

### *Ink Injections*

Ink injections in the present study were vital for data collection through videomicroscopy and were probably the most difficult step in the protocol. Without a contrast-dye, after ligation and reincubation, during which blood drained from the vasculature to pool below the embryo, vessels were invisible. Even in vessels with normal blood flow, a contrast-dye provided for clear delineation of the vessel walls during analysis. While other contrast-dyes were tested such as Evan's blue and methylene blue, neither provided the necessary contrast.

The use of India ink as a contrast material is quite prevalent (eg. Chapman, 1918; Thompson et al., 1989; Seifert, 1994; Hogers et al., 1995; Hogers et al., 1997). Chapman (1918) discussed that he had difficulty injecting ink into embryos much older than HH Stage 12 which had undergone cardiac ablation. Similar difficulties were encountered in the present study when injection was attempted after conontruncal ligation or after cardiac arrest. The best results were achieved when injection occurred with the heart beating and with the vasculature patent. While use of India ink as a contrast dye is widespread, the toxicity of injected India ink is unknown. In the present study, only rarely did mortality occur as a result of ink injection, and in such cases, the cause of mortality was vessel rupture near the heart. While the present protocol only required survival for a maximum

of 7 hr., one injected embryo was reincubated 72 additional hours and survived. Hogers et al. (1997), one of the few studies surveyed to have included reincubation after ink injection, injected volumes of ink in the range of those injected in the present study and reported no mortality after one week as a result of injection. Still, toxicity as well as many of the hemodynamic effects of India ink injection are unclear and may have influenced the results of the present study.

What effects do volume loading and injection of a relatively viscous material have on hemodynamics? Volumes of ink and saline injected in the present study were similar to volumes reported by Wagman et al. (1990) and Hogers et al. (1995). Results of the present study indicated that ink as well as saline injection had no significant effect on blood flow in the dorsal aorta (Figure 7) as measured with a pulse-doppler system. Comparison of flow values obtained in the present study to literature values suggests a discrepancy in the present values of one order of magnitude (0.001 μl/sec versus 0.037 μl/sec (Clark and Hu, 1982 ). Furthermore, heart rate values (Figure 8) for the present study are also on average lower than published values (120 versus 165 bpm (Clark and Hu, 1982)). These discrepancies in flow and heart rate probably exist as a result of hypothermic conditions since hemodynamic parameters are under myogenic control (Culver and Fishman, 1977). As mentioned briefly in the Material and Methods, best efforts to maintain embryo temperature at 38°C produced embryo temperatures 33 to 35.5°C. Wispé et al. (1983) reported a drop in heart rate of HH Stage 18 embryos to 110 bpm when the ambient temperature was lowered to 31.1°C. They also reported a significant drop in mean dorsal aortic flow and stroke volume with temperature. Drawing

from equations and values found in Clark and Hu (1982) and data points from the present study, values from the present study differ by 45% from those in Clark and Hu (1982). Regardless of actual values, however, the data still indicate that the injected volumes did not change mean dorsal aortic flow. Furthermore, literature values for total blood volume per 100 grams of tissue indicate that between days 3 and 4 incubation blood volume reaches a maximum for the entire incubation period of 40 μl (Romanoff, 1960). In the present study the possible maximum injection volume of 0.53 μl (the largest estimated injection volume) is yet minute and probably had no affect on flow or total blood volume.

## Vessel Growth

Angiogenesis (vascular growth) follows many different patterns. Intercalated growth is one of these patterns and involves the growth of vessels in diameter and length by mitotic increase of cell number (Wilting and Christ, 1996). In larger, more well established vessels, extensive remodeling can be a lengthy process. Growth in vessel diameter occurs radially from the lumen with a thickening of the tunica intima, tunica media and tunica adventitia (Hughes, 1942; Skalak and Price, 1996). It seems conceivable, however, that growth could also occur by a thickening of the tunica intima and tunica media to reduce luminal area, but not affect outer vessel diameter. Measurement of the outer vessel diameter, therefore, might not demonstrate that growth were to have occurred. While this hypothesis may be upheld in older embryos and adult animals, in the context of the present study, it most likely does not apply. At 3 days of development, the age of the chick embryos in the present study, the chick vessels consist

of little more than an endothelium and structural mesenchyme cells (Huges, 1942; Skalak and Price, 1996) with makes them transparent. While adluminal growth is a possibility, it seems that to accommodate increasing blood volume, pressure, and flow (Romanoff, 1960; Clark and Hu, 1982), radial or abluminal growth to increase structural integrity and regulatory ability would be most likely to occur. For embryos of the present study, measurement of vessel outer diameter as a meter of growth was most likely accurate.

## Ligation: Oxygen Availability

As Wang and Prewitt (1991 ) mentioned, the use of ligation brings into question whether or not the observed results in the present study were due to a lack or reduction of pulsatile blood flow, or whether the observed results are actually a by-product of hypoxia. Several studies have indicated that hypoxia can produce changes in the circulation at a molecular as well as systemic level, which can lead to increased circulatory pathways. Gu and Adiar (1997) demonstrated that when cultured myocardial vascular smooth muscle cells were exposed to 1% $O_2$ for 24 hr., levels of VEGF, a potent angiogenic stimulator, increased by 5 fold. Höper and Jahn (1995) showed that as a result of exposure to 10% $O_2$ during the first four days of incubation, extraembryonic vascular density increased by 3.8% and the *area vasculosa* expanded by approximately 60 $mm^2$ over control values. Furthermore, in older embryos, short-term hypoxic exposure produced a redistribution of cardiac output that favored the heart and brain at the expense of the liver, the yolk sac, and the rest of the body (Mulder et al., 1998). Within the first four days of incubation, while hypoxia studies by Höper and Jahn (1995) indicated that

vascular changes were made, extended exposure to 13.5% $O_2$ did not produce a significant change in blood volume or the in the number of circulating erythrocytes (Baumann and Meuer, 1992).

Apparently conflicting data exist regarding the importance of a convective oxygen supply in the early chick embryo. Männer et al. (1995) reported that in embryos where the heart had been ablated prior to blood circulation (approximately 48 hr.), corporal deformities were observed within 29 hr. under normoxic conditions. These deformities, including a lack of a cervical flexure and poor cephalic development, were partially corrected if, after cardiac ablation, the embryos were incubated under hyperoxic conditions (50-70% $O_2$), though the forebrain still showed malformation. They, therefore, concluded that the early embryo received $O_2$ by diffusion from the air as well as by convection from the blood. In contrast, Warburton et al. (1996) examined $MO_2$ in the 72 hr. embryo with and without blood flow, due to ligation of the conotruncus arteriosus. They determined that ligation and a lack of systemic circulation did not affect the overall $MO_2$ until day 5 of incubation (48 hr. of reincubation). Their results and reported values agree with those of other studies (Pearson et al., 1996; Pelster and Burggren, 1996; also see Burggren and Territo, 1995). Furthermore, measurements of the eye diameter indicated that growth continued despite a lack blood flow. Growth of the eye as indicated by increased eye diameter was confirmed in the present study.

So then, why in Männer et al. (1995) was a dual method for $O_2$ delivery required, and in Warburton et al. (1996) was a convective $O_2$ supply not required? One obvious reason may be that morphological formations may not equate to $O_2$ consumption; i.e.,

while cells lacked cooperative activity for structural development, their $MO_2$ may not have been affected. Budinger et al. (1996) provided support for this idea by demonstrating that *in situ* chick embryo cardiomyocytes under moderate hypoxic conditions can suppress their ATP demand and $O_2$ uptake by reversible inhibition of cytochrome-c oxidase. Structural development may have also been under the influence of factors related to a pressurized vascular system such as any number of humoral agents modulated by blood flow (Ando and Kamiya, 1996). This may explain why, when more caudal/posterior tissues responded to higher $O_2$ concentrations, Männer et al. (1995) still observed malformations in the forebrain. Furthermore, with regard to tissue response to increases and decreases in $O_2$ levels, Höper and Jahn (1995) reported the inconclusive results that vascular density increased under both hypoxic and hyperoxic conditions. This suggests, therefore, that the influence of hyperoxia on embryonic tissues is unclear.

Ligation (or in many cases equivalent clamping) has been a popular means of regulating blood flow in adult animals as well as embryos. Hudlicka and Price (1990) applied ligation to the external iliac artery of rats to demonstrate the effects of ischemia on capillary growth in fast muscle. Adult dogs have also undergone ligation of the left pulmonary artery (Bloomer et al., 1949) to observe changes in respiratory function, bronchial artery blood flow, and angiogenesis (Charan and Carvalho, 1997), as well as ligation of carotid (Shipley and Gregg, 1944), femoral (Keitzer et al., 1965; Young et al., 1975), and iliac arteries (May et al., 1963, Vol 53 and 54) in an effort to study the effects of stenosis on hemodynamics. Ligation (and clamping) has been used in the chick embryo to study ventricular function (Faber et al., 1974; Keller et al., 1997) as well as

ventricular morphology and aortic arch variances related to blood flow (Rychter, 1962; Clark et al., 1989). Ligation as used in the present study and in Warburton et al. (1996) is a feasible, less invasive means of regulating blood flow than the method of cardiac ablation used by Chapman (1918) and Männer et al. (1995). Furthermore, cardiac ablation described by Chapman (1918) can only be applied in studies where vidoemetric techniques, such as those of the present study are to be used, if ablation occurs before blood flow begins. Otherwise, hemorrhage would obscure data collection. To be able to use Chapman's technique (1918) in embryos where blood flow has begun, ligation or some form of occlusion of the conontruncus arteriosus and the sinus venosus would be necessary to prevent hemorrhage. Such a modification would require much care, however, so as to minimize disruption of the amnion and other membranes to allow for clear videotaping. As mentioned for the present study, a lack of blood flow, combined with minor disruption of the amnion during ligature placement and vitelline membrane during the injection procedure, caused comparably voluminous fluid collection around the embryo and separation of the vascular bed from the vitelline membrane to occur proximal to the embryo. This collection of fluid alone made visualization of vessels near the embryo that much more difficult. In the context of this study, it seems unlikely that factors such as hypoxia, related to the use of ligation, directly influenced the results obtained in this study.

## An Alternative Role for the Embryonic Heart

It was hypothesized and demonstrated that the early embryonic heart begins to

beat and blood begins to flow for reasons other than simple oxygen and nutrient delivery to the tissues (Burggren and Territo, 1995; Warburton et al., 1996). Burggren and Territo (1995) suggested that one of these reasons was to assist in vascular growth. Qualitative evidence has supported their suggestion (Chapman, 1918; Hughes, 1935; Hughes, 1943; Arey, 1963). In fact, results from Chapman (1918) indicated that after 2.5 days of incubation (HH stage 17), blood flow, with its hemodynamic stimuli and endocrine transport, was required for vascular maintenance. Within 18 hr. (approximately 3.5 days of incubation (HH stage 19), significant regression of the vessels which had been established under humoral angiogenic stimuli, was prevalent. This suggests that the vessels of the 3 day (HH stage 18) chick embryo observed in the present study, were established and maintained via cooperative action of humoral growth factors and hemodynamic factors before the experimental elimination of blood flow.

In the present study, only the intraembryonic anterior cardinal vein to grew significantly in the presence or absence of blood flow over the treatment period (Figure 6). All other measured vessels did not grow in the presence or absence of flow over the treatment period. This suggests two things in light of Chapman's results: 1) Once vessels have been established via the cooperative action of both humoral and hemodynamic angiogenic factors, the intercalated growth rate may slow – hence, to gauge angiogenic changes through the measurement of vessel diameter is inaccurate. 2) While the establishment of vessels is under humoral angiogenic control, hemodynamic stimuli may function to stabilize the vessels against rapid degradation (regression). A third point may also be made that intraembryonic humoral growth factors may continue to have a larger

role in vessel growth than extraembryonic humoral growth factors at this stage of development. In summary, therefore, while the results of the present study suggested that vessel growth was independent of hemodynamic stressors resulting from the early heart beat and blood flow, in light of the rate of regression observed in Chapman (1918) in the absolute absence of blood flow, the results of the present study demonstrated that the early heart beat and blood flow did play a role in stabilizing vascular structures against regression.

## A Model for Hemodynamic Influence on Angiogenesis

Given that the chick embryo and its vasculature experience a high rate of growth (Romanoff, 1960), study of the influence of blood flow in the embryo is complicated by the confounding variables which dictate growth. Furthermore, comparatively little data exist regarding the growth and development of embryonic systems. Hence, it seems unlikely that the embryo would be considered a model for the study of hemodynamic influences on vascular growth.

On the contrary, however, because the embryo and its vasculature *do* experience such a high rate of growth, the embryo is the ideal model in which to study the influence of hemodynamic factors on vascular growth. During embryonic development, the vasculature that is being established is the vasculature that allow the adult animal to survive. Experimental manipulation of hemodynamic variables during this period will demonstrate the role of these variables in the various patterns of angiogenesis (Wilting and Christ, 1996) in a shorter period of time and to a greater extent than in an adult

model. For example, manipulation of blood flow out of the heart caused an entire restructuring of the aortic arches in the chick embryo (Rychter, 1962). Also, alteration of intracardiac flow prevented normal intracardiac septation and normal valve formation in the chick embryo (Rychter, 1962; Hogers et al., 1997). Furthermore, in the light of development, the embryo allows the question of developmental trajectory to be explored. More clearly, if alterations to the vasculature are made during development, exactly what physiological and morphological changes will result? And, is there a period of time in which, if an alteration is corrected, embryonic developmental patterns can be returned to their "normal" developmental trajectories? Regarding developmental trajectories, experimental manipulation has been used to study vascular development (Eichmann et al., 1997), and cardiovascular development ( Rychter, 1962; Hogers et al., 1997), as well as development in neural (Matsuno and Nakamura, 1993), skeletal (Bertram et al., 1997), and other systems.

The environment of the embryo in which growth is being stimulated, also provides an excellent model for pathogenic states, such as cancer, where angiogenesis results from the imbalance of positive and negative humoral stimuli (Folkman, 1995). Similar to the embryonic cellular environment, the cellular environment of pathogenic states is also poorly understood. Data from manipulations applied the embryonic systems may closely parallel data that would be collected in actual pathogenic systems, if collection of such data were feasible. The chick embryo is an inexpensive subject which is easily maintained in a laboratory setting, highly accessible *in ovo* for experimental manipulations, and isolated from maternal influences. Further evidence as to the

usefulness of the chick embryo model rests in its use for the CAM assay (Ribatti et al., 1996), as well as a model for the general development of other embryonic systems. The chick embryo as a model for the effects of humor and hemodynamic stimuli on angiogenesis, thus, can be useful for studying pathogenic states and embryonic systems.

## Implications for Future Experiments

Future research is needed to elucidate by results of the present study disagreed with what would be predicted from other studies (Chapman, 1918; Clark, 1918). Further research should address the duration of the treatment period. It is possible that with a longer treatment period a more distinct statement as to the influence of hemodynamic parameters on vessel maintenance and growth could be made. Modifying treatment period could also be used to address the influence of hemodynamic factors on rates of growth or regression. Results from this study suggested that there may be a relationship between the integrity of a vessel and the length of time it had been exposed to hemodynamic, physical angiogenic stimuli such as blood pressure and flow.

The present study examined the actions of humoral and physical stimuli on intercalated growth only (and only changes in diameter, at that). Other vascular changes were observed. An approach in combination with, or in addition to, videometric measurements of diameter to quantify and more fully unveil different patterns of angiogenesis should be applied. Examination of other patterns of angiogenesis may elucidate which pattern is most influenced by blood flow and other hemodynamic parameters.

Examination of other patterns of angiogenesis also demands a closer examination of blood flow in particular. Increased blood flow can induce angiogenesis (Hudlicka et al., 1992). Is a minimum flow required for vessel maintenance over an extended period of time, and at what level of flow does a marked increase in angiogenesis occur? Also, this study indicated exposure to physical stressors resulting from blood flow, may have stabilized vessel structure. Further research should explore the length of exposure required to produce such stabilization, as well as the type of hemodynamic stimulus (i.e., shear stress or circumferential stretch) that is best recognized by the vessels. Other questions to address along this line of examination include what cellular processes occur to promote vessel maintenance or the lack of it, and how do the vessels respond to reperfusion after an extended period of no blood flow?

Finally, if the chick embryo is truly to be established as a model of embryonic systems in general, and more specifically a model of the influence of hemodynamics on angiogenesis, results from chick embryo studies must be replicable to a large extent in other embryonic systems. *In ovo* studies in other vertebrate systems, therefore, should be conducted to underline strengths and weaknesses of modeling in the chick embryo.

## Conclusions

This study did not definitively elucidate whether humoral angiogenic stimuli or hemodynamic angiogenic stimuli had a greater influence on vessel maintenance and growth. This study did, however, open an avenue to more questions by demonstrating an alternative role for the early heart beat and blood flow in the chick embryo – structural

stabilization of vessels against regression. Finally, this study presented the chick embryo as a valid model for study of the influence of hemodynamic regulation in angiogenesis.

Several of the questions arise from the outcome of this study. Future research should address this stabilization effect introduced by blood pressure and blood flow. How do stabilized vessels differ from unstabilized vessels? What is the means by which this stabilization is conferred? How long are vessels stabilized, and what promotes their destabilization in capillary networks where those vessels with increase blood flow are maintained and grow while those vessels which receive decreasing blood flow are degraded and disappear (Clark, 1918)?

Future research should also address how developmental trajectory changes with blood flow manipulations. Do varying levels of flow produce varying levels of stabilization? Do vessels which have begun to regress in the absence of blood flow return to being maintained and to growing upon reinstatement of blood flow, or do they continue to regress? Also, how do collateral pathways develop as a result of regional elimination of blood flow, and how do these collateral vessels then respond to the absence of blood flow?

Finally, future research should address the dichotomous relationships that exist environmentally between intra and extraembryonic vasculature, and structurally between arterial and venous vessels. Does the intraembryonic environment, though not isolated from the extraembryonic environment, promote growth more effectively than the extraembryonic environment? If so, in what way does it promote growth differently? Also, do arteries, which experience different hemodynamic events from those

experienced by veins, respond in different manner angiogenically than do veins? And, are arteries which experience higher blood pressures and flows than veins stabilized to a greater extent against regression and degradation in the absence of blood flow? Answers to these questions will provide a clearer understanding of vascular development in the chick embryo. This stabilization effect introduces a new, longer period of time that a vessel may withstand the absence of blood flow which may elucidate the persistence or presence of some pathogenic states and have application to their treatment. Consideration of this stabilization effect may also have a role in experimental design in a laboratory setting where blood flow is to be manipulated.

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Degree:
Bachelor of Science, Zoology, 1997
Weber State University

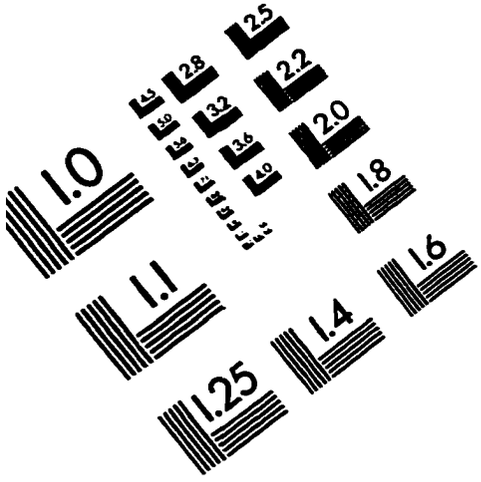
Special Honors and Awards:
Sigma Xi Induction, 1997
Cum Laude, 1997

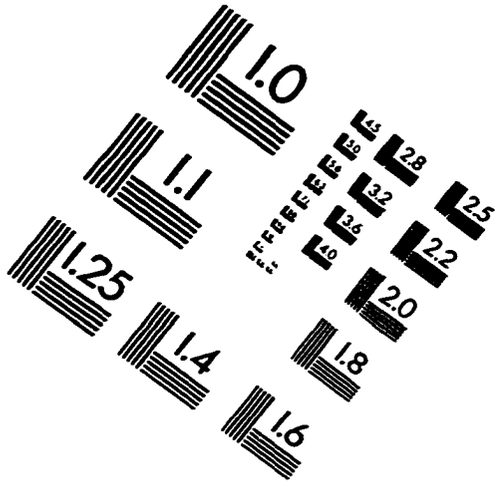
Publications:
Meyers, R.A. and B. M. Clarke. 1998. How do flap-neck chameleons move their flaps? *Copeia* 1998(3): 759-761.

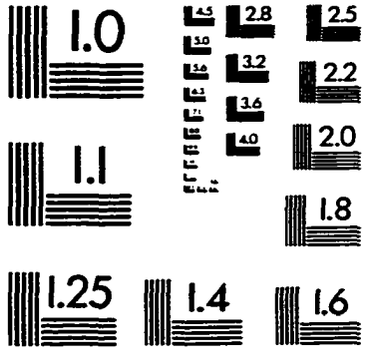
Thesis Title: The Influence of Blood Flow on Angiogenesis in the 3 Day Chick Embryo

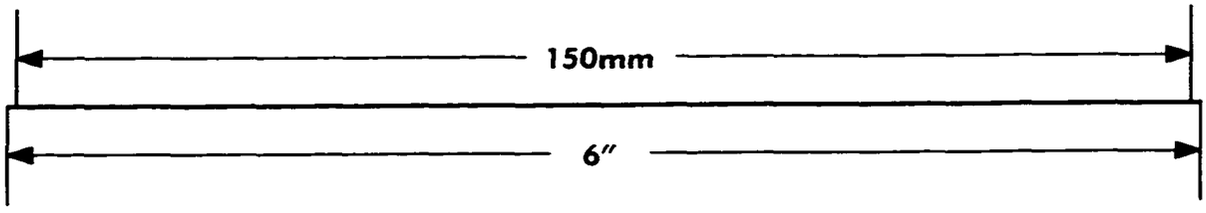
Thesis Examination Committee:
Chairperson, Dr. Carl L. Reiber, PhD
Committee Member, Dr. Warren W. Burggren, PhD
Committee Member, Dr. George Plopper, PhD
Graduate Faculty Representative, Dr. Lawrence A. Golding, PhD

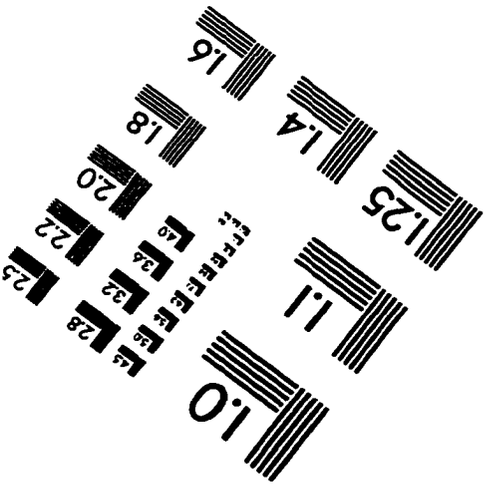
# IMAGE EVALUATION
# TEST TARGET (QA-3)

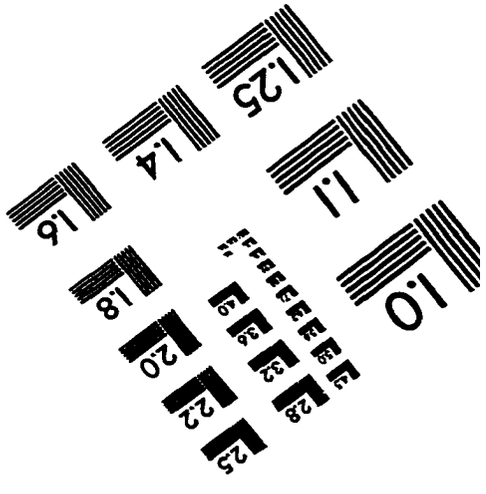
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