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INDUCED BRADYCARDIA EFFECTS ON ANGIOGENESIS, GROWTH, AND DEVELOPMENT IN EARLY DEVELOPMENT IN CHICKEN EMBRYOS, *Gallus domesticus*

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Cardiac performance, angiogenesis and growth was investigated during early chicken development. Heart rate, and thus arterial pulse pressure and cardiac output, were altered with the bradycardic drug ZD7288. Heart rates at 72 h of development of control embryos and those dosed with chicken Ringer were not different at 171 bpm. Acute and chronic application of ZD7288 caused significant bradycardia. Chronic dosing of Ringer and ZD7288 changed neither eye diameter nor development rate. Chronic dosing of ZD7288 did not significantly alter CAM vessel density close to the embryo (2, 3 and 4 mm) but at farther distances (5 and 6 mm) chronic dosing with both Ringer and ZD7288 decreased vessel density by 13 - 16%. Chronic dosing with ZD7288 also reduced body mass by 20%. Thus, lowered heart rate and cardiac output had little effect on vessel density or developmental stage, but did reduce embryo growth.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Cardiovascular Development and Function

Heart development in all vertebrates follows the same general pattern. An ovoid disc appears, that is composed of the endodermal and ectodermal cell layers, between which is situated the mesoderm, the germ cell layer that gives rise to the heart muscle (Gilbert, 2006; Wagner et al., 2007). Soon after the cardiac crescent formation, the flat embryonic disc begins to fold in conjunction with the growth of the cranial neural tube. This folding is channeling the migrating endocardial cells from both sides of the embryo into the developing neck region to form a lumen within the pericardial cavity. Then the endocardial cells become surrounded by mesocardial cells to form a bilaterally symmetric heart tube that is centrally positioned within the embryo. The next step in heart formation is to convert the anterior (ventricular) and posterior (atrial) organization of the linear heart tube into a primitive heart with two atrial and two ventricular chambers arranged in a left to right orientation (Wagner et al., 2007; Kirby, 2007; Hu and Clark, 2010). Following the complete formation of the heart, development of the vascular system begins.

Development of blood vessels begins extra-embryonically in the yolk sac. Angioblasts are generated from hemoblasts that can give rise to both hemoblasts, which form blood cells and angioblasts, which form blood vessels. The vascular system generating blood vessels in vertebrates is patterned after two significant processes, vasculogenesis and angiogenesis. All of the earliest development of blood vessels is

produced by vasculogenesis, in which vesicles of angioblasts form and come together to form tubes (Höper and Jahn, 1994). In angiogenesis, new vessels sprout from endothelial cells in pre-existing vessels (see Ribatti et al., 2001; Ruggiero et al., 2004, Kirby, 2007; Djonov et al., 2009).

Much of the early development of cardiovascular function in vertebrates has been determined using the chicken embryo. Bird embryos are favored for investigating how the vertebrate cardiovascular system development unfolds as they develop in a self contained egg. The chicken heart tube begins to contract at 36 hours of development and at 42 hours of development blood begins to flow. The chicken embryo heart rate is approximately 90 beats per minute (bpm) at onset of blood flow (about day 3) and increases in a non-linear fashion during development to ~210 bpm at hatching (Dunnigan et al., 1987). Heart rate is determined primarily by spontaneous repeating new inward electrical currents carried by sodium ions and potassium ions through hyperpolarization-activated cyclic-nucleotide-gated channels (Borer, 2004). Blood is propelled through the heart by rhythmic contraction and the endocardial and conotruncal cushions act like valves to prevent retrograde blood flow (Dunnigan et al., 1987). There is compelling evidence on many fronts that the convective flow of blood generated by the early embryonic vertebrate heart is not required for transport of oxygen, nutrients, metabolic wastes, or hormones, all of which can be entirely achieved by diffusion (for review see Burggren, 2004). A research area of growing interest is the unexplained ”time lag” between the first onset of the heartbeat, blood flow, blood pressure and the onset of dependence upon convective circulation. During the early stages of development, an embryo’s need for oxygen and nutrients is satisfied by

diffusion (Burggren, 2004) and as the metabolic needs of the growing embryo increase, internal convection by the circulatory system becomes an absolute requirement. One developing hypothesis of why the heart beats so early in development (before it is necessary for transportation of oxygen) is the stimulation of angiogenesis and thus the growth of the cardiovascular circulation (Burggren, 2004).

It has been shown that even with changes in blood flow and pressure in embryos, embryonic development continues for a short period of time during early development. Heart removal in salamander embryos had little effect on growth and development for several subsequent days (Mellish et al., 1994). Similarly, larvae of the frog, *Xenopus*, from which the heart has been surgically removed, also continue to grow and develop well beyond the point at which the heart would normally beat (Grunz, 1999). Short-term disruption of cardiac output in chicken embryos (through surgical ligation of the outflow tract) on day 3 and 4 of development had no negative effect on either growth or development (Burggren et al., 2000). Similarly, partial occlusion of the outflow tract in Hamburger-Hamilton (Hamburger-Hamilton, 1951) stage 16-24 chicken embryos had no effect (Burggren et al., 2004). These studies demonstrate that during the early stages of development, gas exchange for the embryo is largely (perhaps solely) accomplished through diffusion while the embryonic heart begins to beat and loop before it is required for gas exchange, making the heartbeat essential in vascular development rather than oxygen transport.

Along with the cardiac formation, other developmental processes are related to early cardiac performance. Ji et al. (2003) showed that the appearance of the yolk sac-derived erythroblasts is coordinated with the onset of the heart beat suggesting that a

pulsatile blood flow is required for early hematopoiesis. Nowak-Sliwinska et al. (2009) used the chorioallantoic membrane (CAM) of the chicken to study the effects of Avastin (a humanized antibody based compound that neutralizes endothelial growth factors) thereby inhibiting angiogenesis in the capillary plexus. Embryonic development on Days 7 and 9 showed that Avastin® (Genentech/Roche, San Francisco, CA, www.roche.com) strongly inhibited angiogenesis in the capillary plexus. This phase of development showed that the morphological changes observed in naturally occurring angiogenesis are significant when the rate of capillary growth is at its highest. The effect of topical application of Avastin resulted in apparent disorganization of the capillary network combined with the presence of various sizes of avascular zones along the vascular tree. There is little known about adaptation of a developing cardiovascular system to changes in heart rate. This research investigated how changes of heart rate affects physiological processes like angiogenesis, growth and development in chicken embryos.

Angiogenesis in the Chicken Model

Vasculogenesis is the differentiation of precursor cells into endothelial cells and the formation of a primitive vascular network, while angiogenesis is the development of new vessels from preexisting vessels (Semerano et al., 2010). Vasculogenesis is an intrinsic process that does not require blood flow and pressure. On the other hand, angiogenesis can be modified by changes of hemodynamics, especially blood pressure (Isogai et al., 2003). A functional embryonic cardiovascular system must develop both the heart and vasculature. In chicken embryos, blood islands become microscopically visible by HH stage 10, where-as blood circulation between the embryo and yolk sac starts by HH stage 15 (Minko et al., 2003). During early development, the primary

vascular plexus is established through fusion of the blood islands via the process of vasculogenesis. This primary network is ready to receive blood flow once the heart begins to generate it, and the primary vasculature is extended and remodeled into the mature vascular network by angiogenesis (Cleaver and Krieg, 1999).

At a cellular level, angiogenesis is well understood. New vessel formation begins with endothelial cell activation by an angiogenic stimulus, which breaks down the membrane and extracellular matrix so the endothelial cells can migrate and divide, forming the primitive angiotubes (Semerano et al., 2010). Vascular endothelial growth factor (VEGF) is one of the major factors that stimulate angiogenesis that acts early in angiogenesis inducing endothelial cell proliferation and migration (Semerano et al., 2010). The effects of VEGF includes an increase in blood vessel permeability and binds to two tyrosine-kinase receptors (VEGF-1 and VEGF-2) which are mainly expressed by endothelial cells (Semerano et al., 2010). Other factors like fibroblast growth factor (FGF-2), transforming growth factor (TGFβ1), ephrin, and integrins have all been similarly implicated in vascularization, including in the embryonic chicken chorioallantoic membrane and limb bud (Ribatti and Presta, 2002).

Blood flow is an important factor in angiogenesis, generating a frictional force on the vessel wall and shear stress that parallels the direction of the blood flow. Endothelial cells comprise the inner layer of the cardiovascular system and are therefore the first cells that are exposed to shear stress (see review, Groenendijk et al., 2007). The response to changes in wall shear stress in endothelial cells is provided by a mechanosensor for wall shear forces, e.g., by stimulation through the activation of integrins, G-protein receptors, tyrosine kinase receptor, or ion channels (see review,

Groenendijk et al., 2007). On detecting a change in shear stress by endothelial cells, a signal is sent to the nucleus by means of the activation of protein kinase C (PKC), which in turn activates a second messenger cascade, involving the family of mitogen-activated protein (MAP) kinases, most importantly extracellular signal-regulated kinases (ERK1/2), resulting in gene transcription to be up or down-regulated (see review, Groenendijk et al., 2007).

Early pulsatile flow creates shear and stress on endothelial cells lining sprouting blood vessels. In response, endothelial cells proliferate under the influence of VEGF and other secreted factors, resulting in the formation of new vessels with through flow of blood subsequently established. Angiogenesis is crucial to the completion of the capillary network and the connecting of developing arteries and veins. Indeed, failure of angiogenesis in early embryonic development leads to rapid embryonic death, as evident from observations of VEGF knockout mice that fail to develop a complete microvasculature (Carmeliet et al., 1996; Ferrara et al., 1996). Current research and the subject of this thesis concerning angiogenesis and how heart rate affects this process is explored in the early stages of development with the understanding that during this time, growth and stimulation of the vasculature is at its highest.

Is there a Heart Rate – Angiogenesis Relationship?

Could changing arterial pulsatility, by increasing diastolic duration and thus pulse pressure, create sufficient shear stress on the endothelial cells lining the blood vessels and vessel tips to increase angiogenesis? To answer this question, a pharmaceutical drug to was used to reduce heart rate. Especially important for the subsequent interpretation of the data was to use an agent that has “pure” bradycardic effects – that

is, that does not also have inotropic cardiac affects. With this caveat in mind, the bradycardic drug ZD7288, which blocks the I_f cardiac pacemaker channels in the heart, was used. ZD7288 causes a decreased heart rate by specifically affecting the pacemaker cells of the sino-atrial node (SAN) of the heart. The cardiac pacemaker "funny" I_f channel is a mixed sodium and potassium channel. The channel ZD7288 targets the rate of spontaneous activity of sinoatrial myocytes and thus heart rate. The pacemaker potential is a slow increase in voltage across the pacemaker cell's membrane that occurs at the end of one action potential and the beginning of the next. The pacemaker potential drives the self-generating rhythmic firing of these pacemaker cells. This increase in membrane potential causes the cell's membrane to reach the threshold potential and causes the firing of the next action potential, generating repetitive action potentials at a constant controlled rate (DiFrancesco, 2006). ZD7288 specifically blocks this pacemaker current causing a decreased heart rate (Luo *et al*., 2006).

The drug ZD7288 was developed as a part of a search for pharmacological agents that could treat tachyarrhythmia without affecting cardiac contractility or electrophysiological properties (Yusuf and Camm, 2003). In the anaesthetized dog, ZD7288 reduces the beating rate without directly affecting ionotropy (Rouse & Johnson, 1994). The beating rate of the right atria is slowed but the overall contracting force is not affected. There are other studies that specifically study the channels that ZD7288 affect like Strieber et al. (2003) that used HCN4 knockout mice to see how bradycardia affects embryogenesis. HCN4 is a member of the hyperpolarization-activated cyclic nucleotide gated (HCN) cation channel gene family, and is involved in generating the I_f current that

drives cardiac pacemaker activity. The heart rate of $HCN4^{-/-}$ embryos at embryonic day 9.5 was only 63% of the normal heart rate observed in $HCN4^{+/+}$ embryos, and these mutant mice embryos died between day 9.5 and 11.5. There were no obvious cardiac pathologies upon autopsy, so they assumed that the decreased heart rate caused death. Potentially, bradycardia and the changes in blood pressure negatively affected the establishment of a patent vascular network, leading the early embryonic death.

Ample evidence thus exists to consider ZD7288 to be a purely bradycardic drug, and is used in our study to increase in end-diastolic filling enabled by a longer period of diastolic filling produced by bradycardia. An increase of end-diastolic filling is a consequence of inducing bradycardia, while greater pulsatility is the primary effect we are investigating. In this study, we propose to slow the heart rate enough to determine the effect that bradycardia has on angiogenesis, growth and development.

Objectives and Hypothesis

This study aims to examine the relationship between cardiac activity, angiogenesis, growth and development while experimentally perturbing angiogenesis. The specific aim and hypothesis are outlined below:

Specific Aim:

To quantify the relationship between heart rate, angiogenesis, growth and development by reducing the heart rate during the early stages of embryonic cardiovascular development.

Hypothesis:

Oscillating blood pressure, by altering heart rate, and thus arterial pulse pressure and cardiac output, causes changes in vasculature, growth and nutrient delivery, and

consequent changes in growth (mass) and development (eye diameter).

Experiments to be Performed:

Employ the bradycardic drug, ZD7288 to reduce heart rate, affecting pulse pressure and determine if this alters angiogenesis, development and growth of the embryo.

CHAPTER 2

MATERIALS AND METHODS

Source of Experimental Animals

Fertilized white Leghorn chicken eggs (*Gallus domesticus* L.) were obtained from Texas A&M University (College Station, TX) and shipped to the University of North Texas (Denton, TX). The University of North Texas' Institutional Animal Care and Use Committee approved all experimental procedures.

Eggs were separated into groups and randomly placed into a Hova-Bator Styrofoam® (The Dow Chemical Company, www.dow.com) incubator at a constant temperature of 37.5°C ± 0.5 °C and 55-60% humidity. After 48 h of incubation, the eggs were removed from the incubator and transferred into a shell-less culture system.

Shell-less Culture

To facilitate measurement of cardiovascular variables and drug delivery, shell-less embryo cultures were prepared (e.g. Hamamichi and Nishigori, 2001). This method enables normal growth of the embryo while allowing full access to the embryo and its vasculature for both manipulation and observation (Fig. 1). For the purpose of this study, shell-less culture is effective in producing an even distribution of solution and drug to the embryo and its extra-embryonic tissue. Preliminary observations using chicken Ringer solution supplemented with Evans blue dye showed that the excess solution runs off the surface of the embryo and collects remotely at the far edges of the culture dish, thus preventing accumulation of fluid and excess drug on the embryo (Fig. 2).

To prepare a shell-less culture, incubated eggs were removed from the incubator and positioned horizontally at room temperature on an egg crate for 10 minutes to allow the embryo to be positioned correctly during culture. The eggs were sprayed with 70 % ethanol, to reduce contamination from the egg surface, and allowed to air-dry. The eggs were cracked and the whole egg contents were transferred into an autoclaved Kimax crystallizing dish (size 60 mm by 35 mm) under aseptic conditions. The remaining bubbles surrounding the yolk were removed with sterile Kimwipes® (Kimberly-Clark, Irving, TX, www.kimberly-clark.com). The dish was covered by clear polyethylene film and secured with an elastic rubber band. Cultured embryos were then numbered and re-incubated at 37.5°C ± 0.5 °C for further development and use. Care was taken to only include cultures with intact yolks that have the blastodisc positioned on the uppermost side of the yolk.

Experimental Design

Control embryos were not treated with any solution before data was taken, while the Ringer embryos were treated with Pannett-Compton saline for comparison. Pannett-Compton saline solution contains 2.2 M of NaCl and 0.21 M of KCl (see Stern and Holland, 1993), pH = 7. These two groups were useful in determining the effect, if any, that fluid placement on the embryo had on heart rate. Both acute and chronic effects of the bradycardic drug ZD7288 on early development in chicken embryos were determined. In acute experiments, effects of ZD7288 on heart rate were determined by applying 5 µl of 30, 250 and 500 µM ZD7288 topically to the heart of shell-less cultured chicken embryos and heart rate was taken before dosing and then every 5 min after administration of the drug. The concentrations of ZD7288 that was used for acute

dosing was adapted from previous unpublished experiments (M. Fisher and W. Burggren). In this previous study, dose response curves were created by topically applying a 5 μl aliquot of 0 (saline, control), 50, 100, 500 and 1000 μM ZD7288 directly onto the heart of 50-70 h embryos. Heart rate was taken initially and after 30 min, 1h and 2h after application of the drug. These studies revealed that the heart rate reduction occurred 30 min following exposure of 5 μl of 500 μM ZD7288.

In chronic experiments, embryos were dosed for 24 h, starting from 48 h to 72 h of development. A range of concentrations (7.5, 10, 15, 20 and 30 μM) ZD7288 were used for chronic infusion while heart rate, vessel density, eye diameter, wet mass and dry mass of the embryo were measured. In the previous study done by M. Fisher and W. Burggren, chronic treatment of 30 μM ZD7288 at a rate of 200 μl/h (about 40 – 5 μl drops/h) achieved the same degree of bradycardia as the maximum acute bradycardic response produced by a single drop of 5 μl of 500 μM ZD7288. The concentrations used in this study were adapted from those findings, using 30 μM as the maximum chronic dose administered. The parameters measured were used to determine the effect that heart rate has on angiogenesis, growth and development during the early development of vertebrates.

Acute and Chronic Drug Treatment

ZD7288 was obtained from TOCRIS Bioscience, Ellisville, Missouri, USA. A stock solution of 10 mg ZD7288 was prepared with autoclaved distilled water and stored at -20°C. Subsequent solutions of varying concentrations were made using Pannett-Compton saline (Pannett and Compton, 1924), which provides better in vitro growth than standard chick Ringer solutions. This Ringer solution was originally developed to

grow blastodiscs in an artificial setting, so it was useful in this experiment as the vehicle of the drug. Embryos were incubated for 48 h before dosing with either chick Ringer or ZD7288. Hemodynamic data on control embryos was taken for comparison with datum obtained from the ZD7288 treatment and Ringer groups.

Acute Protocol

Heart rate was measured initially at 72 h of development. Treatment groups were removed from the incubator and bradycardia was induced by a single 5 μl aliquot of one of the three concentrations (30 μM, 250 μM and 500 μM) of ZD7288 administered topically, directly on the heart. Embryos were then immediately placed back into the incubator. Heart rate was taken manually by counting the number of heart beats in 15 sec intervals and multiplying this number by 4 to achieve the measurement of beats per min. The measurement was taken twice in a row and averaged to report.

Chronic Protocol

For chronic exposure, embryos at 48 h post incubation were placed into the incubator and the tip of tubing attached to the syringe was placed through the Saran wrap directly over the embryo’s heart. Chronic infusion of ZD7288 (7.5 μM, 10 μM, 15 μM, 20 μM or 30 μM concentration) was administered using a syringe pump (Model 352, Sage Instruments, 1987, Boston, MA) housed in a Lyon Reptilife Incubator (Model RL1&2) that was set at 37°C ± 0.5°C and 55-60% humidity. Three 10 ml glass syringes were secured on the syringe pump and used for smooth infusion simultaneously. The chronic drug delivery system slowly flushed the embryo’s surface with the control, Ringer or ZD7288 solution at a rate of 200 μl per h for 24 h. Total amount of solution administered was 4.8 ml over the 24 h period of dosing. Three ZD7288 treatment

embryos were dosed at the same time and the control embryos were placed next to the pump in the incubator to develop at the same conditions as the other embryos. These control embryos developed for 24 h in the same conditions as the dosed embryos without any solution being administered. After 24 h of dosing, heart rate (bpm), embryo eye diameter (mm), CAM vessel density from a radius of 2mm to 6mm from the umbilical stock (intersections/circle), wet mass (mg) and dry mass (mg) were measured.

Heart Rate

Heart rate was taken manually by counting the number of heart beats in 15 sec intervals and multiplying this number by 4 to achieve the measurement of beats per min. The measurement was taken twice in a row and averaged to report. Heart rate of the chronic experiments was measured in the same manner as for the acute protocol. Percent decrease in heart rate as compared to the control embryos is reported as mean ± 1 S.E. (bpm).

Eye Diameter Measurement

Eye diameter is a parameter that is frequently measured as an indicator of development during early developmental stages. The eye diameter of the embryos was measured with Image-Pro software (Version 4.1). Calibrated images taken with a dissecting microscope and camera at a total magnification of 16X was used for measurement. The diameter was measured and reported in mm.

CAM Vessel Density Index Measurement

Vascularity was quantified using a vascular density index (intersections/circle). Images of each embryo were taken at 3.5X using the Image-Pro software. To measure the CAM vessel density index of the extra-embryonic vasculature of embryos as a

function of distance from the embryos, five concentric circles (2, 3, 4, 5 and 6 mm) were placed directly over the image of the umbilical stock of the embryo using the Image Pro drawing feature. These circles were placed directly over the umbilical stock of the embryo each time pictures were taken to ensure accuracy in counting. The CAM vessel density was calculated by counting the number of clearly visible vessels that intersect the entire way around the drawn circle. Each circle was counted separately, starting with the 2mm circle moving up to 6mm (Fig. 3). The measurement protocol was adapted from other techniques used by Strick *et al.* (1991); Höper, J. (1994); Corona *et al.* (1999).

Embryo Dry and Wet Mass Measurement

Embryos were removed after the vasculature measurements were taken to acquire the wet and dry mass. The embryo was carefully cut away from the surrounding vessels and moved into a small amount of chicken Ringer in a small plastic dish, which was initially weighed. The membrane was pulled off of the embryo with forceps, blotted with filter paper to absorb the liquid and placed into a plastic dish to be weighed for wet mass. The dish containing the embryo was put into the oven at 80°C for 48 h and then weighed to obtain dry mass.

Statistical Analyses

The effects of development and chronic exposure to ZD7288 on heart rate, eye diameter and wet and dry mass were assessed using one-way ANOVAs, followed by Tukey post-hoc tests to determine pair-wise differences. Two-way ANOVAs were used to assess statistical differences in CAM vessel density and acute heart rate caused by chronic exposure to ZD7288 and distance from the embryo, and by acute exposure to

ZD7288 and development, respectively. If significant differences were found, Tukey post-hoc tests were used for pair-wise comparisons. All variables are represented as mean $\pm$ 1 S.E. $\alpha = 0.05$. All statistical analyses were performed using SigmaStat® and SigmaPlot® software (Systat Software, Inc., Chicago, IL, www.systat.com).

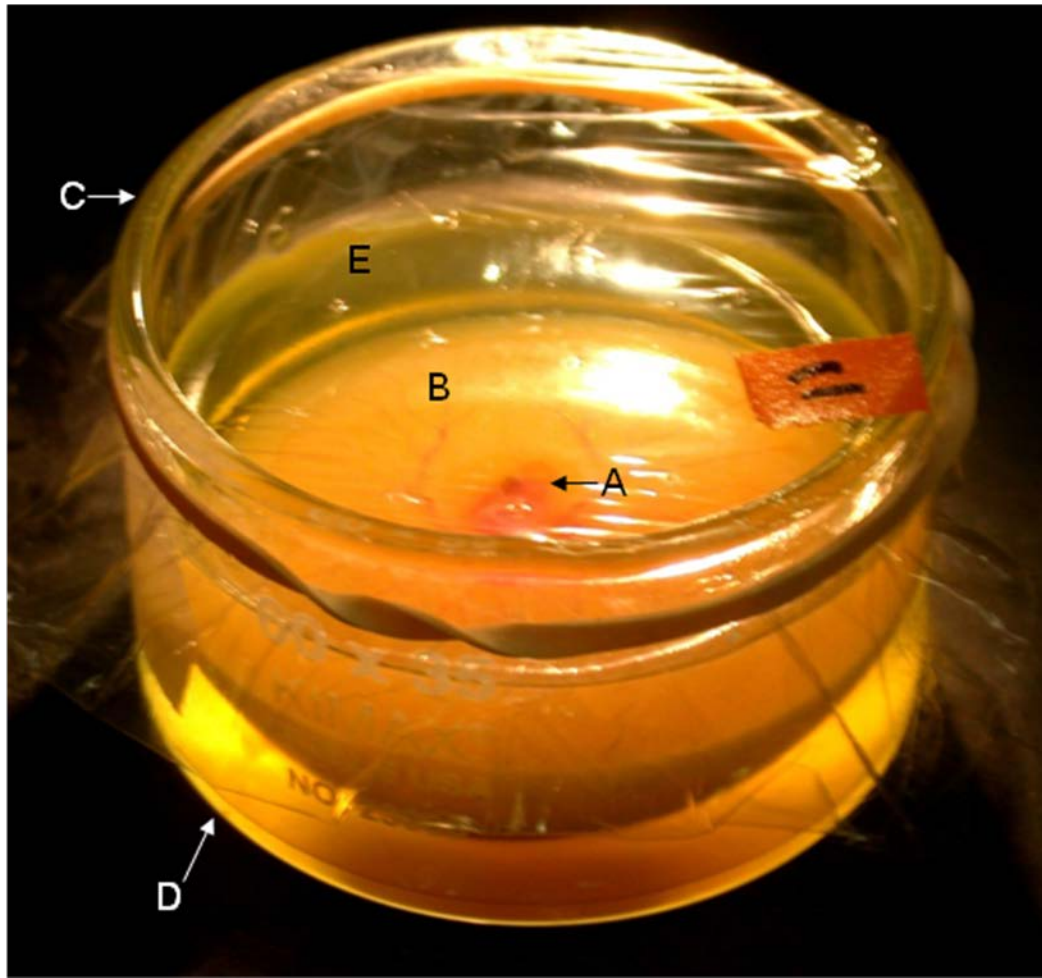

Fig. 1. 4 day old shell-less cultured chicken embryo. (A) developing chicken embryo; (B) yolk; (C) Saran wrap covering the dish; (D) Petri dish holding egg culture ; and (E) albumin. Petri dish is 60mm (diameter) by 35mm (height).

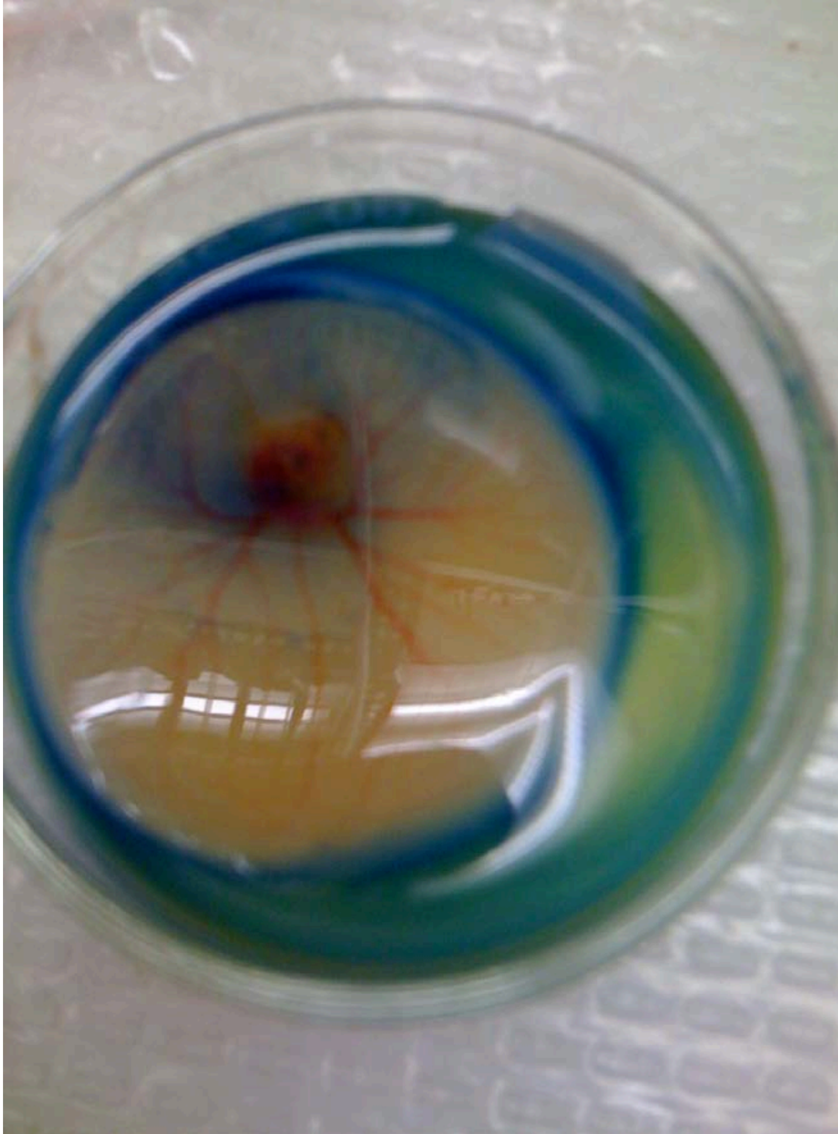

Fig. 2. Preliminary experiment using chicken Ringer infused with Evans blue dye. Note that excess solution runs off the embryo and collects remotely at the edges of the dish, preventing accumulation of fluid and drug on the embryo. Petri dish is 60 mm in diameter.

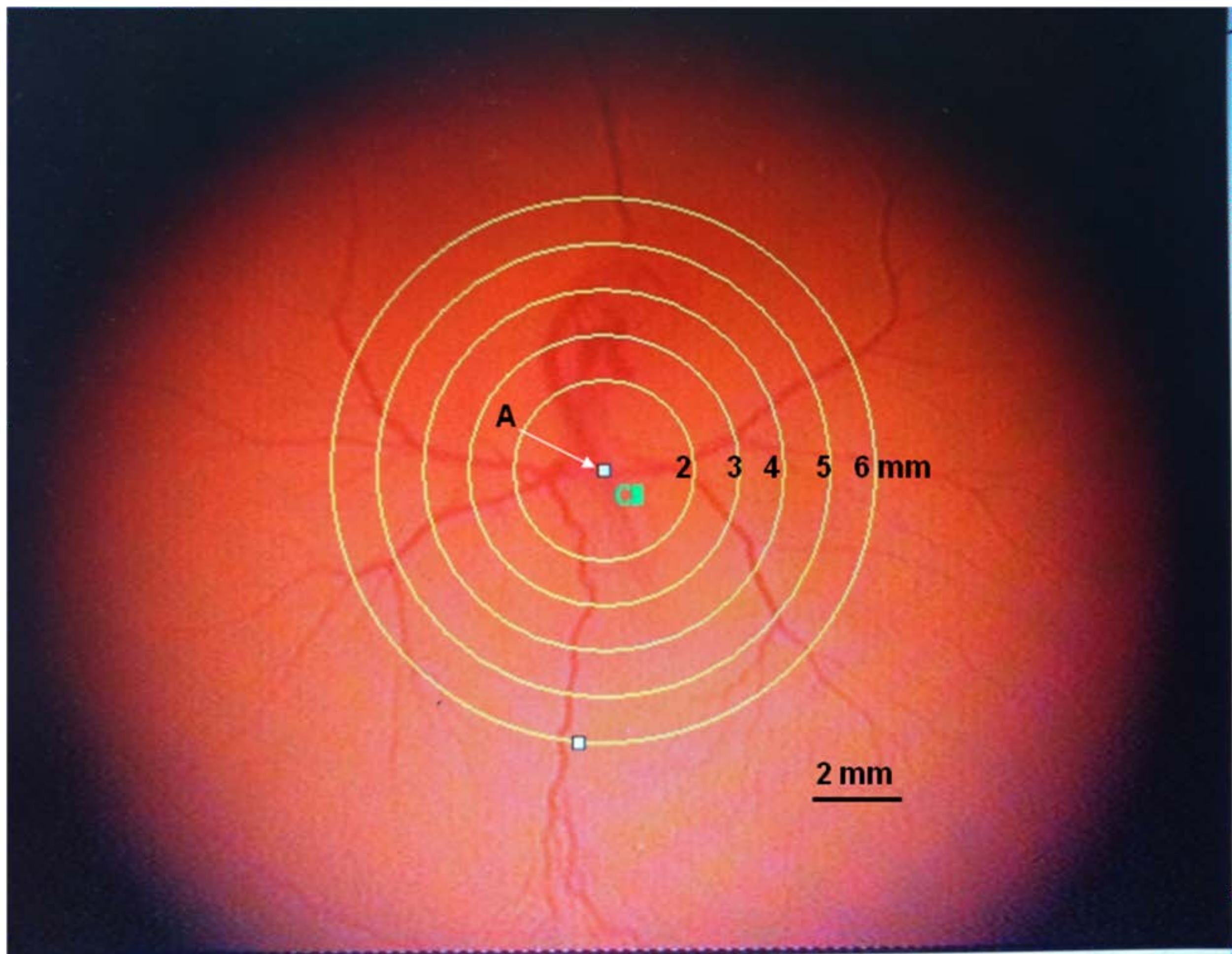

Fig. 3. CAM vessel density index measurement concentric circles. The label (A) indicates the embryo umbilical stock. Numbers show radius of circles measured (mm). Vessels intersecting each entire circle were counted to report. Symbol “C” in the center was used to assure placement of circles was aligned correctly.

CHAPTER 3

RESULTS

Acute Chronotropic Effects

To validate the drug vehicle, embryos acutely dosed with chicken Ringer were compared to control embryos. Mean heart rate was 174.7 ± 0.8 bpm in Ringer-treated embryos compared to 174.5 ± 0.3 bpm in control embryos, and these values were not significantly different ($p > 0.05$) (Fig. 4). At 48 h of development, acute dosing with 30 μM ZD7288 produced a heart rate that was significantly lower than the control and Ringer groups ($p < 0.001$) but significantly higher than the 250 and 500 μM ZD7288 groups ($p < 0.001$) (Fig. 4). Following acute dosing of 48 h old embryos with 5 μL of 250 and 500 μM ZD7288, heart rate was significantly lower for both concentrations than the heart rate of the control and Ringer groups ($p < 0.001$) (Fig. 4). However, heart rate of the 250 and 500 μM ZD7288 groups were not significantly different from each other ($p = 0.830$) (Fig. 4).

The effects of development on the heart rate responses to ZD7288 were investigated at 72, 96 and 120 h of development. A single aliquot of 5 μl chicken Ringer (acute treatment) produced no significant change from control heart rate at any of the three developmental stages tested ($p > 0.05$) (Fig. 5). However, there was a significant decline in heart rate of embryos acutely dosed with 500 μM ZD7288 at the same three stages of development, indicating that ZD7288 causes bradycardia throughout different stages of early development ($p < 0.001$) Treatment and time both caused significant differences in heart rate ($p < 0.001$) (Fig. 5).

Chronic Chronotropic Effects

Heart rates of control (169 ± 3 bpm) and embryos chronically treated with Ringer (172 ± 3 bpm) at 72 h of development were not significantly different ($p > 0.05$). Comparison of the different concentrations of ZD7288 (7.5 μM, 10 μM, 15 μM, 20 μM, 30 μM) showed that the heart rate of chronically dosed embryos was significantly different from both the control and Ringer groups ($p < 0.001$), yet not significantly different from each other within the ZD7288 group ($p > 0.05$) (Fig. 6). Chronic dosing of ZD7288 decreased heart rate by 33 % from 169 ± 3 to 116 ± 3 bpm at 72 h of development. The lethal dose was 30 μM ZD7288, as 50 % of the embryos did not survive 24 h during application of ZD7288.

Eye diameter at 72 h of development was 0.52 ± 0.02 mm in control embryos and 0.58 ± 0.02 mm in embryos chronically dosed with Ringer. Embryos chronically dosed with ZD7288 had an eye diameter of 0.50 ± 0.01 mm at 72 h of development. No significant difference was found between eye diameter between the three groups: control, Ringer and any of the ZD7288 doses ($p = 0.061$) (Fig. 7).

As there was no significant effect of ZD7288 concentration on heart rate in chronic experiments, CAM vessel density datum for all embryos chronically dosed with ZD7288 was grouped together to form a total of three groups for statistical analysis (i.e. control, Ringer–treated and ZD7288-treated groups). CAM vessel density index of control embryos at 2, 3 and 4 mm distance from the umbilical stalk was 3.2 ± 0.1, 6.2 ± 0.3, and 9.5 ± 0.4 intersect / mm, respectively. There was no significant difference in CAM vessel density between all the groups ($p > 0.05$) proximally at 2, 3, and 4mm from

the embryo umbilical stalk (Fig. 8). However, chronic application of ZD7288 induced a significant decrease in CAM vessel density at a distance of 5mm from the umbilical stalk, 12.2 ± 0.4 intersects / mm, compared to the control, 14.1 ± 0.7 intersects / mm ($p < 0.001$). Surprisingly chronic application of chicken Ringer <u>also</u> induced a significant decrease in CAM vessel density at 5mm, 12.5 ± 4 intersects / mm compared to the control, 14.3 ± 0.7 intersects / mm ($p = 0.020$). At a distance of 6mm from the umbilical stock, there was also a significant decrease in the CAM vessel density of both the ZD7288, 16.6 ± 0.6 intersects / mm and Ringer embryos, 18.5 ± 0.6 intersects / mm as compared to the control group 19.7 ± 0.7 intersects / mm ($p < 0.001$) (Fig. 8).

Wet mass of the control (14.1 ± 0.9 mg) and Ringer-treated embryos (14.8 ± 0.8) mg were not significantly different ($p = 0.131$) and therefore, were grouped together in subsequent analyses. Wet mass of embryos dosed with different concentrations of ZD7288 (7.5, 10, 15, 20 and 30 μM) was also not significantly different ($p = 0.053$). However, wet mass (11.6 ± 0.8 mg) was 22% smaller (compared to the Ringer-treated group (14.8 ± 0.5 mg) in the pooled ZD7288 embryos ($p < 0.001$) (Fig. 9)**.**

Dry mass of control (1.2 ± 0.1 mg) and Ringer-treated embryos (1.0 ± 0.3 mg) was also not significantly different ($p = 0.335$) and therefore, were grouped together for subsequent analysis. Dry mass of embryos chronically dosed with different concentrations (7.5, 10, 15, 20 and 30 μM) of ZD7288 were also not significantly different ($p = 0.175$) from each other. Dry mass showed a 33% decrease in the ZD7288 embryos from 1.2 ± 0.4 mg to 0.8 ± 0.04 mg as compared to the control ($p < 0.001$) (Fig. 9).

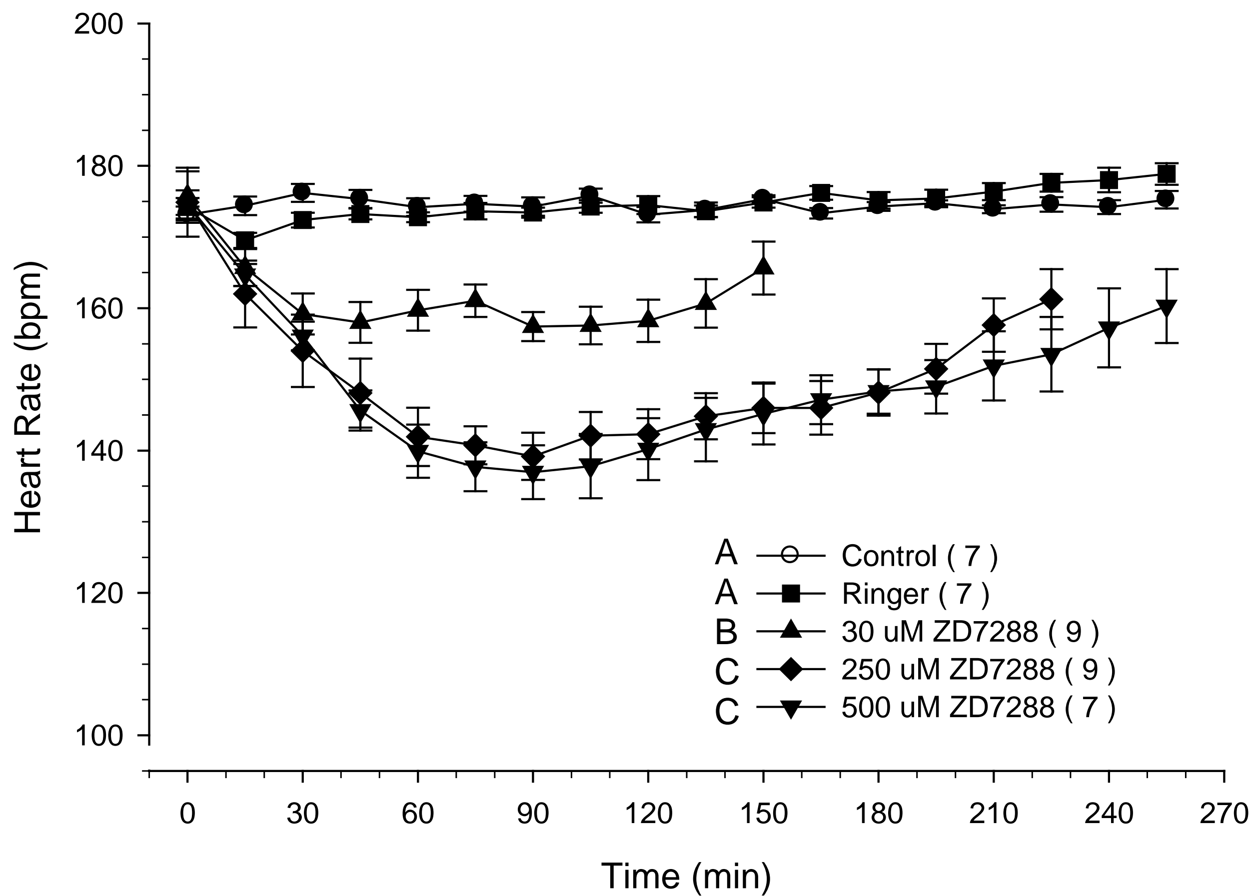

Fig. 4. Effect on heart rate as a function of acute dosing ZD7288 at three different concentrations. Data is shown as mean ± S.E. Every point indicates a 15 min period of mean heart rate that has been averaged, *n* values are in parentheses. Letters indicate overall difference among groups.

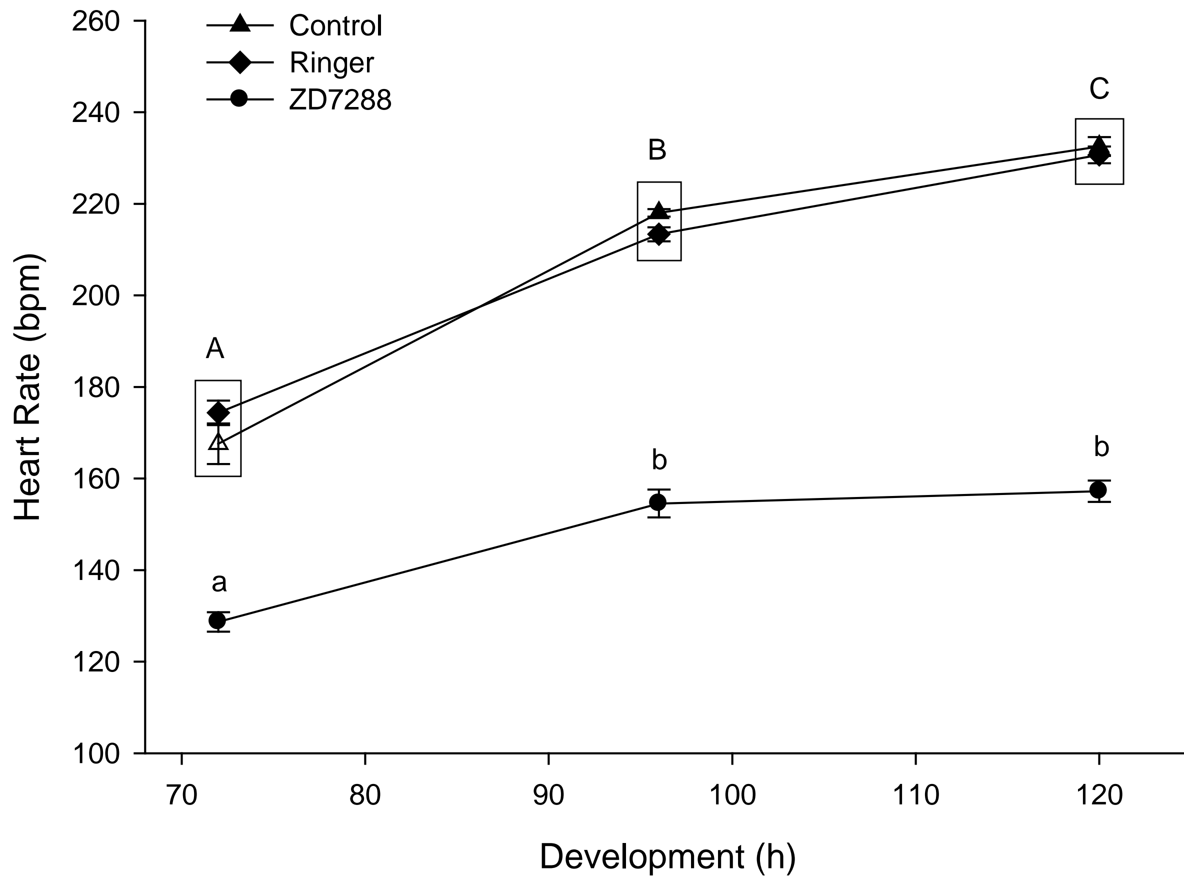

Fig. 5. Heart rate between hours of development and control, Ringer-treated, and ZD7288 acutely treated embryos (5 µl of 500 µM ZD7288). Means ± S.E are plotted, *n* = 6 for each group. Boxes enclose statistically identical mean values. Letters indicate differences in heart rate over development. Uppercase letters show differences in control and Ringer groups while lowercase letters show differences between ZD7288 groups.

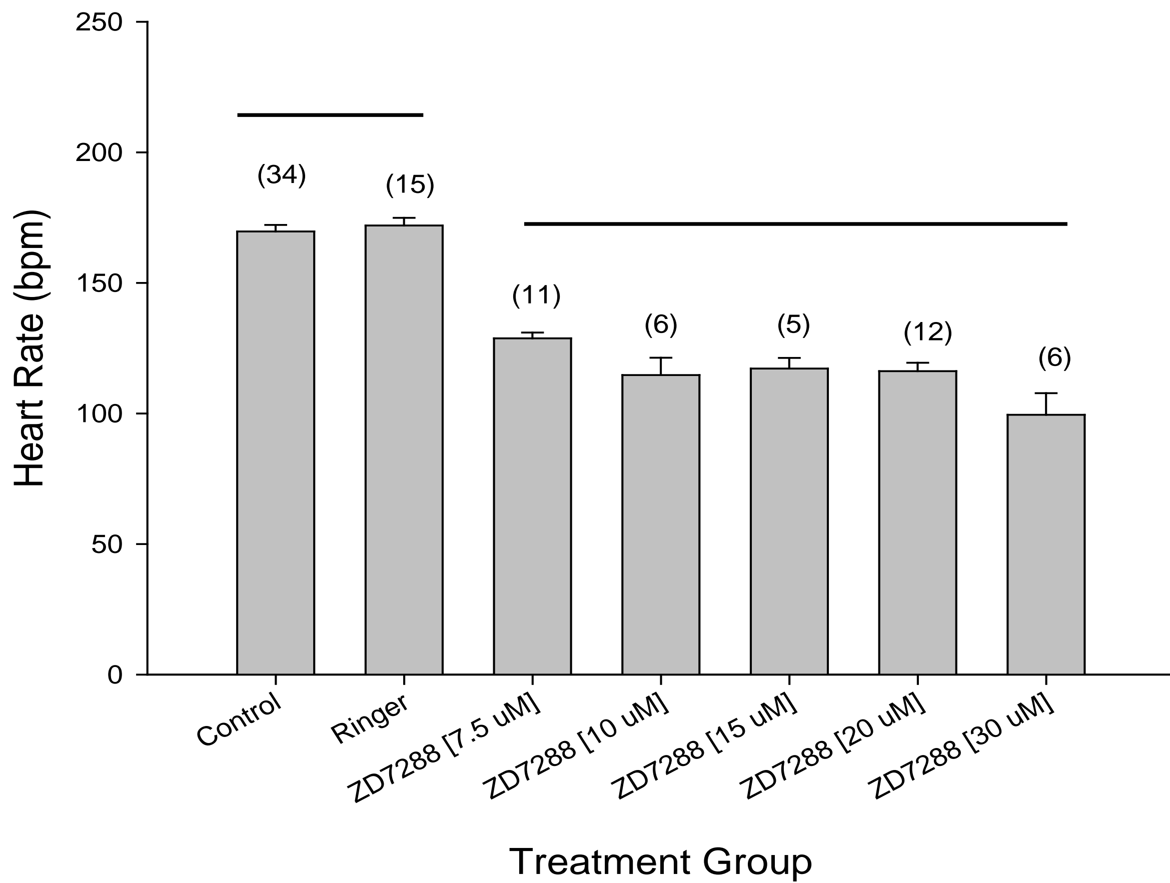

Fig. 6. Effects on heart rate at 72 hours after 24 h of chronic dosing beginning at 48 h of development. Horizontal lines indicate statistically identical groups. Mean ± 1 S.E., are plotted, *n* values are in parentheses.

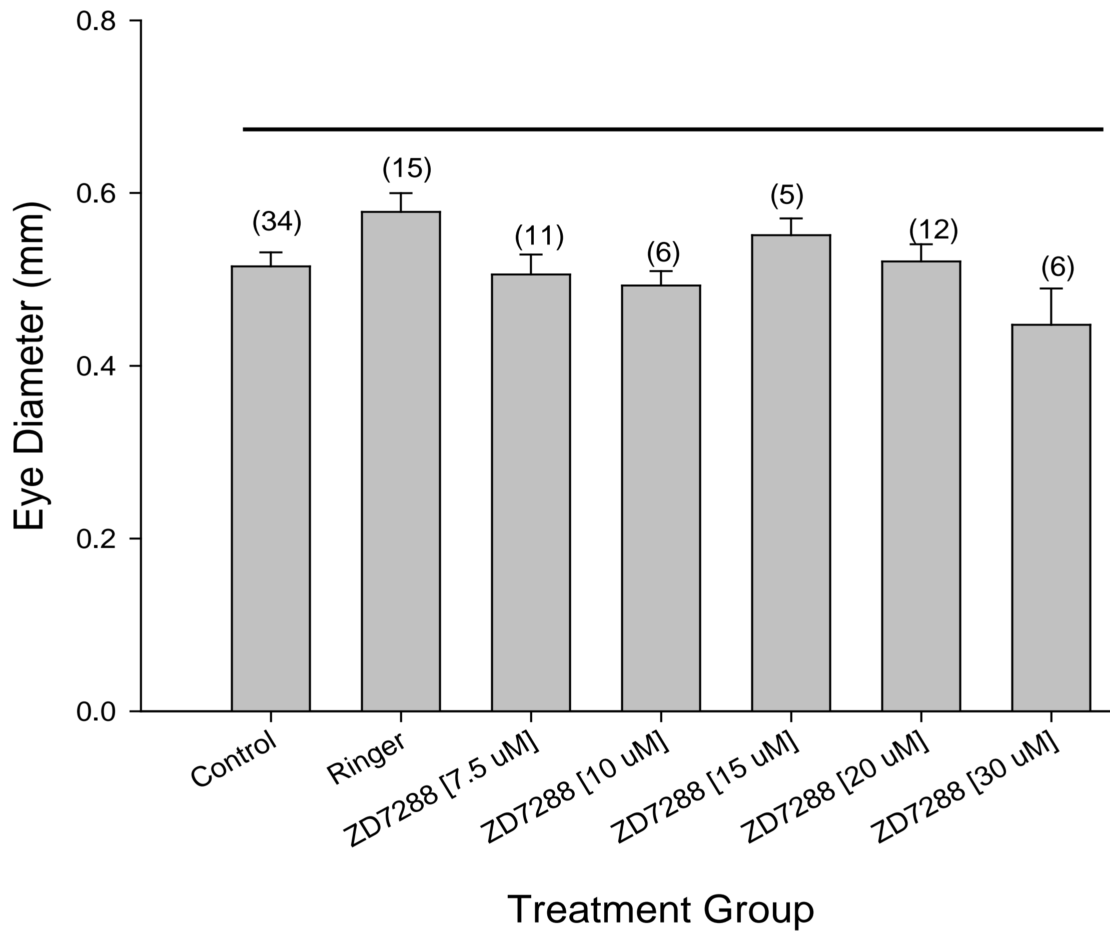

Fig. 7. Effects on eye diameter at 72 hours after 24 h of chronic dosing beginning at 48 h of development. Horizontal lines indicate statistically identical groups. Mean ± 1 S.E., are plotted, *n* values are in parentheses.

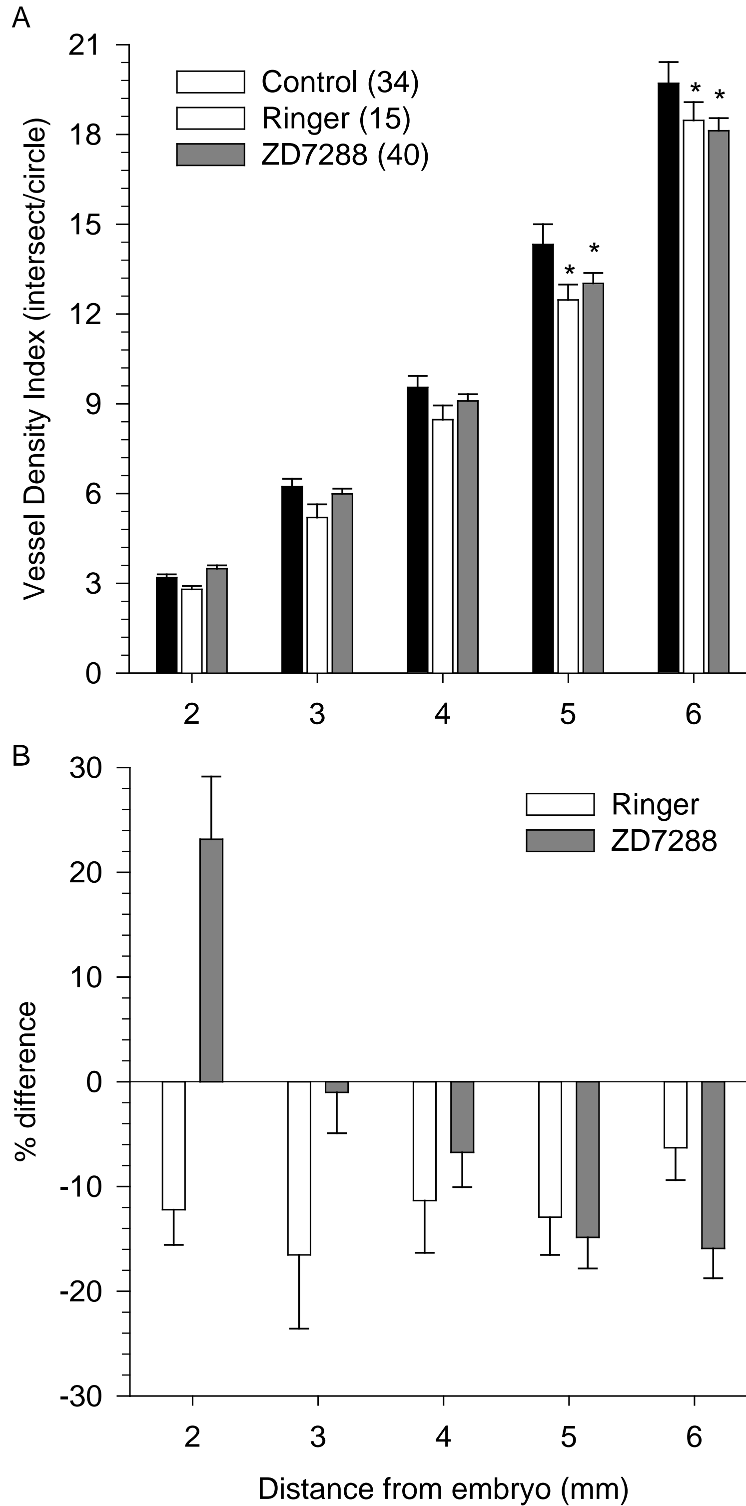

Fig. 8. Chronic effects on CAM vessel density index (A) and percent difference as compared to the control (B) at 72 h of development. Asterisk indicates groups that are significantly different. Data are represented as mean ± 1 S.E., *n* values are in parentheses.

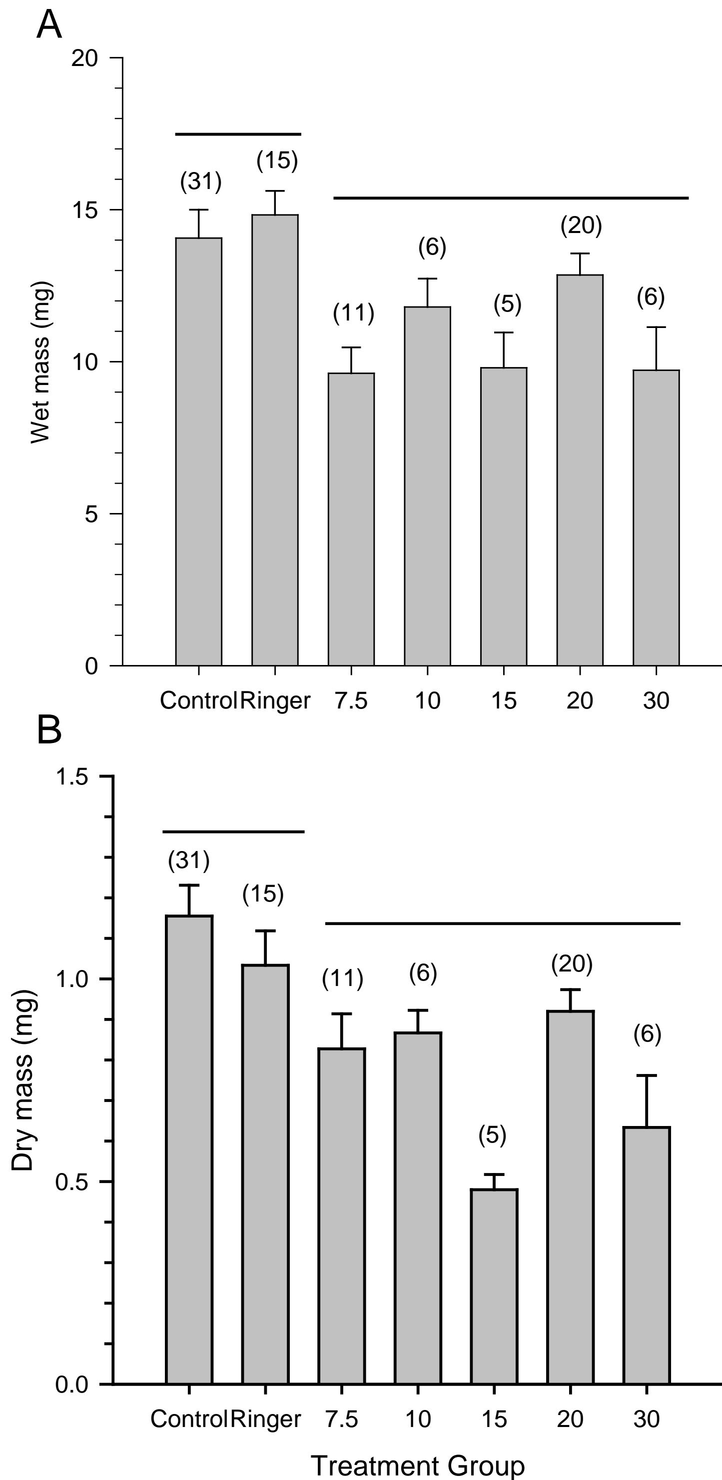

Fig. 9. Wet mass (A) and dry mass (B) as a result of chronic dosing for 24 h of different concentrations of ZD7288 at 48 h of development. Data are represented as mean ± 1 S.E. Horizontal lines indicate groups that are statistically identical, *n* values are in parentheses.

CHAPTER 4

DISCUSSION

This thesis investigated the relationship between cardiac performance, angiogenesis, growth and development during the early stages of chicken development. We altered heart rate, and thus arterial pulse pressure, to see if it caused changes in vasculature and consequent changes in growth (mass) and development (eye diameter).

Use of the Bradycardic Agent ZD7288

Heart rate of control embryos and embryos dosed with chicken Ringer were not different (Fig. 4, 5, 6). This indicates that the bradycardic effects caused by the application of ZD7288 were strictly pharmacological and not altered by the physical application of chicken Ringer - i.e. the vehicle by which the drug was administered. Acute dosing of ZD7288 at 30, 250 and 500 µM concentrations caused bradycardia that was dependent on dose and time (Fig. 4). The lowest concentration of 30 µM ZD7288 caused a significant decrease in heart rate but recovered much more quickly than the higher concentrations. Acute dosing at 48 h of development, of 250 and 500 µM ZD7288, caused the same degree of bradycardia (Fig. 4). Similarly, the bradycardia induced by chronic ZD7288 at 72 h of development was independent of dose above 7.5 µM (Fig. 6). This could possibly indicate that, early in development, the embryos were exhibiting a “saturation” effect in which even the lowest doses were affecting all membrane channels in the embryonic heart. Small doses of ZD7288 (1 µM) have been shown to be sufficient to produce stable and profound bradycardia (67% heart rate

decrease) within 10 min of drug exposure in isolated mouse hearts, with a similar saturation effect (i.e. no further increase in heart rate) of ZD7288 at the higher concentrations (Stieber et al., 2003). In the current study, the highest concentration of 30 μM ZD7288 used in the chronic experiments (which did not produce a significantly greater bradycardia than the lower concentrations) proved to be a lethal dose, as many of the embryos did not survive the full 24 h of dosing before death occurred. Additional studies to determine the effects of ZD7288 in older chicken embryos would help characterize this drug for potential use in future studies.

Angiogenesis and Heart Rate

A decrease in heart rate will increase diastolic duration and pulse pressure in the heart and central arterial vessels. This in turn is likely to cause greater blood vessel wall distension, increase the degree of shear and strain on the endothelial cells lining the blood vessels, and potentially affect angiogenesis. Yet, induced bradycardia did not alter angiogenesis in the present experiments, as quantified by the CAM vessel density index, at the closest distances of 2, 3 or 4 mm distance from the umbilical stock of the embryo (Fig. 8). Indeed, at the farthest measured distances from the embryo (5 and 6 mm) there was a significant *decrease* of CAM vessel density between the ZD7288 and control treatment.

These data do not support the original hypothesis, that decreased heart rate would stimulate angiogenesis and thus, create a greater number of CAM blood vessels. There are several possible reasons for this finding. Perhaps early on in embryonic development the degree of shear and strain mechanically produced on the endothelial cells lining the forming vessels is not great enough to stimulate angiogenesis and create

a greater CAM vessel density. Alternatively, endothelial cells at these early stages of development may be less sensitive to mechanical shear and stress than later in development. Either of these scenarios may have failed to stimulate angiogenesis at 72 h of development. Under this scenario, the original hypothesis might have been supported if we had employed older chicken embryos.

Unexpectedly, there was also a significant difference in CAM vessel density index between the Ringer-treated embryos and controls (Fig. 8). Sharp gradients in metabolic activities and bioelectric potential exist within the developing blastoderm of the embryo (Romanoff, 1960). A sharp pH gradient exists between the acidic yolk and basic albumin, which then helps establish the embryonic dorsal-ventral axis (for review see Stern and Cannings, 1988). Chronically applying chicken Ringer to the embryo's surface, as in the protocol used in the present study, may be disturbing the natural metabolic and bioelectric gradients that surround the heart and CAM vessels, even by simply washing out molecules important to normal CAM vessel development. There also may be unknown hormonal factors or stimulatory angiogenic agents during these early stages that are diluted or washed away by continuous application of Ringer. Since the application of both Ringer and ZD7288 decreased CAM vessel density, this suggests there is a specific environmental balance that maintains normal CAM blood vessel development. Application of ZD7288 was also combined with chicken Ringer as the vehicle solution to dose the embryos. It is possible that this washing away of necessary factors – rather than a pharmacological effect of ZD7288 - could have affected the development of vessels in the ZD7288-treated embryos as well, and the changes in CAM vessel density were actually not related to heart rate and pulse

pressure changes in the embryos as originally hypothesized.

Development, Growth and Cardiac Output

Development is the complex interplay of growth and maturation in the embryo, which is directed by the coordinated expression of various genes such as maternal and embryonic genes. Eye diameter is a very useful method to track such development in the chicken embryo because the eye shows large, consistent increases during hours 72 to 120, and is also straight-forward to measure (Romanoff, 1960). In the present experiments, eye diameter (mm) was measured for each embryo in each group for comparison of the embryos rate of development. Any difference in eye diameter presumably would indicate that ZD7288 was altering the normal developmental trajectory of the embryo. That there was no difference in eye diameter between any of the chronically treated groups indicates that the development, *per se*, of the embryos was not altered by chronic dosage of ZD7288 or by Ringer for 24 h in the present study (Fig. 7).

Burggren et al. (2004) reported that at no developmental stage was there a difference between the means of the eye diameters of the control embryos and the embryos that had their outflow tract of the heart partially-ligated for 4 h. This study showed that the development of the eye within any state is not dependent on any particular level of cardiac output. In the present study, cardiac output was not measured, but there is evidence that heart rate is a strong indicator of stroke volume and cardiac output in the chicken embryos. For example, Bowers et al. (1996) demonstrated that embryonic cardiac output is linearly related to heart rate and preload while end-diastolic volume is linearly related to stroke volume. In their study, embryos

vasodilated with nitrous oxide showed that a decrease in end-diastolic volume had clearly a reduction in heart rate and preload, which then led to a reduction in stroke volume. We assume, then, that the decreased cardiac output associated directly with the decreased heart rate in the present study was not necessary for eye development of the treated embryos.

Wet and dry mass of the whole embryo are indicative of whether an embryo experiences changes in overall growth, through cell division and/or cell hypertrophy. Chronic exposure to ZD7288 resulted in a significant decrease in wet and dry body mass than either control or Ringer-treated embryos (Fig. 9). Thus, the decrease in heart rate (and presumably cardiac output) created by ZD7288 slowed absolute growth, as measured by reduced body mass, but not development *per se*, as measured by sustained increase in eye diameter.

Collectively, these findings suggest that changes in heart rate at 48 to 72 h in development do not greatly affect vessel development, but through its presumed action on cardiac output, heart rate changes appear to alter the transport of nutrients and materials required for normal embryonic growth. This finding is significant, because previous studies have suggested that the early heart beat and the convective flow of blood it generates is not required for continued oxygen consumption or development (Burggren et al., 2000; Burggren, 2004). However, the present study suggests that convective blood flow is required for growth of the embryo. Burggren et al. (2000) reported that embryos on day 3 and 4 of development, that had their ventricular outflow tract completely ligated, had decreased embryonic body mass. Thus, it would appear that while diffusion of oxygen across the embryo body wall can maintain oxygen

consumption, diffusion of nutrients and or waste products must be supplemented by blood convection in early embryonic states.

Future Directions

There are great similarities in the cardiovascular system of all vertebrates at the start of their development, making research at such early stages transferable between species. The goal of this research was to understand how heart rate affects the physiological processes of angiogenesis, growth and development in chicken embryos and, by extension, potentially all vertebrates.

Future applications of this research could possibly be in fetal surgery which is becoming more and more commonplace and now includes the use of interventional cardiac procedures (Michelfelder et al., 2008; McElhinney et al., 2010). Up to 15% of all clinically recognized pregnancies are miscarried during the first 12 weeks of gestation (Wilcox et al., 1988; Wang et al., 2003), in part due to abnormal cardiovascular development. By looking at the links between cardiac performance, development and heart rate, we could reveal a possible cause of early pregnancy loss. Along with this, finding ways to stimulate angiogenesis could possibly contribute to the development of new interventions for embryonic viability and help aid stoke victims and people with myocardial infarction.

One question that might be asked is “Are studies of embryos transferable to adults”? The hypothesis that the study was investigating is well known to function in adults. In adults, lower heart rate is associated with elevated systolic pressure and reduced diastolic pressure. This results in a larger degree of stretch/relaxation of the endothelium lining the blood vessels with each cardiac cycle and in adults, is

responsible for the up-regulation of angiogenesis. The mechanism for embryos may be different but the comparison between embryos and adults can still be used in furthering our understanding of how these relationships interact.

The most challenging obstacle of this study was to obtain reliable and reproducible physiological values from the fragile and small early stage chicken embryos. Relatively few physiological studied have been performed on early stage embryos for this reason (see Hu and Clark, 1989; Hu et al., 1991). Along with this challenge, the specific side effects of these “pure bradycardic agents” like ZD7288 is not fully yet known. These drugs are designed to slow heart rate specifically by depressing diastolic depolarization rate, with limited side effects on action potential duration and inotropic state (DiFrancesco, 2006). These claims of limited side effects need to be researched as to be included in studies like this one to remove any ideas that this could be a variable on how the data is interpreted.

Future aims of this project are to measure exact stroke volume, cardiac output and mean velocity using a 20 MHz Pulsed Doppler flowmeter on the dorsal aorta on the treatment groups compared to the control. Ventricular contractility also needs to be measured, to see if chronically dosing the embryo is depressing ventricular contractility in the heart. Histology of the heart can also be done to see if there are any differences in the cardiac wall thickness between the control and treatment groups. The pressures at all of the distances from the embryo need to be measured using a Micropressure system to see if there are any pressure changes. Also, inducing tachycardia (increasing heart rate) by pacing the heart will give us further insight into the effect of heart rate on angiogenesis. For future analysis of angiogenesis, it would be very interesting to use

fluorescent microscopy which labels endothelial cells to evaluate how either bradycardia or tachycardia has affected the vessel growth. Also it would be of interest to determine the genes, such as VEGF and FGF expression level that are involved in the changes of angiogenesis at this stage of development by RT-PCR.

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