BETA-ADRENERGIC BLOCKADE VIA ATENOLOL AND ITS EFFECTS ON BLOOD

PRESSURE, HEART RATE, AND RENAL MORPHOLOGY IN THE

DEVELOPING CHICKEN *Gallus gallus domesticus*

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Chicken embryos were chronically exposed to the $\beta 1$- blocker atenolol during one of three stages: mesonephros (E7-E9), mesonephros-metanephros (E11-E13), or metanephros (E15-E17). Mesonephros group hearts were larger than all other groups ($P < 0.01$). Mesonephros and metanephros group kidneys were larger than all remaining groups ($P < 0.0001$). The mesonephros group nephron number was ~40% lower than control values ($P = 0.002$). Glomerular areas were 26% and 18% larger than the control group in the mesonephros and metanephros groups, respectively ($P < 0.001$). These data suggest an E7-E9 critical window of cardiovascular and renal development for atenolol. Acute atenolol exposure in E15 embryos showed an increase in mean arterial pressure with all but the highest dose. All doses significantly decreased heart rate.

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CHAPTER 1

INTRODUCTION

1.1.   Rationale for the Study

Many physiological studies adhere to the paradigm in which adult physiology is

analyzed to infer the mechanisms taking place during the embryonic or fetal condition.

The information gathered from such studies is often very useful in understanding the

corresponding mechanisms involved in prenatal development. Although this has proven

instrumental to physiological discovery, exchanging this methodology for a more direct

one could prove more effective in elucidating the development of physiological

processes. The object of this study centers on direct observation of the early effects

taking place in the embryonic chicken as cardiovascular parameters are depressed by

decreasing heart rate and blood pressure using the β blocker atenolol. We then

characterized morphological changes in the renal microstructures and the extent to

which these deviated from the normal developmental trajectory. Atenolol causes a

selective β_1-adrenergic receptor (β_1-AR) blockade in mammals (Thorley et al. 1981). In

the chicken embryo, atenolol is less specific and there is both β_1 and a partial β_2

blockade (Kamimura et al. 1995). β_1-AR blockade on heart tissue reduces the force of

contraction, heart rate, and conduction velocity in the atrio-ventricular (AV) node

(Triposkiadis et al. 2009). Additionally, β_1-AR blockade affects the renin angiotensin

aldosterone system (RAAS) to reduce renin secretion by the juxtaglomerular cells of the

kidney which results in a reduced systemic blood pressure (Ahmed et al. 2010). In this

study, these regulatory systems were manipulated by treating with atenolol during one

of three renal stages of development. This was followed by a morphological evaluation of the kidneys to isolate critical periods of exposure to atenolol.

A primary aim of this work was to explore the interaction between the developing cardiovascular and renal systems. Aside from the heart's clear mechanical effects of blood pressure upon all tissues of an organism, there are more subtle extant mechanisms. Cardiovascular-renal interaction studies must necessarily view the heart/vasculature and kidney as functioning endocrine organs secreting multiple regulatory hormones. These include atrial natriuretic peptide (Cheung et al. 1998) by the heart, nitric oxide and endothelin by the vasculature (Durieu-Trautmann et al. 1993), as well as renin and renalase by the kidney (Wheeler-Schilling et al. 2001).

Currently, much of the research carried out involving cardiovascular-renal communication is focused in the biomedical context of adult-onset pathology. The vast majority of those studies investigate adult animals with the goal of developing better treatments and or cures to chronic endemic illnesses such as hypertension and coronary artery disease. However, growing knowledge is beginning to challenge the premise that studying adults is the best method to decipher and prevent adult illness. Fetal programming, whereby an insult is introduced sometime in embryonic or fetal life at a critical period of development, has permanent ramifications upon the adult's metabolic or physiologic phenotype (Kensara et al. 2005). For instance slow growth during fetal life and even early infancy is correlated with rapid weight gain during childhood (Barker 2002), which can predispose the individual to type 2 diabetes mellitus and hypertension. Both of these chronic conditions then place the individual at a high

risk of coronary heart disease. A better understanding of fetal physiology can help researchers explore the mechanisms involved in such effects.

Barker (2002) hypothesizes that such mechanisms could include the development of insulin resistance *in utero*, reduced nephron number at birth, and/or altered micro-architecture and function of the liver. Of particular interest is the idea that a reduced nephron number can have long term effects on adult health, particularly in the programming of hypertension. In rats maternal under-nutrition (withholding protein) can produce intra-uterine growth retardation (IUGR) and reduced nephron numbers in the adult (Merlet-Bénichou et al. 1994). The rat nephron deficit seems to be induced before birth, sometimes occurring as early as pre-implantation without postnatal compensatory strategy even though nephrogenesis continues for 8 days after birth (Moritz et al. 2003). An insult such as maternal corticosterone treatment can impair development of the intra-renal RAAS, leading to reduced nephron number and adult-onset hypertension in those offspring (Singh et al. 2007). In sheep, prenatal exposure to dexamethasone, a potent synthetic glucocorticoid, reduces nephron number and results in higher glomerular corpuscular volumes in animals at 7 years of age (Moritz et al. 2003). This reported reduction in nephron numbers in rats and sheep via undernourishment and RAAS disturbance raises the question of whether disrupting the RAAS via β-adrenergic blockade could parallel these studies and yield similar morphological effects in the kidneys.

Another means to study RAAS disturbance is to use angiotensin II selective AT1 receptor blockers (ARB). An ARB known as Losartan, was used to explore the effects of offspring kidney morphology in rat dams (pregnant rats). Offspring sacrificed on

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embryonic Day 18 had abnormal renal capsule formation, enlarged Bowman's spaces, and an increased amount of induced and underdeveloped glomeruli with reminiscent apoptotic bodies (Akil et al. 2005). Curiously, a common concern in the medical community is the danger associated with pregnant hypertensive patients taking angiotensin-converting enzyme (ACE) inhibitors. Treatment with this class of drugs has been correlated with neonatal hypotension, anuria, oligohydramnios, and renal failure in fetuses exposed during the second and/or third trimesters (Quan 2006). While the studies imply that ACE inhibitors and ARBs are inducing a direct pharmacological effect via disruption of downstream RAAS components, we must not discount the idea that a hypotensive effect specifically in the fetuses could be disturbing the renal development.

The current study used the chicken embryo *Gallus gallus domesticus* to study cardiovascular-renal relationships for several reasons. The chicken provides the researcher with three general advantages. Primarily, the chicken embryo is easily accessible for manipulation as it exists outside of the mother during incubation. Incubation environment is then easily controlled and altered. Second, due to its external incubation, experiments conducted on the embryo need not consider direct maternal physiological influence. Since the chicken chorioallantoic membrane (CAM) and vasculature is easily accessible, the experimental drug atenolol can be administered and its effects recorded without having to consider maternal influence as a confounding variable. In addition to these advantages, chickens have a relatively short incubation period of 20-21 days, (Hamburger et al. 1951) versus the commonly used sheep mammalian model which has a gestation period of about 147 days (Gopalakrishnan et

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al. 2005). This allows for reasonable time to conduct stage-specific experiments and collect higher sample sizes more quickly.

To better understand the effects of β-blockade in the chicken embryo, there must first be a discussion about blood pressure regulation.

1.2. Blood Pressure Regulation in the Chicken Embryo

1.2.1. Renin Angiotensin Aldosterone System

Extensive research has been carried out on the renin-angiotensin aldosterone system (RAAS) as it pertains to adult-onset pathologies such as hypertension, congestive heart failure, and disruption of electrolyte homeostasis (Wehlage et al. 2011). In adult mammals, birds, and other vertebrates, the RAAS plays a direct role in blood pressure regulation via sympathetic activation of the β_1 receptors of the juxtaglomerular cells, upon signaling from the appropriate arterial mechanoreceptors. Stimulation of the β_1 receptors of the juxtaglomerular cells can be elicited by stretch of the local renal glomeruli, detection of low blood salt in the macula densa cells, or sympathetic stimulation (Burns et al. 1993). Stimulation causes secretion of the hormone renin. In the circulation, renin binds angiotensinogen and cleaves it into an inactive decapeptide called angiotensin I. Angiotensin I then binds to a dipeptidyl carboxypeptidase known as angiotensin converting enzyme (ACE) and is cleaved into the active octapeptide angiotensin II (ANG II) (Wheeler-Schilling et al. 2001). ANG II itself is a vasoconstrictor and can in this way increase systemic blood pressure. ANG II also mediates the release of aldosterone from the adrenal cortex and antidiuretic hormone from the posterior pituitary which both serve to ultimately increase or conserve

blood volume. Thirst and the desire for salt consumption increase as ANG II interacts with the CNS (Fyhrquist et al. 2008). Finally ANG II is also involved in decreasing the effects of the baroreflex (Guo et al. 1984).

The kidneys are arguably the most important organs involved in blood pressure regulation in the higher vertebrates. In the adult chicken, each kidney consists of many conical macrostructures called lobules (Wideman 1989). Each of these lobules contains numerous nephrons or the basic functional units of the kidney. The chicken kidney contains different forms of nephrons known as the "looped nephrons" which contain a loop of Henle and are located in the juxtamedullary region of the kidney and the "loopless nephrons" that lack a loop of Henle and are located in the cortical region (Casotti et al. 1995). This presence or absence of the long loops of Henle determine the chicken's capability to concentrate or not concentrate urine respectively (Wideman 1989). In the adult, blood to be filtered reaches the kidneys and must then enter via the afferent arteriolar vessels of the nephron's glomerulus. Here the blood is filtered as it enters the Bowman's capsule and continues to be filtered throughout the nephron proximal tubule, loop of Henle (if looped nephron), distal tubule, and collecting duct. Waste products then leave the kidney via a straight narrow ureter and enter the terminal portion of the gastrointestinal tract called the cloaca. If the bird is normally hydrated, the waste contents are then moved via reverse peristalsis into the colon. The urine composition is then further modified by coming in contact with these gastric epithelia. Finally, waste is excreted from the body (Braun 1993).

The capability of the chicken kidney to regulate blood pressure during embryonic life is not fully understood. The aforementioned mechanisms may begin to fully function

during late embryonic development or even in post-natal life. Many of the RAAS components are recognized in the early chicken embryo but tend to be described in an independent context and with independent purpose from blood pressure such as organogenesis or hematopoiesis (Park et al. 2009). For instance, expression of ACE, renin, angiotensinogen, and ANG II receptors has been detected as early as Hamburger Hamilton Stage 8 (HH8) in the yolk sac of the chicken embryo just as blood islands were differentiating in the extraembryonic area adjacent to the site of RAAS component production. Blocking the RAAS at two levels by use of an ACE inhibitor and chicken angiotensin (cAT) receptor blocker, showed that ANG II was required for primitive erythropoiesis (Savary et al. 2005). ANG II behaves as a trophic factor for cardiac and vascular development as well (Baker et al. 1990). In humans, angiotensinogen mRNA shows prevalent expression in the yolk sac, liver and kidney from days 30-35 of gestation. ACE mRNA expression is observed in the chorion, kidney, and heart and allows for the production of ANG II. Human ANG II receptor types AT1 and AT2 are both expressed by 24 days of gestation, suggesting a role in organogenesis before taking on the duty of blood pressure regulation (Schütz et al. 1996).

The RAAS is involved in promoting production of extracellular matrix, potentiating cellular growth factors, and promoting expression of proto-oncogenes (Huckle et al. 1994). Mutation in genes encoding components of the RAAS in mice and humans causes severe congenital anomalies of the kidney and urinary tract (Yosypiv 2011). Addition of ANG II to a whole embryo culture of embryonic 9.25 or 10.25 day old Sprague-Dawley rats increased ventricular growth and myocyte hypertrophy (Baker et al. 1990). Rat embryos treated with Losartan, an ANG II receptor blocker, showed

reduced ventricular development and cardiac dilation (Price et al. 1997). Although AT1 receptors are responsive to ANG II, there is no indication that the RAAS is actually functioning on its own in this stage of early development (Price et al., 1997). Components of the RAAS and their role in growth and differentiation are well established, but information on direct blood pressure and osmotic regulation during fetal life is limited. When does the RAAS actually begin to regulate blood pressure and blood osmolality? In late gestation fetal sheep, when hypotension was induced via obstruction of the vena cava, renal blood flow decreased and plasma renin activity increased from 2.5 ± 0.8 ng Ang-I/mL to 4.6 ± 1.2 ng Ang-I/mL (Lakhdir et al. 2001). However, aldosterone synthesis by the fetal adrenal gland seems to be unresponsive to exogenous ANG II until close to term (Wintour et al, 1998). A separate study revealed that if a sheep was given ANG II during mid-gestation on a 3 day dosing regimen, there was an increased expression of an aquaporin 1 water channel. Apparently ANG II functions late in gestation in fetal sheep via AT1 receptors to promote salt and water excretion by the metanephros to keep fluid-filled fetal sacs at appropriate volumes for growth and development (Wintour et al. 1998). In a study using chicken embryos, ANG II produced a dose-dependent increase in arterial pressure from embryonic Day 13 to 21 (Crossley et al. 2010). These experiments confirmed at least a partial functioning of an immature RAAS in fetal chicken and sheep.

1.2.2. Neural Regulation and β-Adrenergic Receptor

β-adrenergic receptors (β-AR) belong to the G-protein coupled receptor family and play a large regulatory role in myocardial function. Three subtypes are generally

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recognized: β1, β2, and β3 (Wallukat 2002). However, it has been shown pharmacologically that a 4th β-AR may exist (Kaumann et al. 1997). β-ARs are found in many places in mammals. This study focuses on the receptors populating mainly the heart and juxtaglomerular cells of the kidney. In the human heart, adrenergic β_1 and β_2 receptors predominate. These receptors are found in a β1:β2 ratio of 70-80%:30-20% in the ventricles and 60-70%:40-30% in the atria (Brodde 1991). In the chicken embryo, a similar ratio of receptors exists where the β1-AR predominates by 75-80% over the β2-AR (Asano et al. 2001). Receptors can be stimulated by catecholamines such as epinephrine and norepinephrine to produce positive inotropic and chronotropic effects (Brodde et al. 1999). The current study will exploit this action in the chicken embryo to induce via β-blockade a hypotension and bradycardia. β-ARs seem to populate different tissues of the embryo at different times. For instance, α and β receptors are present in the vascular tree by 30% development or Day 6 of incubation (Koide et al. 1989; Saint-Petery et al. 1974).

The isolated chicken embryo heart is sensitive to β-adrenergic drugs earlier than embryonic Day 6. Studies show epinephrine increases arterial pressure as early as Day 3 of development (Hoffman Jr et al. 1971; Girard 1973). A second study claims that the β-adrenoceptor in the chicken embryo begins to display sensitivity to catecholamines on embryonic Day 5 (Lipshultz et al., 1981). This coincides with when the mesonephros has begun to function (Romanoff 1960) and thus allows use of embryonic Day 7 to safely proceed with β-blockade in the heart with an existing temporary kidney. Lipshultz et al. (1981) demonstrated that embryonic Day 5 chicken hearts showed dose dependent increases in chronotropic contraction and cAMP levels in response to

epinephrine. This effect could be blocked by the β-antagonists propranolol and practolol. Another study published around the time of Lipshultz et al. (1981) showed that the ventricle of the chicken embryo responded to the β-agonist isoproterenol as early as embryonic Day 4. Although tissues are able to respond to β-adrenergic activation and blockade at such an early embryonic stage, this fact may not reflect a real physiological action analogous to that observed in the adult. Clearly, sympathetic innervation has not taken place by Day 3, and therefore there may be unknown functions of having β-ARs present so early on. Adrenergic nerves are first capable of stimulating cardiac contractility on Day 16 (Higgins et al. 1981). Vagal nerve branches reach the truncus and atria at 20% of development (Kuratani et al. 1990) and connect with all cardiac chambers by 35% of development. Sympathetic cardiac nerves from the sympathetic ganglia reach the heart region at 50% of development (Higgins et al. 1979; Kirby et al. 1980). Since endocrine constrictor responses are typically attained before neurogenic responses, the sympathetic control of arterial vascular resistance is limited to the late phases of fetal life in chickens (le Noble et al. 2000). This developmental pattern allows for manipulation of the cardiovascular system of the chicken embryo throughout the majority of development with only minimal neurogenic counteractions to pharmacological insult.

1.3.   Medical Applications to Current Atenolol Study

Maternal disease-states or pharmacological treatment of those diseases can pose a tremendous risk on the health and stability of the mother and developing fetus. Hypertension is present in approximately 10% of all pregnancies (Podymow et al.

2008). These patients fall under one of four specific hypertensive conditions (Moretti et al. 2012):

    (i)   Chronic hypertension: pre-pregnancy hypertension that persists into pregnancy.

    (ii)   Preeclampsia and possible eclampsia: Pre-eclampsia occurs in 5% of all pregnancies and is characterized by hypertension, proteinuria, edema, and other symptoms appearing after 20 weeks gestation (Nayak et al. 2012). Eclampsia results from progression of preeclampsia to include seizures, and higher maternal and fetal mortality (Duley 2009).

    (iii)   Chronic hypertension with superimposed pre-eclampsia:

    (iv)   Gestational hypertension: Hypertension usually presenting after 20 weeks gestation or into 3rd trimester.

For the patient experiencing mild symptoms, the decision to receive drug therapy or not is dependent upon their potential risk of organ damage, severity of hypertension, and the presence or absence of preexisting cardiovascular disease (Sibai 1996). Physicians are faced with the difficult task of deciding what is safe enough to prescribe and what medications should be eliminated during particular trimesters while minimizing risks to both mother and fetus. Additionally, 50% of all pregnancies are unplanned (Al-Maawali et al. 2012) and many may remain undiagnosed for a substantial duration of the gestational period. Many of the women that fall into this category and suffer from chronic hypertension may therefore unknowingly expose the developing fetus to either of the anti-hypertensives such as angiotensin converting enzyme (ACE) Inhibitors, β-blockers, and angiotensin II receptor blockers (ARBs) (Moretti et al. 2012).

Many studies have shown that ACE inhibitors and ARBs are contraindicated during the 2nd and 3rd trimester of pregnancy (Ratnapalan et al. 2002; Cooper et al. 2006; Caton et al. 2009). Alarmingly, at least one study showed that ACE inhibitor usage during the 1st trimester was correlated with and increased risk of cardiovascular malformations. See Cooper et al. 2006 for references. Subsequent studies have failed to duplicate the findings and attribute the congenital heart malformation risk to the underlying hypertension in the mother (Li et al. 2011). Usage of atenolol during pregnancy is associated with intrauterine growth retardation (IUGR) probably due to increased fetal and uteroplacental peripheral vascular resistance and decreased placental blood flow (Stephens et al. 2009). Often, any of the three RAAS-exploiting drugs continue to be prescribed to pregnant women, although none have been proven safe for first trimester use.

The safest first choice for anti-hypertensives is methyldopa which works as an α_2 agonist. Second line agents include nifedipine (calcium channel blocker), hydrochlorothiazide (diuretic), labetalol (α & β antagonist), hydralazine (vasodilator), β blockers , ACE inhibitors, and ARBs (Podymow & August, 2008). Sadly, the latter three drugs have been linked to intrauterine growth restriction (Stephens et al. 2009), congenital malformations (Cooper et al. 2006), and severe renal malformations if mothers were exposed during the 2nd or 3rd trimesters, respectively (Quan, 2006). Although numerous worrisome fetopathies associated with these drugs have been elucidated in recent years, a more subtle and perhaps substantially more relevant issue is that of intrauterine growth restriction induced by exposure to β blockers or other *in-utero* insults that ultimately result in low birth weight (Stephens et al. 2009); (Symonds

et al. 2007). The Brenner Hypothesis holds that low birth weight is associated with a reduced nephron number which later on in life can predispose the individual to an increased arterial pressure. Also, the RAAS appears to be more active in low birth weight individuals (Dotsch 2009).

1.4.   Research Objectives and Hypotheses

 Our study seeks to explore the implications of β-blockade during particular stages of renal development and how that relates to nephron number in late embryonic life. The reduction in nephron number and hyper-activation of RAAS components is a major concern for a developing fetus, because any number of mechanisms could be in progress to program eventual hypertension in the later adult individual.

This study has been designed to provide a better understanding of how the cardiovascular and renal systems affect one another during embryonic and fetal development. Pharmacological insult was applied to chicken embryos using the β-blocker atenolol during three specific periods of renal development. The chicken embryo CAM was injected with atenolol either within a three day period when the mesonephros is the only functioning kidney, within a three day period when both the mesonephros and metanephros simultaneously function, or within the three day period when the metanephros is the only functioning kidney. By using atenolol at these different developmental periods to alter the cardiovascular system, we can identify when the embryo is most susceptible to cardiovascular fluctuation and experiences a critical window of nephrogenesis.

This study is designed around the testing of three hypotheses:

- Hypothesis 1: B-blockade via a pseudo-chronic regimen of CAM injections with atenolol in chicken embryos during the earlier renal developmental period will result in smaller total body, heart, and kidney masses than in embryos treated during a later renal developmental period, and all embryos exposed to β-blockade will have smaller body, heart, and kidney masses than control embryos.

With these pseudo-chronic CAM injections, we aimed to determine how atenolol exposure could affect body, heart, and kidney masses. Atenolol has been associated with intrauterine growth restriction and reduced birth weight (Karanam et al. 2012). This is consistent with our finding that whole body and organ masses were indeed affected by atenolol exposure.

Therefore, a next aim of this study was to determine how doses above and below our chosen CAM injection dosage were altering heart rate and blood pressure. We tested how atenolol exposure immediately after intra-arterial dosing changed heart rate and blood pressure while also observing the duration of the effects. Antihypertensives like ACE inhibitors produced hypotension in the neonate (Quan 2006). Beta-blockade could similarly effect the embryonic chicken long term.

- Hypothesis 2: Acute β-blockade via intra-arterial atenolol injections will induce a hypotension and bradycardia.

While the physiological effects of β blockers on the chicken embryo have been mapped previously, we sought to describe how defined doses worked to affect the embryo. Once it was determined how treatments altered the embryonic cardiovascular physiology, any resulting changes in kidney morphology were analyzed. Maternal

and/or fetal hypertension can reduce the total nephron number. Our next aim was to determine if atenolol exposure via CAM injection had altered the nephron number and average size of the glomeruli.

- Hypothesis 3: The transient hypertension and bradycardia induced by atenolol will disrupt renal development and alter nephron numbers when compared to control embryos.

This analysis can then be used to determine the implications of possible pathologies the embryo would have experienced post-hatch and potentially in adulthood.

CHAPTER 2

MATERIALS AND METHODS

2.1.   Egg Source and Incubation Conditions

Fertilized white leghorn eggs (*Gallus gallus domesticus*) were obtained from Texas A&M University, College Station, and shipped to the University of North Texas, Department of Biological Sciences.   All eggs were incubated under normoxic conditions at 21% O_2, 37.5 ± 0.5 °C and 55-60 % relative humidity and turned automatically every three hours (Bamelis et al. 2002). Day 18 eggs were removed from incubation, sacrificed, and dissected for organ mass collection. Day 18 was chosen for analysis in all mass and histology experiments to document morphological and physiological changes occurring before air-breathing begins, which could occur as early as Day 19 when internal pipping can begin (Altimiras et al. 2000; Villamor et al. 2002).

2.2.   Arterial Catheterization

On Day 15, embryo blood pressure and heart rate data were collected via cannulation of an arterial vessel. A chorioallantoic membrane (CAM) artery was located and marked through candling. The artery site was then identified using a pencil to mark the shell at the appropriate entry point as well as to note the blood flow direction. Each egg was prepared for surgery by placing it onto a cushioned surface that sat inside a temperature controlled chamber that kept the embryo's environment regulated at about 38°C. To prepare the catheter, polyethylene tubing (PE-90) was heat-pulled to produce a catheter tip of approximately a 0.5 mm diameter. The tip was cut at a 45° angle to allow for easy insertion into the CAM vessel. The catheter was hooked up to a needle

connector which was connected to a 15 cm length of PE-90 tubing connected to a 23 gauge needle connected to a 1mL syringe. The syringe and tubing was filled with heparinized saline (0.9%) and was inspected to ensure no air bubbles were present. This prepared the catheter for proper use with the pressure transducer system.

To begin the catheterization surgery, an 18 G needle was used to puncture the egg shell. Fine tip forceps were then used to remove the egg shell while care was taken to leave the inner eggshell membrane intact. The exposed membranous area was then swabbed with heparinized saline to keep it moist and preserve the structural integrity of the CAM. Using fine forceps, the shell membrane was slowly peeled off of the CAM, exposing the CAM and making it accessible for artery cannulation. Size 4-0 silk suturing thread was placed at three approximately equidistant spots along the artery to prepare for fastening of the catheter. The upstream suture served to temporarily impede blood flow. The middle suture secured the catheter. Finally, the downstream suture served to better manipulate the vessel and keep it taut during cannulation. After a semi-firm fastening of the upstream suture, small surgical scissors were used to snip a small slit in the artery between the middle and downstream sutures. The catheter was slipped into the artery and inspected for acceptable blood flow and pressure. The exposed catheter was secured to the shell with cyanomethacrylate glue accelerated by application of Zip kicker (Pacer Technology, PT-29). Finally, the middle suture was used to hold the catheter in place (Crossley et al. 2000).

2.3.   Atenolol Injection

Atenolol, the drug used for pharmacological treatments in this study, is a

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cardioselective β_1-Adrenergic receptor antagonist in mammals (Robinson et al. 1978). Atenolol is one of the most widely used β blockers clinically and is mainly used to treat primary hypertension (Carlberg et al. 2004). Atenolol was chosen as a suitable drug for this study due to its β_1-selectivity, lack of partial agonistic activity, and stability in the face of drug-metabolizing enzymes in humans (Sasaguri et al. 2011). These properties increased the likelihood that the drug remained active in the chicken embryo's system for a sufficient amount of time to induce chronic effects.

Atenolol in the form of powder was purchased from Sigma-Aldrich. The drug was prepared by dissolving the powder in autoclaved saline. Atenolol was injected just under the CAM. Each egg was placed on its side and the injection site swabbed with 70% ethanol for disinfection. An 18 gauge sterile needle was used to break a small hole in the egg shell measuring approximately 2 mm in diameter. Care was taken to preserve the integrity of the CAM by candling, which ensured no tearing of visible blood vessels during injection. After injection of the drug, a small amount of wax was melted onto the shell opening to seal it aseptically. Eggs were then returned to normal incubation until they were ready for dissection on embryonic Day 18.

2.4.   Atenolol Treatment Protocol

2.4.1. Acute Dosing

One group ($n = 9$) of Day 15 embryos received arterial atenolol injections of 0.75 µg/mg, 1.5 µg/mg, 3.0 µg/mg, 6.0 µg/mg, and 12 µg/mg of embryonic body mass, to document the acute blood pressure and heart rate response (see below) as well as the duration of the effect of a single acute dose. Due to atenolol's minimal metabolism,

these doses are expressed alternatively in the figures to represent the cumulative atenolol concentration in the embryo. The cumulative doses are therefore 0.75, 2.25, 5.25, 11.25, and 23.25 µg/mg of embryonic mass.

Chicken embryo body and extraembryonic membrane mass for each developmental day treated was approximated by using previously recorded mass values (Romanoff 1967).

2.4.2. Chronic Dosing

There were four treatment groups, each consisting of 15 embryos which received 3.0 µg/mg of body mass corresponding to their respective renal treatment period (total n = 60):

- Group 1 (control) served as the control group and received no injections.

- Group 2 (mesonephros group) underwent atenolol treatment during embryonic Days 7, 8, and 9, which correspond to the period when only the mesonephric kidneys are functioning.

- Group 3 (mesonephros-metanephros group) underwent treatment during embryonic Days 11, 12, and 13, which correspond to the period when both the mesonephric and metanephric kidneys are both functioning together.

- Group 4 (metanephros group) underwent treatment during embryonic Days 15, 16, and 17, which correspond to the period when only the metanephric kidneys are functioning (Romanoff 1960).

The three treatment groups received one injection a day for three consecutive days during the appropriate developmental stage to administer a pseudo-chronic dosing regimen.

2.5.   Heart Rate and Blood Pressure Measurement

Cannulated eggs were removed from the surgery station and placed inside a temperature controlled chamber fitted with a flow-through warm water system to maintain eggs at 37.5 ± 0.5 °C. This system contained six separate compartments, allowing measurement of data on six eggs simultaneously. Each compartment was covered by a flat stainless steel lid to maintain chamber temperature and humidity. Air was fed into each chamber to maintain normal gas availability. The free end of the CAM catheter of each egg was led out from the chamber and was connected to a MLT0699 Disposable Blood Pressure transducer (ADInstruments). Pressure transducers were calibrated with static water columns to calibrate for a zero value and for a hydrostatic pressure of 1 kPa. The zero level was set level to the highest point on the egg shell (catheter entry point) as it sat in the chamber, after Crossley and Altimiras (2000). Each pressure transducer was connected to an Octal Bridge Amplifier (ADInstruments) which in turn was connected to a PL3516 PowerLab data acquisition system. The PowerLab connects to a computer using LabChart 7 software to display and analyze arterial pressures and derived heart rates.

2.6.   Tissue Collection

On embryonic Day 18 embryos were sacrificed for measurement of body, heart,

and kidney mass. Day 18 was chosen for analysis in all experiments to document morphological and physiological changes occurring before the extra variable of air-breathing can be introduced, which could occur as early as Day 19 when internal pipping can begin (Altimiras et al. 2000; Villamor et al. 2002). Eggs were removed from the incubator and placed in a closed glass jar containing isoflurane vapor for 15 min to anesthetize the embryo, and were then removed and quickly decapitated (Copeland and Dzialowski, 2008). The embryo was then freed from its extra embryonic membranes and weighed for whole body wet mass. Hearts were excised from the body, blotted and weighed. Kidneys were removed and sat for approximately 5-10 min to allow the blood to drain but not blotted as the tissue was too fragile for such manipulation. Hearts and kidneys were then placed in open petri dishes and allowed to dry in a desiccator oven at 60°C for 48 h. Dry mass was measured for all sampled organs.

2.7.   Histology and Stereology

Excised kidneys were immediately placed in microcentrifuge tubes containing 4% paraformaldehyde solution and stored at 4°C for 48 hours of fixation (Nishimura et al. 2007). After fixation, whole kidneys were processed in a microwave rapid histoprocessor (Milestone RHS I) where they underwent a standard cycle of dehydration in ethanol, vaporization, and embedding in paraffin wax. Kidney sections (5 µm) were then created with a microtome (Leica RM2245). Sections were put on glass slides and placed in a 60°C dessicator oven to maximally affix them to the glass. Sections were

then cleaned of residual paraffin by soaking in a xylene bath. Next, the tissue underwent a standard rehydration and staining with Hematoxylin and Eosin.

Stereological analysis (Akil et al., 2005) was carried out for each kidney section. Kidney sections were photographed using an Olympus DP70 digital camera that was mounted onto a light microscope. Images were captured at 100X total magnification and manipulated for optimal visualization using the DP70 software.

Total numbers of glomeruli were counted across a whole kidney section to give a relative nephron number or nephron index for each embryo (Dziarmaga et al. 2006). The section used was approximately the largest cross sectional area that could be obtained from an individual kidney. The same kidney section was evaluated for an average glomerulus area value. Only nephrons with a clear glomerulus and Bowman's Capsule were included in the analyses. Measurements were taken using Image J software which was downloaded from the National Institute of Health website.

2.8.   Statistical Analyses

Body mass was analyzed using a one way ANOVA. Heart and kidney organ masses of the four treatment groups were analyzed via both an ANOVA and an ANCOVA, using embryo mass as the covariate. Nephron numbers were compared using an ANOVA and Student Newman Keuls post-hoc analysis. Glomerular average areas were analyzed similarly. Blood pressure and heart rate responses to various doses of atenolol were analyzed using matched pairs t tests that compared at least 30 sec of readings before each injection to at least 30 sec after each injection. This analysis determined whether each individual dose produced a significant change in

physiology. A one way repeated measures ANOVA was used to determine whether the different doses caused significantly different percent change responses in blood pressure and heart rate. Analyses done on blood pressure refer to mean arterial pressure (MAP). Significance threshold was set at $P = 0.05$. Data are presented and analyzed as mean ± standard error.

CHAPTER 3

RESULTS

3.1.   Body and Organ Masses

Whole body wet mass was significantly smaller in the treatment group embryos

than in control (21.5 ± 3.6 g) embryos (Fig. 1, ANOVA and Student Newman Keuls test

[SNK], $P = 0.0008$). The earliest treated Mesonephros group had the smallest mean

whole body mass (16.3 ± 2.4 g). For dosed embryos, means had a direct relationship

with treatment time. That is, the later in development the treatment period, the greater

the whole embryo mass.

Mean heart wet mass of the Mesonephros-Metanephros group was significantly

smaller than the control group (Fig. 2, ANOVA and SNK, $P = 0.03$). None of the

experimental groups' heart dry mass means were significantly different from the control

group (Fig. 2, ANOVA and SNK, $P = 0.06$). Whole embryo wet mass showed the pattern

mentioned earlier, so using embryo mass as a covariate showed the heart wet mass

mean of the Mesonephros group was significantly greater than any other group mean

(Fig. 3, ANCOVA, $P = 0.009$).

Kidney wet mass mean of the Mesonephros-Metanephros group (106 ± 32 mg)

was significantly smaller than all other groups (Fig. 4, ANOVA and SNK, $P = 0.03$). The

kidney dry mass mean of the Mesonephros-Metanephros group was also significantly

smaller than all other groups (Fig. 4, ANOVA and SNK, $P = 0.002$). If whole embryo wet

mass is considered as a covariate, an ANCOVA reveals that both the Mesonephros and

Metanephros group means are significantly larger than the control groups (Fig. 5, $P = $

0.0004).

3.2.   Blood Pressure and Heart Rate

3.2.1. Saline Injection

To determine the effects of saline injection per se on blood pressure, measurements were made after injecting embryos with 120 µL of saline, and then followed by injection of various doses of atenolol. Saline injection had no significant ($P >$ 0.05) effect on mean arterial pressure or heart rate (Fig.9).

3.2.2. Atenolol Treatment

Fig. 8 shows two representative blood pressure and heart rate traces of the Day 15 embryos used to measure responses to acute atenolol exposure via intra-arterial injection. Panel A shows a representative trace of a saline injection. As expected, the 120 µL of saline did not cause a significant change in MAP or heart rate. Panel B shows a representative trace of the first and least concentrated dose of atenolol or 0.75 µg/mg of embryonic mass. Mean arterial pressure (MAP) responses to atenolol doses of 0.75, 1.5, 3.0, and 6.0 µg/mg of embryonic mass were each significantly different (matched pairs t tests, $P = 0.001$, $P = 0.002$, $P = 0.04$, $P = 0.01$) from pre-injection values (Fig. 9A).The most concentrated dose tested, 12.0 µg/mg, did not significantly ($P > 0.05$) increase MAP.

Atenolol in the present study produced a short-lived rise in MAP for the smallest 0.75 µg/mg dose and then showed a long term rise in MAP as a result of the 1.5 µg/mg dose. The third atenolol dose of 3.0 µg/mg then produced only a short-lived rise in MAP, followed by a return to the previous baseline. Next, the 6.0 µg/mg dose showed a short-lived rise in MAP along with a long term eventual slight drop in MAP. The last and

strongest dose of 12.0 µg/mg showed a non-significant rise in MAP immediately after injection and then dropped back down to baseline values for the remainder of the experiment (Fig. 9A). This 3.0 µg/mg dose of atenolol is the starting point where baselines are being reduced as noticed visually.

Fig.10 shows MAP and heart rate as a percent change from each pre-injection baseline to its corresponding peak response. The percent changes are plotted against the cumulative atenolol doses. No atenolol doses caused a significantly different response in MAP. Cumulative atenolol doses of 5.25, 11.25, and 23.25 produced the only significantly different responses from saline injection (one way repeated measures ANOVA, $P = 0.002$, $P = 0.001$, $P < 0.001$).

Mean heart rate showed a significant decrease from baseline in all atenolol doses tested (Fig. 9B, matched pairs t test, $P = 0.01$, $P = 0.001$, $P = 0.0001$, $P = 0.005$, $P = 0.005$). The largest bradycardia occurred after injection with the highest dose of 12.0 µg/mg of atenolol (-34 beats $\cdot$ min^{-1}).

Fig.11 shows the dose effects upon pre-injection values (Fig.11A) and the dose effects upon the post-injection values (Fig.11B).

Plotting the rise in MAP as a function of the falling heart rate (HR) yields Fig.12. MAP correlates to a fall in heart rate ($P > 0.001$, r=0.99). The equation of the line of best fit for this correlation is MAP = -0.00273(HR) + 0.133.

3.3.   Kidney Morphology

Nephron density, expressed as number of nephrons/mm² of kidney section area, is shown in Fig. 6. Of the three treatment groups, only the Mesonephros group had a

significantly smaller nephron number from the control group, having approximately 40%

less nephrons (Fig. 6, one way ANOVA, $P = 0.004$). The Mesonephros group

glomerular areas were however approximately 26% larger than the control group, while

the Metanephros group had glomerular areas 18% larger than the control group. Both

the Mesonephros and Metanephros groups had significantly larger average glomerular

areas than the control group (Fig. 7, one way ANOVA, $P < 0.001$).
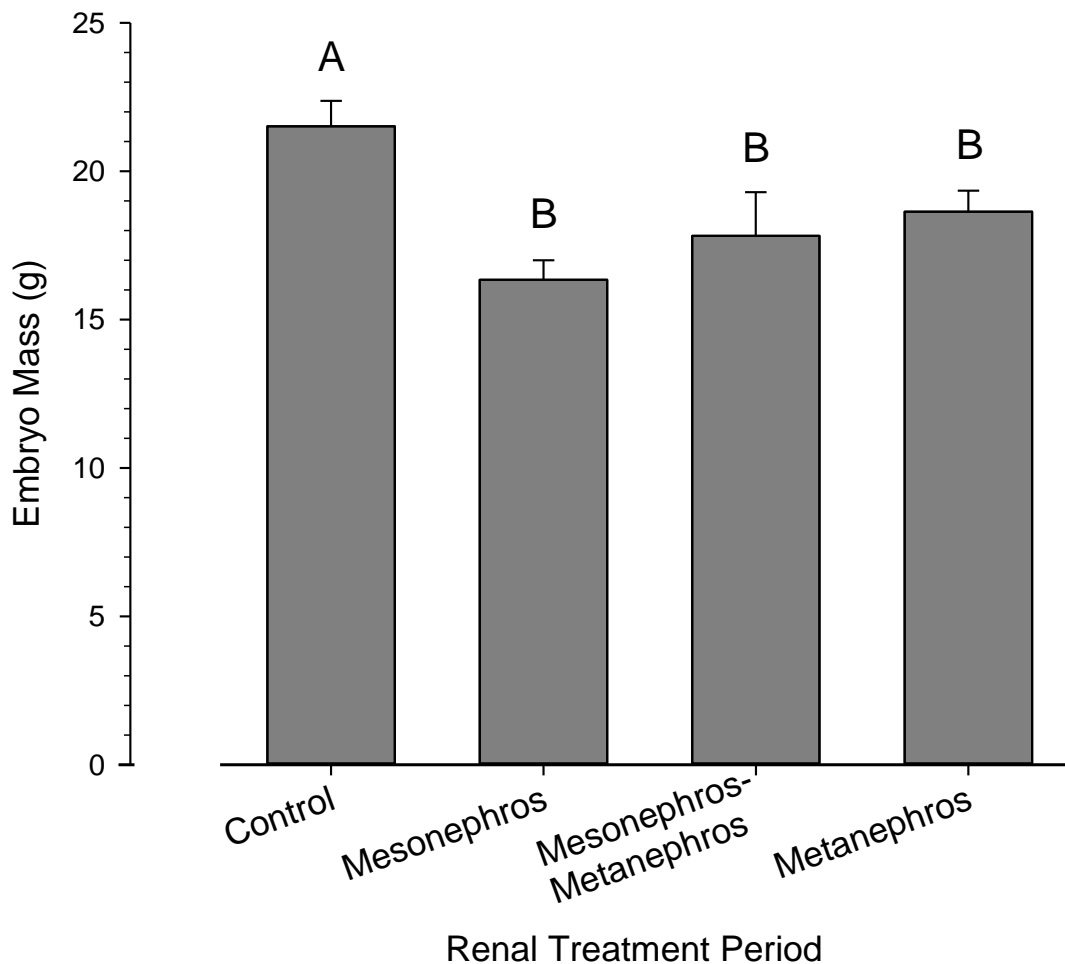
Fig. 1. Atenolol effects on whole embryo wet mass collected on Day 18. Different letters indicate significant differences according to an ANOVA. Data are presented as mean ± SE. See text for additional statistical analyses and findings.
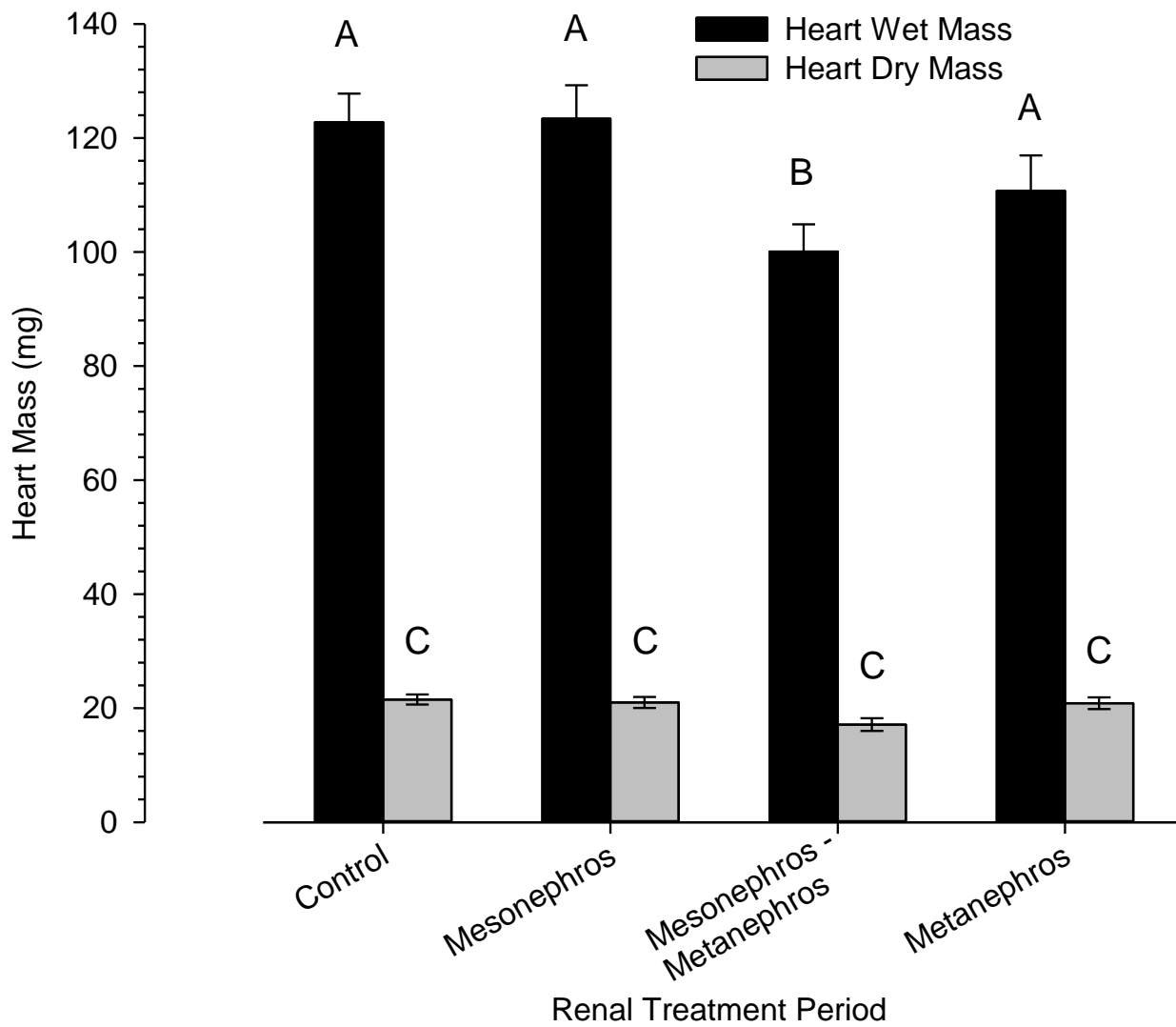
Fig. 2. Atenolol effects on heart wet and dry mass, determined on Day 18. Different letters indicate significant differences according to an ANOVA. Data are presented as mean ± SE. See text for additional statistical analyses and findings.
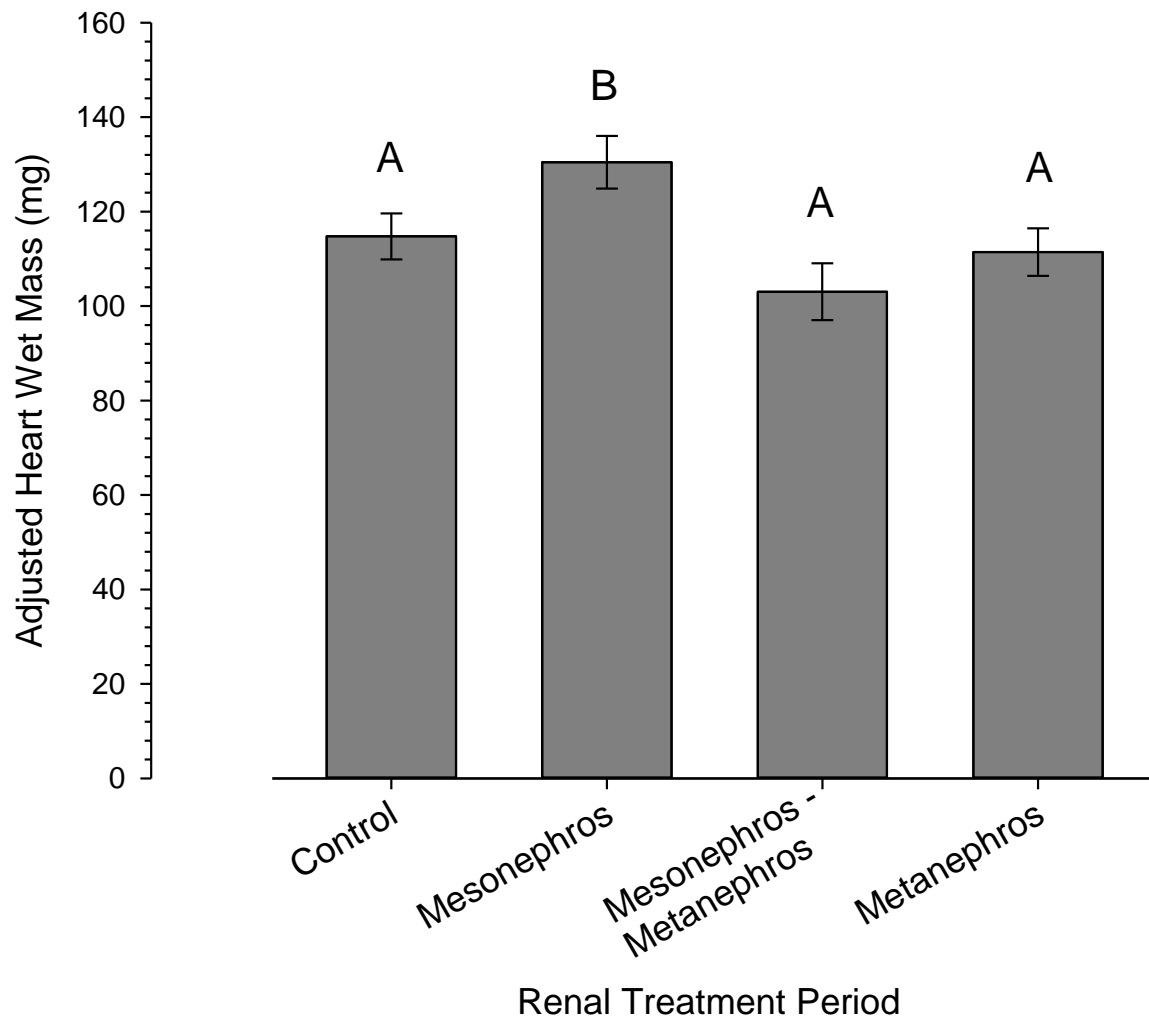
Fig. 3. Atenolol effects on heart wet mass with whole embryo mass as a covariate. Different letters indicate significant differences according to an ANCOVA. Data presented as mean ± SE. See text for additional statistical analyses and findings.
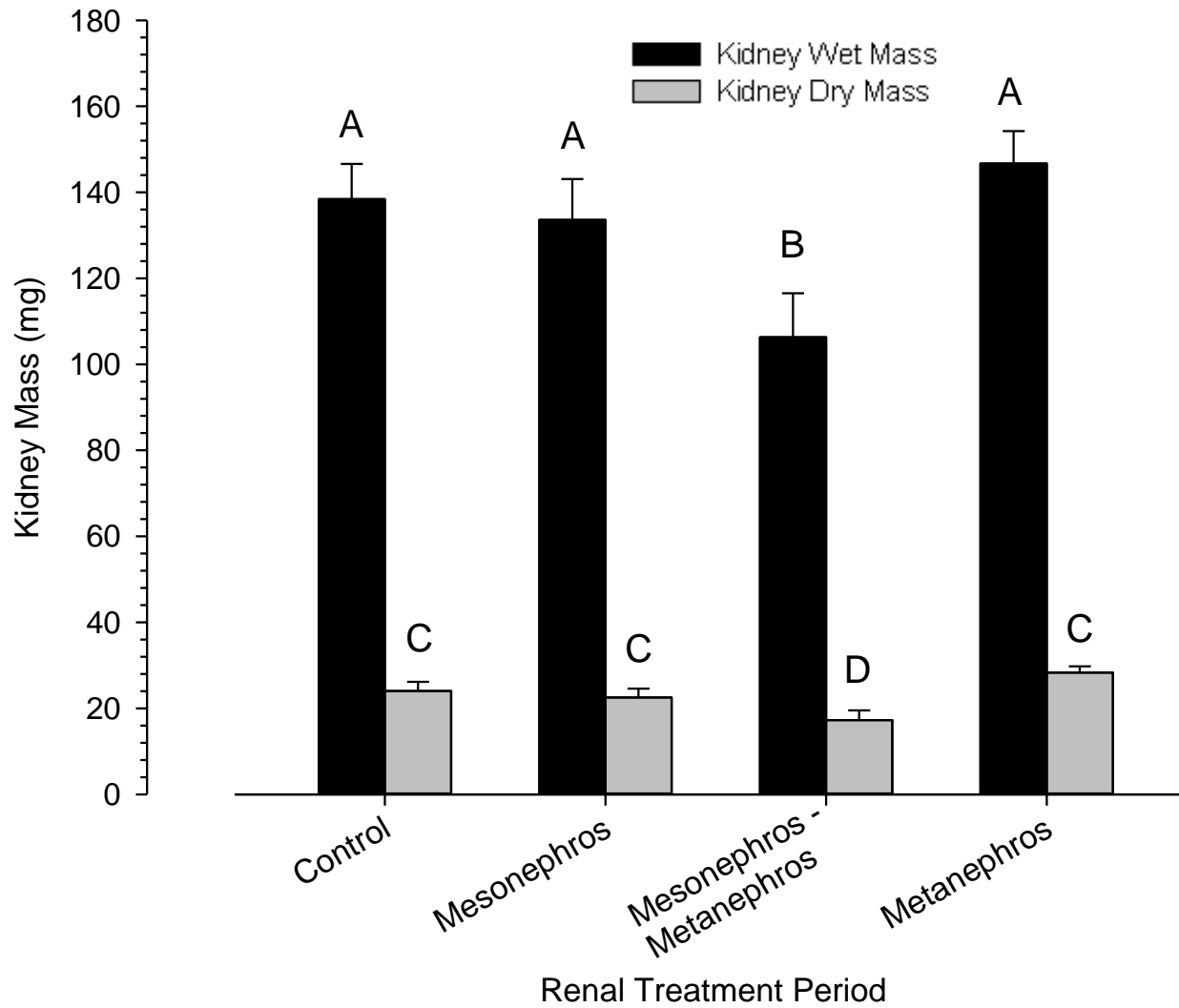
Fig. 4. Atenolol effects on kidney wet and dry mass determined on E18. Different letters indicate significant differences according to an ANOVA. Data are presented as mean ± SE. See text for additional statistical analyses and findings.
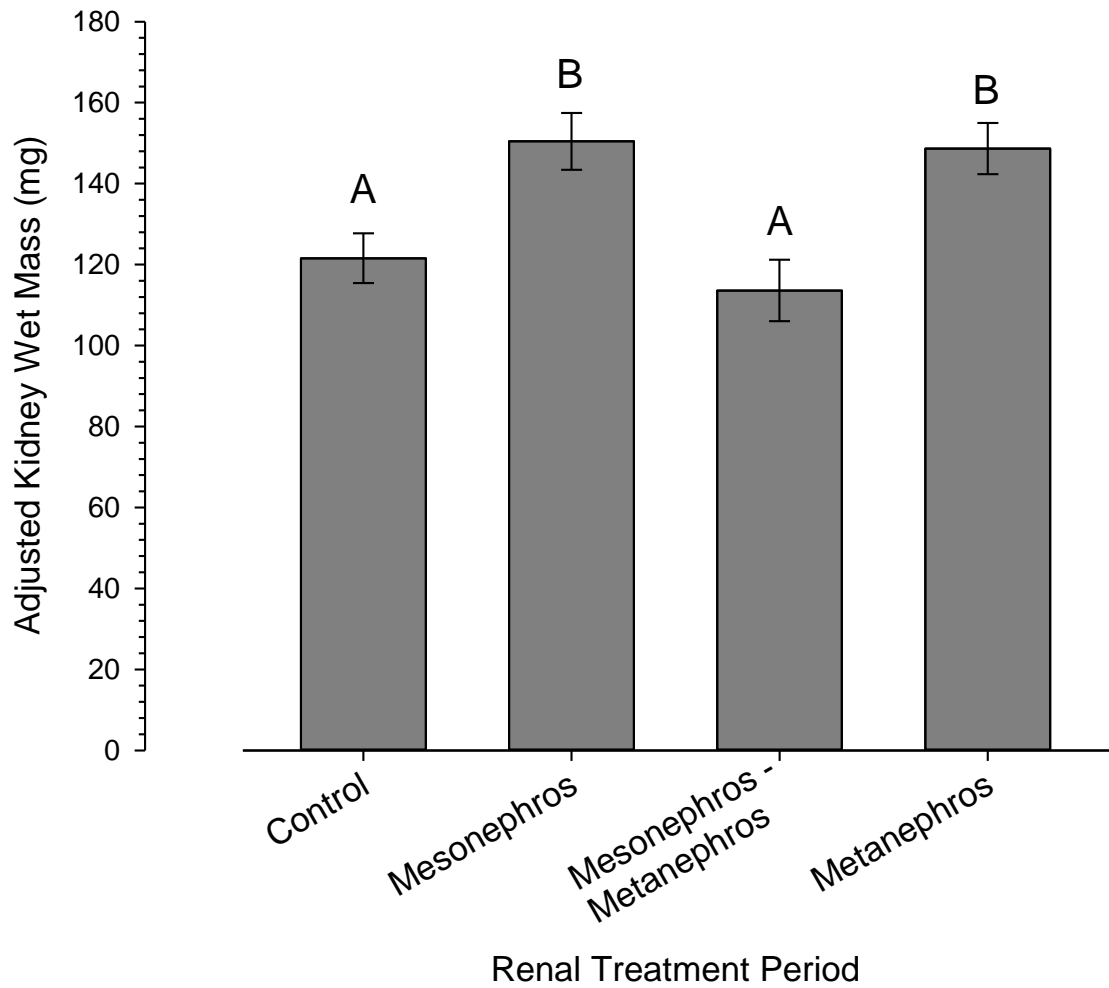
Fig. 5. Atenolol effects on kidney wet mass with whole embryo mass as a covariate. Different letters indicate significant differences according to an ANCOVA. Data presented as mean ± SE. See text for additional statistical analyses and findings.
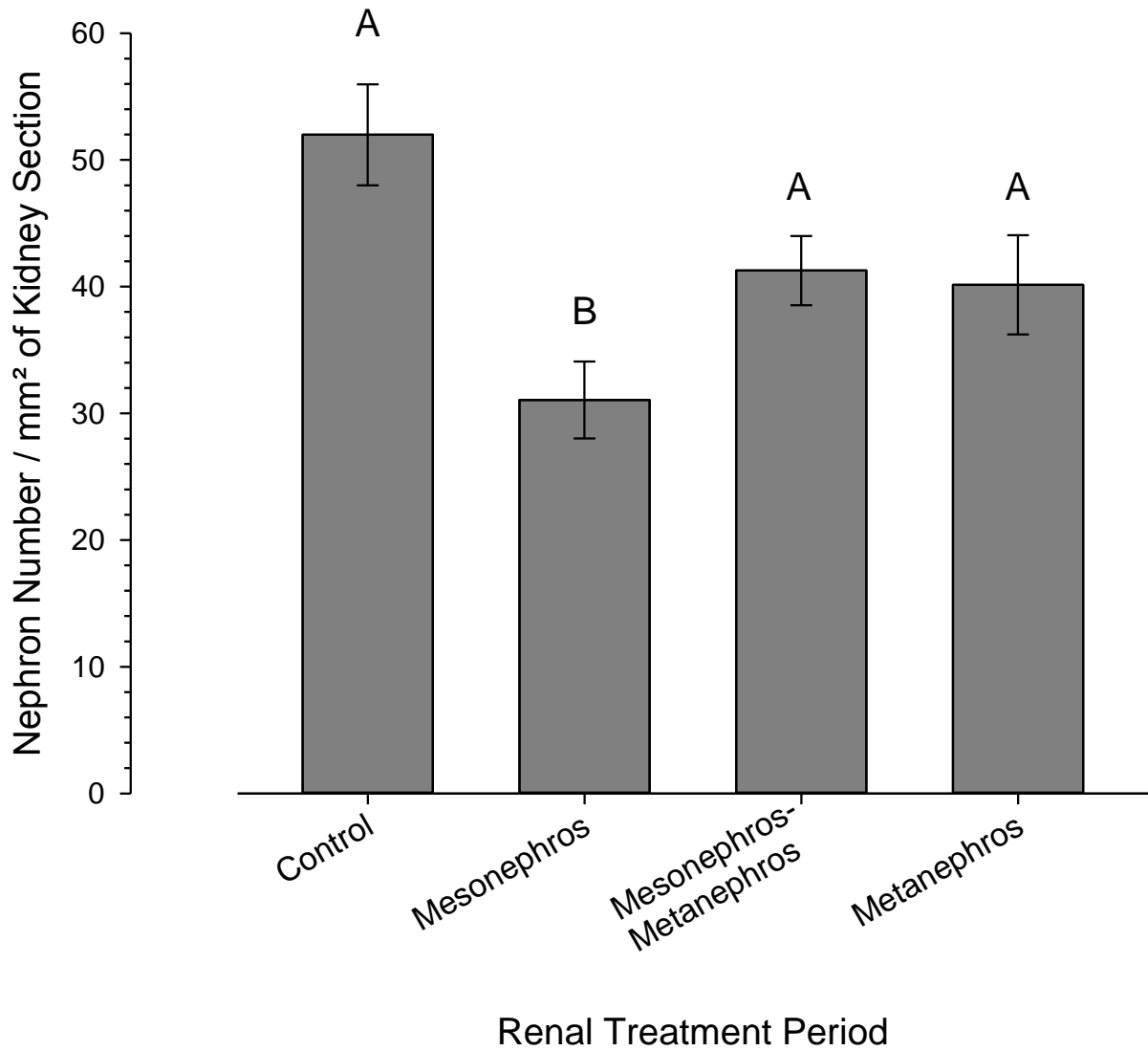
Fig. 6. Effect of atenolol treatment on nephron density in Day 18 chicken embryos. Different letters indicate significant differences according to an ANOVA. Data presented as mean ± SE. See text for additional statistical analyses and findings.
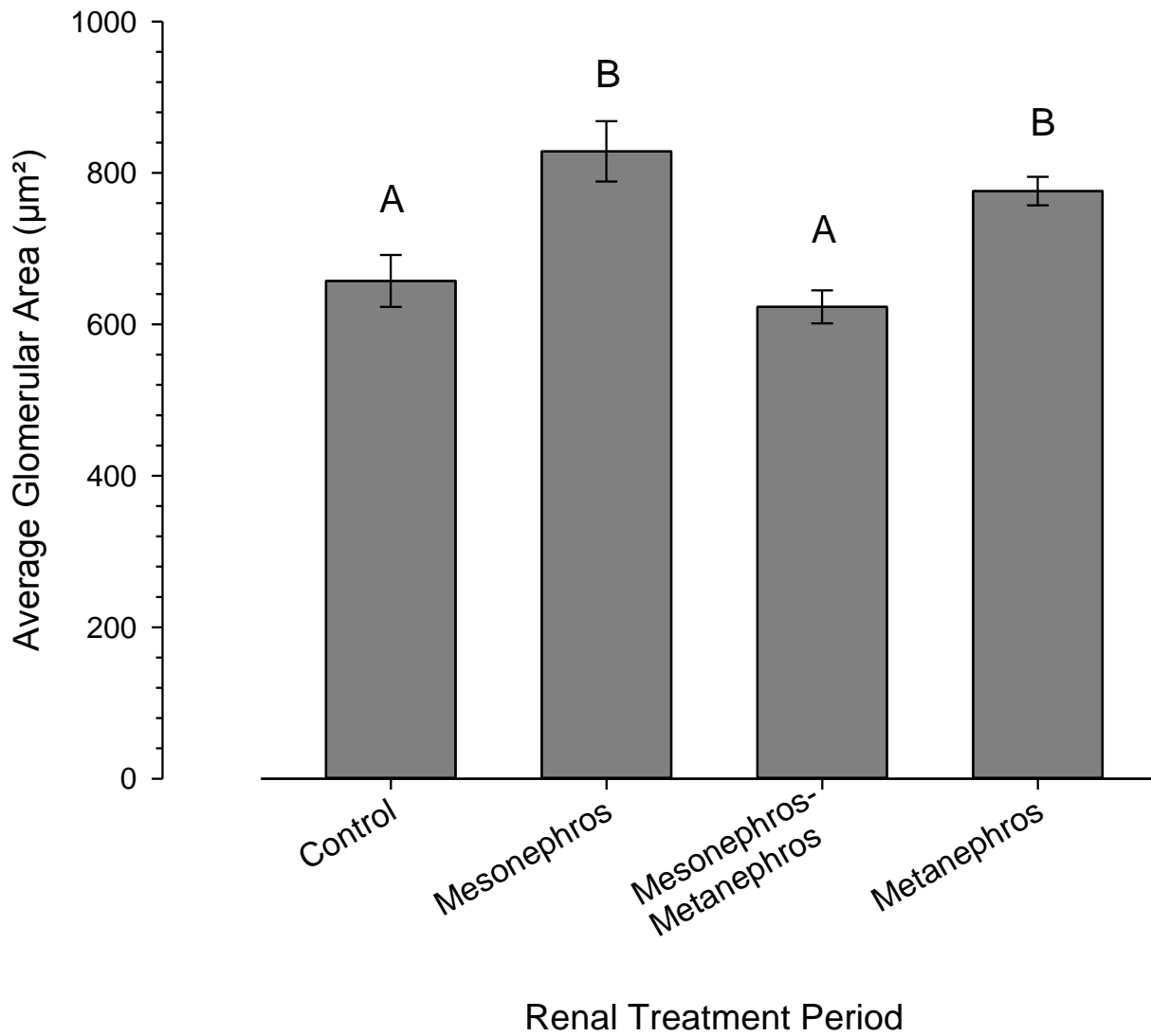
Fig. 7. Effect of atenolol treatment on glomerular area. Different letters indicate significant differences according to an ANOVA. Data presented as mean ± SE. See text for additional statistical analyses and findings.
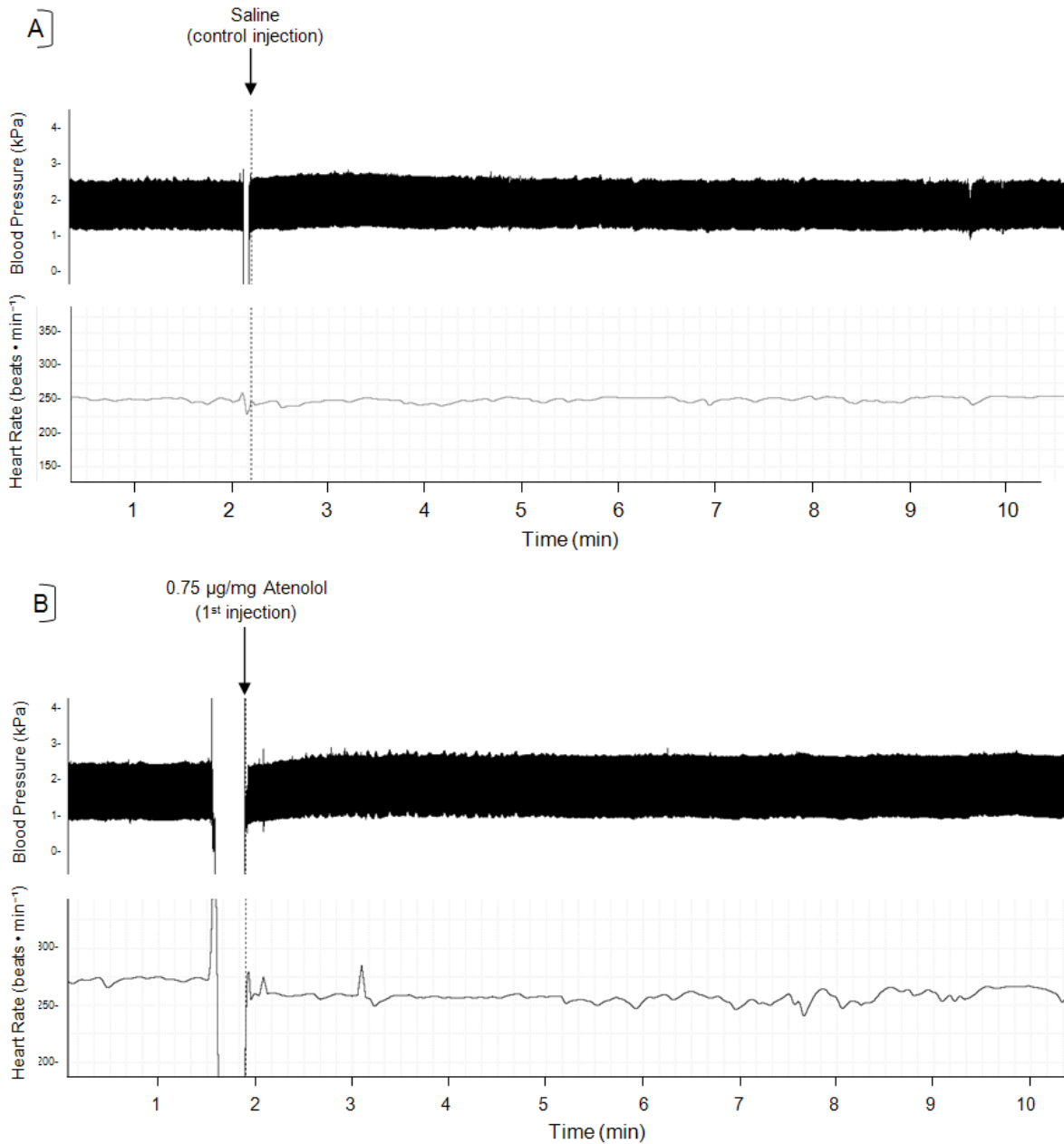
Fig. 8. Representative traces showing blood pressure and heart rate for a Day 15 chicken embryo. Panel A shows a blood pressure and heart rate trace after a control saline injection of 120 µL. Panel B shows a blood pressure and heart rate trace after the first atenolol injection of 0.75 µg/mg of embryo mass.
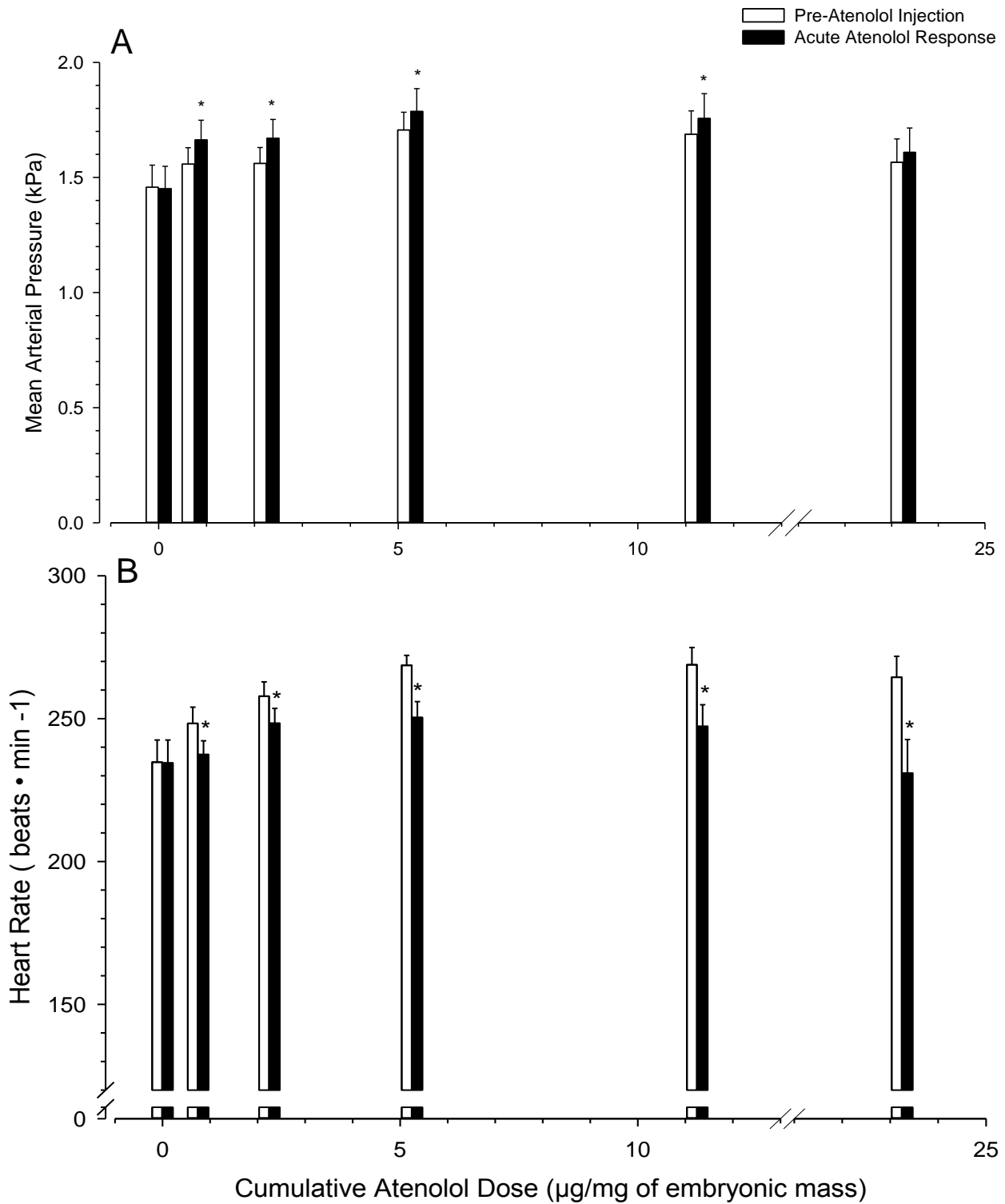
Fig. 9. Response to atenolol immediately before and after each intra-arterial injection. Panel A shows mean arterial pressure response. Panel B shows heart rate response. Asterisks show statistical significance. Data are presented as mean ± SE.
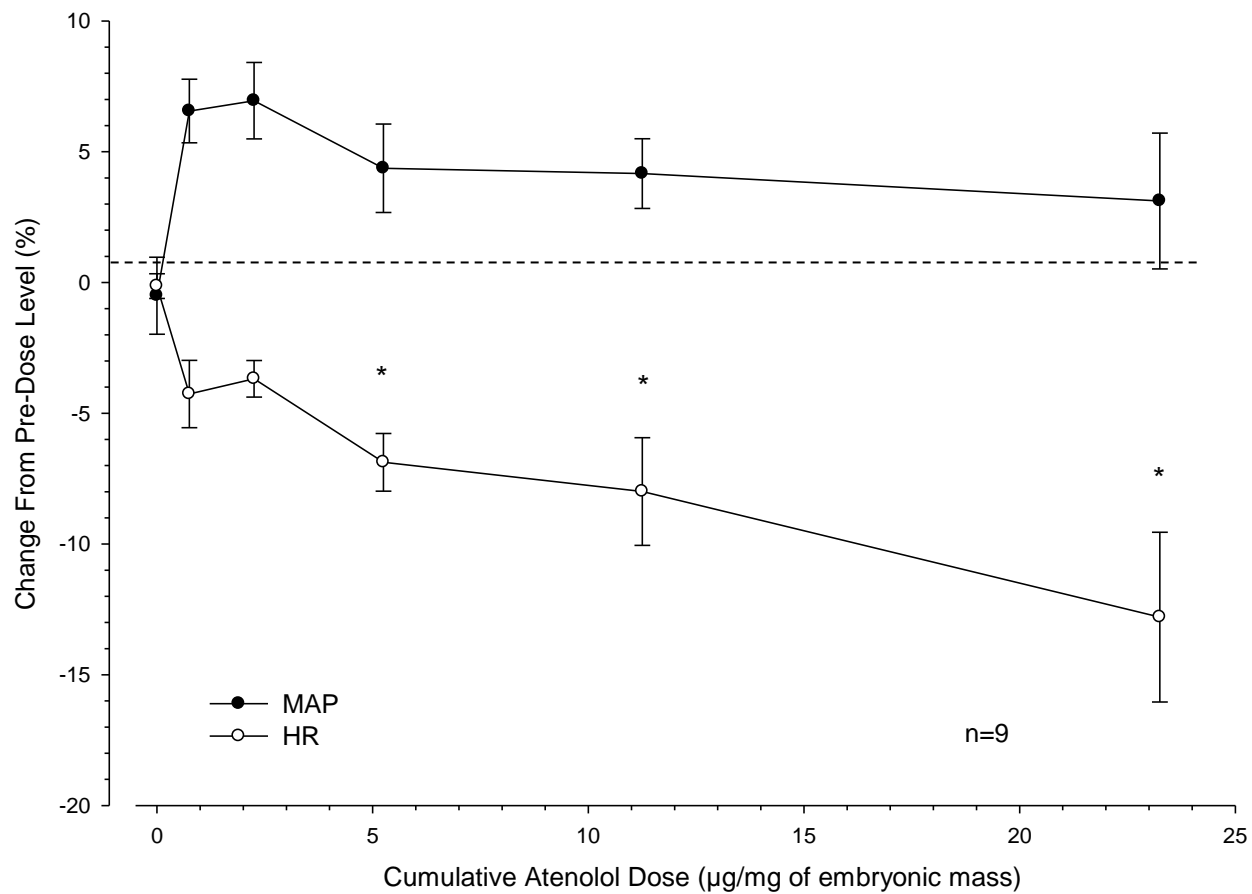
Fig. 10. Effects of atenolol on blood pressure and heart rate expressed as percent change. Black circles represent mean arterial pressure. Open circles represent heart rate. Data are presented as mean ± SE. Asterisks indicate statistical significance. See
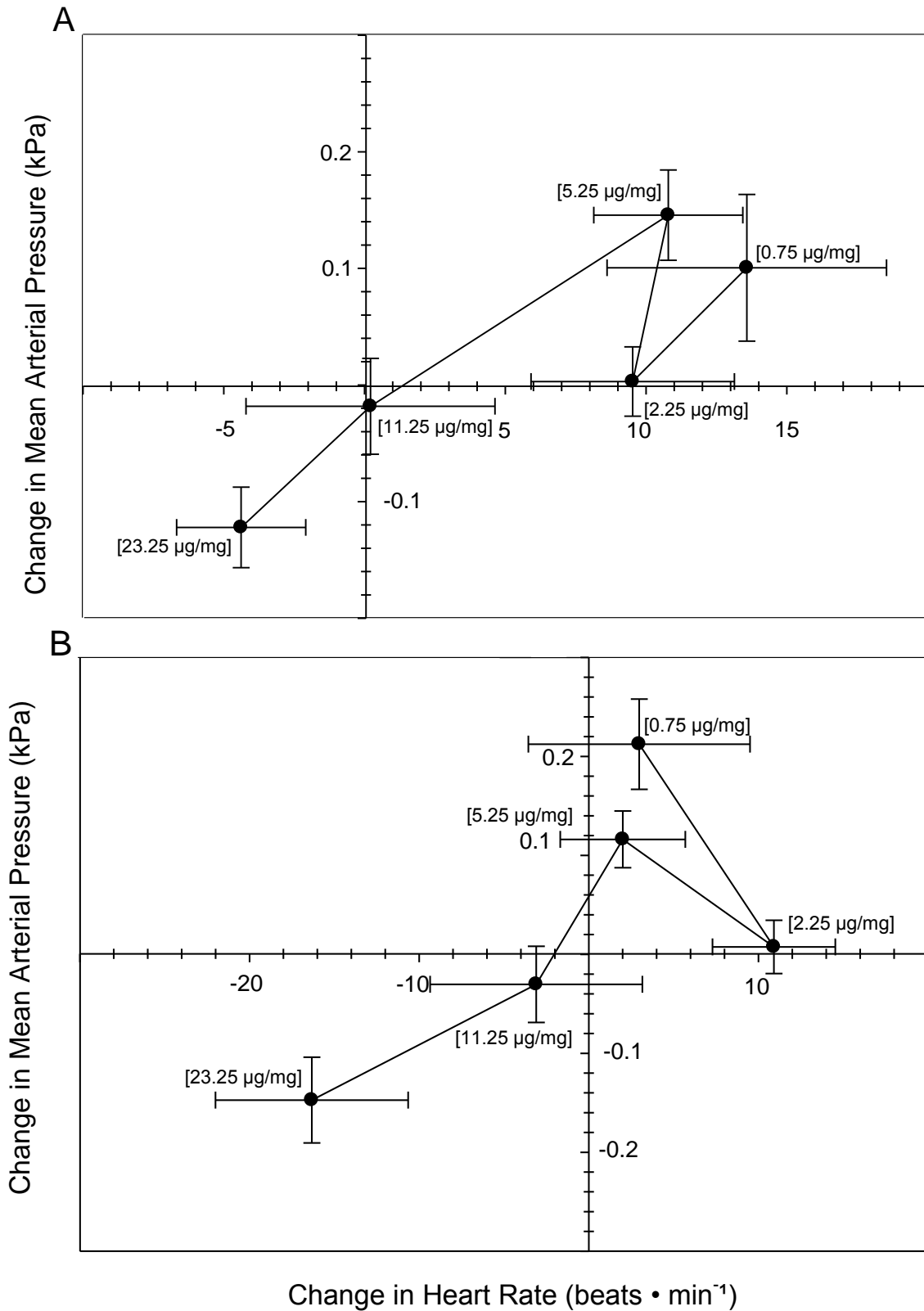
Fig. 11. Effects of atenolol on mean arterial pressure and heart rate. (A) Dose effects on pre-injection values. (B) Dose effects on post-injection values. Data presented as mean ± SE. Cumulative atenolol concentrations are shown in brackets next to each data pt.
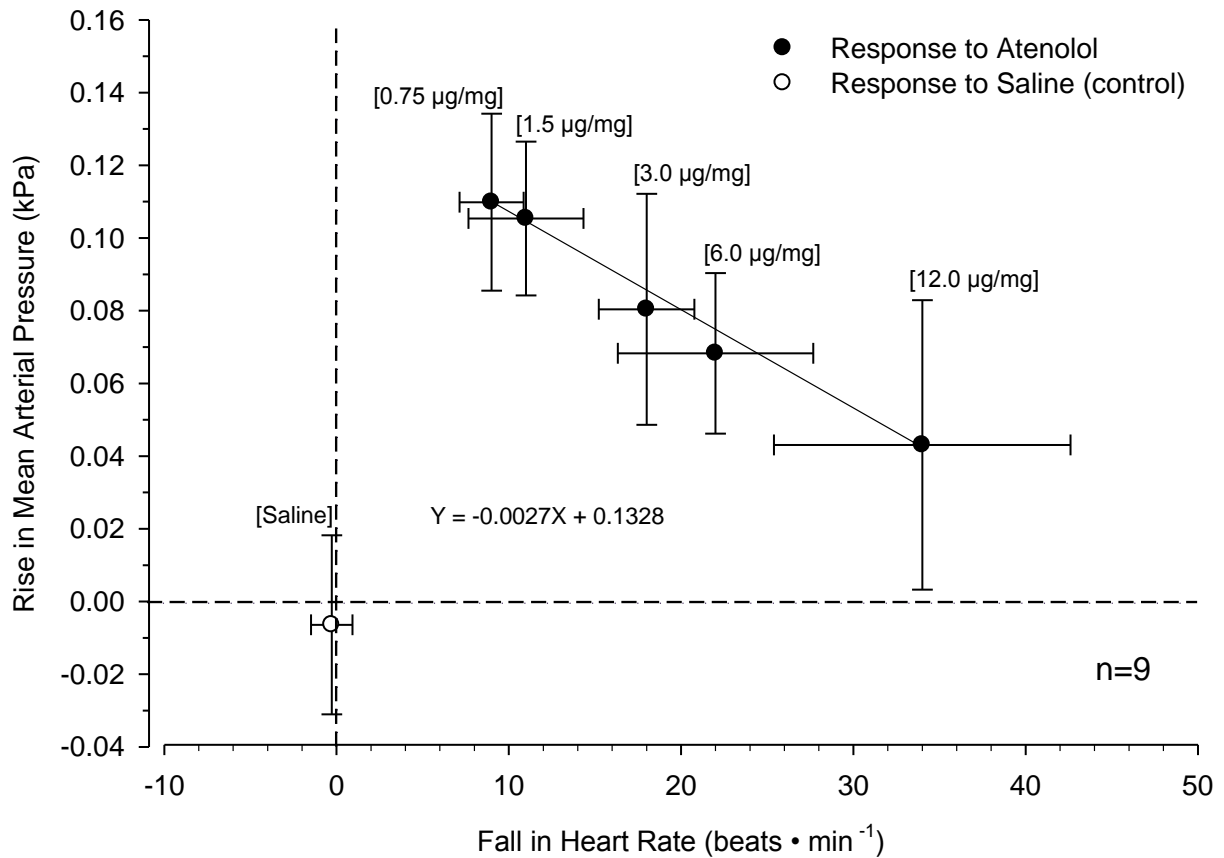
Fig. 12. Effect of Atenolol on mean arterial pressure (MAP) and heart rate on Day 15 chicken embryo. Relationship shows how a fall in heart rate correlates to the rise in MAP for each dose of Atenolol given. Data are presented as mean ± SE. n=9, equation is shown.

CHAPTER 4

DISCUSSION

4.1. Embryo and Organ Mass

Treatment of hypertensive pregnant human mothers with atenolol is heavily associated with low birth weight infants (Lip et al. 1997). Atenolol crosses the placenta (Thorley et al. 1981). Therefore, atenolol likely infiltrates fetal circulation and can negatively affect the fetus in potentially multiple ways. In adult humans, atenolol appears to increase risk of stroke and negatively affects other factors such as insulin sensitivity and ventricular hypertrophy (Bangalore et al. 2007). Similar vascular mechanisms may occur in the fetus, and could explain some of the growth restriction issues encountered in the clinical setting and in animal research laboratories. In fact, Thorley et al. (1981) found that treating pregnant women with atenolol did result in neonates having elevated blood glucose. Fetuses exposed to atenolol showed a significant increase in pulsatility index, suggesting that the peripheral vascular resistance of both mother and fetus increased during this short term exposure (Montan et al. 1987). It is reasonable to make analogous connections between what is observed *in-utero* in mammals and what can occur *in-ovo* in the chicken embryo. Atenolol has a high potential to affect many circulatory factors in the chicken embryo. Any of these factors can be considered as possibilities for the reduced embryonic weight observed in the present chicken embryo study.

The Mesonephros group contained a significantly larger heart wet mass than all other groups. However, heart dry mass means across groups were not significantly different. Montan's study (1987) suggests that outflow tract sculpting may have been

compromised and could contribute in the current study to the heart mass discrepancy. This discrepancy between wet and dry mass could also point to heart edema as a result of atenolol exposure (Stanton et al, 1968). The bradycardia may have proven to be most severe in animals treated in the mesonephric period. This suggests a critical window for β-AR blockade. No data has been gathered concerning contractile protein production, DNA synthesis changes, or individual cardiomyocyte morphology. Histological studies can reveal if cardiomyocyte hypertrophy has taken place. Assigning a developmental stage for the extent of contractile protein maturation could contribute tremendously to understanding atenolol's effects.

Stimulation of the β-ARs via isoproterenol causes a down regulation of receptor activity via loss of high-affinity binding in the receptor and not receptor number (Marsh et al. 1985). So alternatively, β-AR blockade could have conversely prompted cardiomyocytes to up-regulate receptor function via either adenylate cyclase stimulation of coupling, increasing binding affinity, or increasing protein synthesis and over-sensitize the cell to catecholamines. If over-stimulation by baseline catecholamines was taking place, this may have simulated the occurrence of early sympathetic innervation. β-AR stimulation in rats inhibited further cell division and DNA synthesis in the atria thereby causing the transition in heart development from hyperplasia to hypertrophy-mediated growth (Tucker & Gist, 1986). If a similar effect occurred in the chronically atenolol-treated embryos, it could mean that Mesonephros group hearts were larger due to pathological hypertrophy. This excess growth in cardiomyocytes can cause less efficient pumping as seen in older human patients suffering from heart failure. Inefficient pumping causes insufficient filling and thus decreased cardiac output but can also

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cause back up of fluids due to insufficient pumping and consequently edema (Mohrman et al. 2010).

Kidney wet mass of the Mesonephros-Metanephros group was significantly smaller than all other groups (Fig. 4). Kidney dry mass of the same Mesonephros-Metanephros group was not significantly different from the control group but was significantly smaller than the Metanephros group. This suggests that the transitional period when the mesonephros and metanephros are both partially functioning in the embryo is the most critical period for embryonic renal development. Because morphological analyses reveal that both nephron number and glomerular area of the Mesoneprhos-Metanephros group are not significantly different from the control group, the reason for the decreased renal mass likely comes from alteration of other renal microstructures.

On the other hand, the adjusted kidney wet mass figure (Fig.5) shows that the Mesonephros and Metanephros groups are significantly larger than the control group. This suggests that these periods rather than the transitional renal period are most affected and thus more critically vulnerable to β-AR blockade.

Angiotensinogen is one of the many components of the RAAS and a member of the serine protease inhibitor or serpin family. In one study, the angiogenic effects of its cleaved derivatives were investigated, where angiotensin I inhibited angiogenesis. Specifically, a 1μg dose of angiotensin I produced a 20-35% decrease in small vessel density in the chicken CAM (Célérier et al. 2002).

Depending on how active or inactive the RAAS is during these two renal stages (Mesonephros and Metanephros stages), atenolol's β-blockade may be responsible for

glomerular blood vessel over-proliferation. If the blood vessels are displaying an exaggerated hyperplasia over development, it could explain the difference in kidney masses. This claim however, needs confirmation of tissue mass differences between tubules, vessels, and remaining renal microstructures as well as a histological confirmation of this difference in vessel density as well as glomerular vessel thickness.

Kidney tubule damage and interstitial space alteration should also be considered as possible sources of reduced kidney mass. In a recent study, rats that were prenatally treated with dexamethasone to induce hypertension experienced post-natal physiological changes in their proximal convoluted tubules' ability to reabsorb sodium and hydrogen ions (Dagan et al. 2007). Proximal convoluted tubules of the dexamethasone treated rats reabsorbed these ions at a 50% higher rate, suggesting that tubule physical alterations could follow.

If we consider the highly interactive signaling nature of embryonic tissue, the mesonephric-metanephric transitional period is likely the most crucial to normal organ development. In the mammalian kidney, a complex series of inductive and morphogenetic events are heavily involved in ureteric bud branching and induction of mesenchyme to form the epithelia of nephrons (Burrow 2000). Embryonic studies show that the metanephric mesenchyme secretes diffusible factors that sculpt the ingrowth and branching of the ureteric bud while the ureteric bud branches out from the mesonephric duct (Grobstein 1955). If we extrapolate this information to the chicken embryo and should this back and forth communication be disturbed, it is reasonable to expect an overall underdevelopment of the renal tissues.

4.2.   Physiological Response to Acute Atenolol Exposure

Day 15 chicken embryos showed expected control values of mean arterial pressure (MAP) as per an average of 1.69 ± 0.49 kPa obtained from three studies reporting blood pressures on Day 15 (Tazawa et al. 1992; Altimiras et al. 2000; Crossley et al. 2003). In the present study, MAP baselines began to drop between the 3.0 and 6.0 (5.25 and 11.25 cumulative) µg/mg doses of atenolol. This indicates a critical dosage exists between these doses that could better be used to depress cardiovascular parameters, were this study repeated or elaborated upon in the future. Also, when comparing the delta values between pre-injection and post-injection values, only the weakest doses of atenolol (0.75, 1.5, and 3.0 µg/mg) showed a change in MAP significantly different from saline. However, none of the percent changes in pre and post-injection values in MAP were significantly different from one another (Fig.10).

Heart rate patterns (Fig. 9B) started rising within the expected average range of 252 ± 10 bpm after the saline dose and before the 0.75 µg/mg dose (Tazawa et al. 1992; Crossley et al. 2000; Crossley et al. 2003). All atenolol doses produced a significant drop in heart rate but the heart rate baseline did not always remain depressed in each embryo. The highest atenolol dose of 12.0 (23.25 cumulative) µg/mg was the most damaging and caused the most significant drop in heart rate.

Baseline values of both MAP and heart rate began to drop chronically as expected due to β-AR blockade after the 12.0 (23.25 cumulative) µg/mg dose of atenolol was administered. This suggests full blockade did not occur until this dose was given.

Atenolol is expected to cause a bradycardia and hypotension upon exposure. The bradycardia was observed acutely as expected but a seemingly paradoxical simultaneous hypertension occurred as well. This may be explained by a simple mechanical phenomenon known as the Frank-Starling Law of the Heart (Mohrman et al. 2010). The law states that all factors being equal, stroke volume increases as cardiac filing increases. Since atenolol caused an immediate bradycardia in each dose tested, the decrease in heart rate allows more time for increased diastolic filling. A greater end-diastolic volume causes greater distention of the cardiomyocytes. Then, a greater shortening during subsequent contraction leads to an increased blood pressure.

Another way hypertension could have been caused is via unexpected blockade behavior. Atenolol does not behave as fully β-1 selective in avian species (Kamimura 1985), therefore its partial β-2 antagonism could be causing a vasoconstriction that produced the temporary rise in blood pressure. In another study, the chicken β-ARs are thought to behave pharmacologically different from mammalian β-1 and β-2 receptors. Low and high affinity sites were found for β-antagonists which could be altering the actions of coupling ligand binding to adenylyl cyclase activation and thus points to a slightly modified mechanism of action that could explain the unexpected blood pressure response and expected heart rate response (Nakao 1987). If atenolol is binding to β1-ARs in their low affinity sites and β2-ARs in their high affinity sites, there may be a modest bradycardia via cardiac antagonism and slight hypertension due to peripheral vasculature vasoconstriction.

Another aspect of this study to consider is that the entire adrenergic system which includes both α and β receptor regulation must be taken into account. If β-

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blockade is applied, this leaves the α-ARs as the only adrenergic regulatory system free to be affected by catecholamines. In the current study, since the α-ARs were left available, catecholamines could have been left to activate only the α_1 -ARs thereby causing a widespread vasoconstriction in the peripheral vasculature and consequent hypertension (Hitner et al. 2012).

To have ensured optimal data collection, surgery duration should have been shortened and recovery time lengthened to allow embryos a minimally stressful environment during injections. Also, to better understand the true mechanism of atenolol in the chicken embryo, studies are needed to show the receptor affinity as well as the true nature of β selectivity.

4.3.   Nephron Number and Morphology

In a study performed by inducing hypertension via dexamethasone (DEX) treatment of fetal sheep, treated sheep had lower nephron numbers, higher glomerular volumes, and more dilated proximal tubules than control animals (Wintour et al, 2003). The present study shows that only the earliest treated Mesonephros group had reduced nephron numbers (Fig. 6). However, when glomerular area was considered, both the Mesonephros and Metanephros groups had significantly larger areas than the control group (Fig. 7). The kidney wet mass ANCOVA (Fig. 5) showed that the Mesonephros and Metanephros groups were significantly larger than the control group while kidney dry masses (Fig. 4.) were not significantly different from the control group. This suggests that these two groups were more massive due to their overall increased corpuscular sizes which held more filtered blood. Avian nephrons consist of three types

which include two mammalian type nephrons (MTN) or looped juxtamedullary nephrons and one reptilian type nephron (RTN) or loopless cortical nephrons. All three nephron types are present on embryonic Day 18 with the MTN having the largest glomeruli (Gambaryan 1992). As corpuscular diameters were larger in the Mesonephros and Metanephros groups, the β-blockade with atenolol may have stimulated the development of the looped nephrons over the loopless nephrons in these two groups.

Atenolol was used to induce a hypotension, but observations on the kidney instead showed morphological results expected with an induced hypertension. This may actually suggest that the chronic CAM injections produced a similar response to the observed responses during the acute atenolol injections. Chronic CAM injections of the middle concentration (3.0 µg/mg) were likely sufficient to induce a response and affect the long term embryonic outcome of renal morphology. To induce the desired bradycardia and hypotension however, the dose that must be used should be at least as great as our highest concentration dose of 12.0 µg/mg of atenolol. When considering that chronic CAM injections can allow the healthy embryo a longer time to react to insult, it may have been that β-blockade this early in embryonic development produced a stronger up-regulation of β-ARs (Gilbert et al. 1993). If so, it is possible that more receptor availability combined with baseline catecholamines caused a compensatory hypertension in the embryo to combat the unnatural blockade. However, since a hypertension was noted in the acute intra-arterial atenolol injection experiments, there may be reason to think atenolol produces a hypertensive response in low doses in the chicken embryo. More data and references are needed to verify this pharmacological behavior.

Assessing atenolol effects on hatchling and adult physiology would help decipher the true nature of atenolol's effect on the embryo. More experiments are also needed to collect a full work up of embryo physiology throughout chronic and acute atenolol treatments.

4.4. Future Directions

The findings of the present study suggest several avenues for future research as now will be considered.

4.4.1. Glomerular Filtration

A primary and basic future experiment would be to repeat our experiments and take into account whether kidney filtration efficiency changed as a result of larger and lesser glomeruli. Glomerular filtration rate (GFR) can be measured by infusing a radio-labeled molecule such as inulin and taking cloacal urine samples to measure the clearance of inulin as a way to observe kidney function (Cooke et al. 1970).

4.4.2. β-Adrenergic Agonism

This study's experiments used a β-AR blocker. Exploring whether a β-AR agonist would result in either equivalent or inverse effects would be helpful in determining the mechanisms of the physiological and/or morphological changes taking place. Using β1-AR agonists such as dobutamine (Tuttle et al. 1975) or isoproterenol, could provide more information about the effects on kidney development that occur with what could be considered an opposite treatment.

4.4.3. Cardiovascular Measurements Post-Chronic Exposure

An especially useful experiment that would contribute to our understanding of atenolol on kidney development involves repeating the experiments with the chronic CAM injections and then cannulating these embryos on Day 18. While embryonic Day 15 cannulation helped to understand how the embryo reacted to atenolol, Day 18 cannulation post-chronic exposure would reveal whether the chronic regimen continued to take effect in late embryonic life. This experiment would document whether the embryos' cardiovascular system remained affected through life *in-ovo*, and if embryos could develop an altered physiological reaction to insult with β-blockade, were they to be dosed again.

4.4.4. Expansion of Atenolol Exposure Timeframe

Another simple but valuable addition to the puzzle would be to add earlier CAM injections to the analysis, going back to embryonic Day 5 when the mesonephros just begins to function (Romanoff 1960) and when studies have first claimed β-ARs respond to stimuli (Lipshultz et al. 1981). Would heart proportions be even larger? Would nephron number and glomerular size still show the same pattern as found in this study or would earlier insult induce larger effects? In addition to changing dosing times, using lesser and greater atenolol doses in separate experiments, would provide a matrix of data to better understand at just what time and with what quantity of drug cardiovascular depression changes physiology, morphology, and lethality to the embryo.

4.4.5. Blood Pressure Changes and Mechanotransduction

Since there was success in finding pronounced morphological changes in the stereology of the kidneys, it would be interesting to learn about the expression of the Polycystin-1 and 2 genes (Pkd1 and Pkd2). These genes code for the primary cilia. These cilia are transmembrane proteins in endothelial cells that act as mechanosensors of shear stress and can affect differentiation, proliferation, and apoptosis of a cell (Dalagiorgou et al. 2010). Polycystin genes are mutated in human pathologies such as Polycystic Kidney Disease (PKD). Patients with PKD suffer from renal cysts that essentially threaten the structural integrity of the kidneys. It is believed that constriction of the renal architecture by these cysts, elicits an over exaggerated response from the RAAS and can cause hypertension (Nauli et al. 2011). So the question arises, how would these cilia behave in a genetically normal but pharmacologically stressed individual?

4.4.6. Renalase in Birds

A newly discovered hormone called renalase acts as a monoamine oxidase that travels in the bloodstream and metabolizes catecholamines (Xu et al. 2005). By exposing the chicken to atenolol we are reducing the RAAS activation and may be also reducing renalase expression. Renalase infusion in rats decreases cardiac contractility, heart rate, and blood pressure, and prevents a compensatory increase in peripheral vascular tone (Xu et al. 2005). Renalase has been identified as an attractive target of study for chronic hypertension and congestive heart failure. Currently, no published studies describe renalase actions in chicken. Birds tend to have proportionately larger

hearts to mammals of comparable size as well as a lower heart rate and larger stroke volume, which is attributed to higher blood pressures in birds than their mammalian counterpart. This makes the adult chicken a logical choice for studying cardiovascular disease (Grubb 1983). If birds use renalase for this additional endocrine function to regulate their cardiovascular system, it would be very interesting to identify and report this mechanism in the chicken.

4.4.7. Expression and Distribution of β-Adrenergic Receptors

The presence and pattern of β1-AR in the chicken mesonephros and metanephros is largely unknown. To learn more about the nature of β blocker treatment in the chicken embryo or any fetal animal, studies must be done to map expression of these receptors throughout embryogenesis by immunohistochemistry. No studies have been found describing such an endeavor. Achieving this would allow researchers to learn just how sensitive or resistant these receptors are to embryonic insult. Such a study would also reveal what physiological role if any β-ARs play in development, independent of neural stimulation.

4.4.8. Cardiac Morphology

Studying the morphology of the atenolol- treated chicken embryo heart would contribute clues to cardiomyocyte development. β-AR stimulation by isoproterenol in newborn rats was found to inhibit further cell division and DNA synthesis in the heart (Claycomb et al, 1976). In fetal and newborn rats, the heart grows primarily by increase in cell number. At approximately the 2 week postnatal period, this method of hyperplasia

is abandoned for hypertrophic growth. This transition happens to coincide with sympathetic innervation. Catecholamine stimulation could therefore influence growth and differentiation mechanisms in the cardiomyocyte (Tucker et al. 1986). Our study would benefit from seeing if atenolol-treated chickens are experiencing an accelerated transition into hypertrophic heart growth. Another study that would contribute greatly to learning the effects of atenolol on the cardiovascular system concerns elastin. Elastin is a fiber that develops and is deposited in the aorta and large conduit arteries in the very early embryo. It gives vessels the flexibility to minimize systolic blood pressure while maintaining diastolic blood pressure. It has been hypothesized that impaired fetal development can disturb elastin synthesis, stiffen the arteries in the future adult, and thus be a causative agent of hypertension (Godfrey et al. 2001).

In conclusion, the findings of the present experiments with atenolol suggest numerous potentially productive avenues for future experimentation.

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