RETINOIC ACID TREATMENT AFFECTS KIDNEY DEVELOPMENT AND OSMOREGULATORY SYSTEM IN THE DEVELOPING CHICKEN (*Gallus gallus*)

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Development is a dynamic process characterized by critical periods in which organ systems are sensitive to changes in the surrounding environment. In the current study, critical windows of embryonic growth and kidney development were assessed in the embryonic chicken. All-trans retinoic acid (tRA) influences not only organogenesis and cell proliferation, but also targets metanephric kidney nephrogenesis.

Embryonic chickens were given a single injection of tRA on embryonic day 8. tRA decreased embryo, kidney, and heart mass from day 16 to day 18. However, mass specific kidney and heart masses showed no differences. Whole blood, plasma, and allantoic fluid osmolality were altered in tRA treated embryos from day 16 to day 18. In addition, hematocrit, red blood cell count, and hemoglobin concentration were altered in tRA treated embryos. The results suggest that although nephrogenesis was not affected by tRA, the developing osmoregulatory system was altered in tRA treated embryos.
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By

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I. Critical Periods in Development

During vertebrate embryonic life, physiology is highly conserved among species (Burggren and Fitsche, 1997; and Keller, 1997) with many cardiovascular variables early in development being similar amongst fish, amphibians and birds (Burggren, 1998). Thus, understanding the developmental processes and trajectories of whole organisms or specific organ systems in lower vertebrates can serve as a framework for basic mechanisms of higher vertebrate physiology. Development is not composed of a series of discrete unrelated events; rather, development proceeds as a sequence of intricate signaling events with many organ systems becoming co-dependent with other organ systems. Timing of such interactions are critical for the developing embryo and insults during this critical time could have lasting consequences for the developing embryo.

Critical periods in development are time points in any organ or organ system during which it is most susceptible to insults that may alter the normal developmental trajectory of the organ or organ system (Spicer and Burggren, 2003). The developing avian kidney is a perfect model for studying critical periods in development because it proceeds through three distinct developmental stages; each of which likely has its own critical period in development, and could also influence the preceding kidney stage. Moreover, the developing kidney is very sensitive to insults during development, especially during nephrogenesis, which takes place in both embryonic mammals and birds after the heart has fully developed. A reduction in nephron number has been implicated as the primary (Hostetter et al., 1981; Garcia et al., 1988; Rennke et al., 1989; Brenner, 1998; Hughson et al., 2003; and Hoy et al., 2005), or more recently a secondary cause of adult onset diseases
such as hypertension, renal failure, and diabetes (Ruta et al., 2010). The idea that early embryonic life is critical and may program disease that does not manifest itself until later life has lead to further exploration of the developmental environment; more specifically, how altering the developing environment at specific critical periods in development, will alter normal organ system maturation.

II. Renal Fetal Programming
A. Timing is Everything

The period of development is a very dynamic and critical period for any organism. Understanding how changes in developmental trajectories of an organism or individual organ systems affect overall development may reveal basic mechanisms about adult human diseases. Fetal programming is the process by which adverse effects in the developing environment during critical periods result in irreversible changes in gene expression, tissue structure, and/or physiological function that has the potential to predispose the embryo to adult diseases later in life (Barker, 1995). A key aspect to fetal programming is the timing of the insult. For example, intra-uterine growth restriction (IUGR) due to twinning leads to a reduced nephron endowment, whereas late gestational IUGR does not, suggesting that reduced nephron endowment is dependent on the timing of the growth restriction (Mitchell et al., 2004). If any insult during development occurs after nephrogenesis is complete, the developed nephrons will not be affected and the embryo will develop a full complement of nephrons. The insult may not affect the kidney specifically, but has the potential to disrupt other organ systems that are in the midst of their critical developmental time frame. Thus in the above case late gestation IUGR occurs after nephrogenesis is complete.
B. Fetal Origins Hypothesis: Significance of Low Birth Weight

Towards the end of the 20th century, a landslide of debate and research began about fetal programming and its effects and consequences later in life. Barker proposed what is known as the “fetal origins hypothesis” which states that fetal under-nutrition in middle to late gestation leads to disproportionate fetal growth, and programming later coronary heart disease (Barker, 1995). Due in part to Barker and the fetal origins hypothesis, researchers began to link poor nutrition during prenatal life to risks for other adult onset diseases such as hypertension, heart disease and renal failure. Several studies have documented an association between low birth weight and blood pressure in humans (Law et al., 1993; Taylor et al., 1997; and Yiu et al., 1999).

Because blood pressure is largely regulated by the kidney through reflexes and hormonal interactions such as angiotensin II (Goodfriend et al., 1996; Griendling et al., 1996) researchers began to investigate how normal kidney development, specifically nephron numbers is affected by low birth weight. Low birth weight has been shown to be directly correlated with a reduction in nephron number (Hughson et al., 2003; Hoy et al., 2005). Moreover, an inverse relationship between glomerular volume and glomerular number was found in low birth weight subjects, in addition to low nephron numbers (Manalich et al., 2000; Hoy et al., 2003). Although much progress has been made since Barker’s fetal origins hypothesis the mechanisms and the actual relationship between low nephron numbers and the incidence of hypertension and renal failure remains unclear.

C. Brenner Hypothesis: Nephron Number Matters

Given the complexities of development and nephrogenesis, it is unlikely that low birth weight is the only significant factor that would predispose embryos to develop adult
onset diseases later in life. To that point, many studies have since disputed that there is an association between low birth weight and development of any forms of adult onset diseases (Huxley et al., 2002 and 2004; Williams et al., 2002; Falkner et al., 2003). There is further evidence that programming of adult onset diseases can occur across a range of birth weights, including those that are considered normal (Barker, 2008). Therefore, reduced overall fetal growth may not be seen as causing the long-term pathologies seen in adult life, but rather, could be an indication of a poor intrauterine environment. Taking the place of the fetal origins hypothesis is the idea that low nephron endowment, caused by insult during critical periods of nephrogenesis, may contribute to the prevalence of adult onset diseases. When embryos are faced with nephron loss, the remaining nephrons may undergo compensatory hypertrophy and hyperfiltration to maintain normal levels of renal function. The “brenner hypothesis” states that there is an inverse relationship between total nephron number and the risk of developing arterial hypertension (Brenner et al., 1988).

D. The Danger of Compensatory Renal Growth

Given certain situations, the developmental path of the embryo may be altered, potentially resulting in changes in the physiology of the embryo. These physiological changes could be either beneficial or destructive to the fitness of the embryo. Some organ systems have the ability to compensate for such a disturbance, whereas the developing kidney is tremendously sensitive to changes in utero during critical periods of nephrogenesis (Bagby, 2007). Although ultimately a low nephron number is characteristic of adult onset diseases, the danger of developing adult onset disease is a consequence of two compensatory actions of the remaining nephrons. First, several human and rat studies
have demonstrated that renal injury associated with nephron loss produces high glomerular capillary pressures that constitutes a major driving force for continuing glomerular injury. These sustained elevations in glomerular capillary hydraulic pressures can cause focal glomerular sclerosis leading to a self-perpetuating viscous cycle of glomerular loss leading to chronic renal failure and hypertension (Hostetter et al., 1981; Garcia et al., 1988; Rennke et al., 1989). Second, low glomerular number produces a decrease in filtration surface area causing the remaining nephrons to be subjected to increased single nephron filtration rates to maintain adequate glomerular filtration rate and proper blood volume. This increase in filtration rate causes subsequent renal sclerosis, and eventually chronic renal disease (Moore et al., 1999). It is likely that there is not one single factor that is responsible for predisposing individuals to develop hypertension and renal failure. On the contrary, development of adult onset disease is probably a result of complex interactions between the in utero and ex utero environment.

E. Low Nephron Number: The First Strike

Many studies support the hypothesis that a deficit in nephron number increases the risk of hypertension and renal diseases later in life. However, the methodologies used in these studies to produce insults during critical periods of nephrogenesis usually resulted in a variety of secondary consequences for the embryo in addition to a reduction in nephron number. The majority of proven methods to disrupt nephrogenesis, e.g. increase stress related steroids, and food depravation, result in smaller embryos, heart defects, and overall developmental abnormalities to the embryo. Essentially, because of these possible confounding variables, the extent to which a reduced nephron endowment directly contributes to hypertension and renal diseases remains largely unclear (Ruta et al., 2010).
Genetic models, such as glial cell line-derived neurotrophic factor (GDNF) are being engineered to remove low birth weight as a confounding variable i.e. to directly target the kidney. GDNF is critical for the initiation of ureteric bud and kidney development (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) as well as inducing the branching of ureteric epithelium, essentially determining the number of nephrons in the adult kidney (Cullen-McEwen et al., 2001). No relationship between nephron number and total glomerular volume with mean arterial pressure or renal function was found in aged GDNF knockout mice despite moderate and marked levels of nephron deficiency (Ruta et al., 2010). Mean arterial pressure and renal function were susceptible to an acute high-salt diet secondary insult. However, demonstrating that deficits in nephron number or glomerular volume alone may not directly translate to hypertension or renal disease, even when examined well into adulthood (Ruta et al., 2010). Although the current study goes against the bulk of the literature, it may influence a new trend of research that not only looks specifically at fetal programming in utero, but also examines the importance of the extra-utero environment as well. Models that have the ability to produce low nephron numbers while avoiding some of the secondary effects, namely low birth weight, may provide an opportunity to identify underlying mechanisms of disease.

Altered programming during critical periods of development not only occurs in utero but also can occur in postnatal life. If reduced nephron endowment constitutes the first insult towards higher incidence of adult onset diseases, controlling diet, nutrient intake, and monitoring growth trajectories may be just as important in prevention of adult diseases. Studies have shown that adults who were born with low birth weight and underwent compensatory, or catch-up growth as infants who are considered low birth
weight during the first two years of age have higher incidences of hypertension and cardiovascular-related events as adults compared to those who remain short and thin throughout childhood (Barker et al., 2005; Eriksson et al., 2007). Potentially, such compensatory growth may direct necessary resources away from processes necessary for maintenance and development and funnel them towards growth; which may ultimately lead to deleterious effects later in life. Both intrauterine and extra-uterine programming works in tandem to shape the developmental outcomes that may eventually lead to adult onset diseases.

F. Potential Mechanisms for Nephron Deficiency

Although much is known about nephron deficit and its consequences, the overall mechanisms that mediate this process is not well understood. Currently, proposed mechanisms include the following: nutrition, oxidative stress, glucocorticoid exposure, epigenetic, apoptosis regulation, renin-angiotensin system, and multiple environmental factors. Probably one of the most studied and a critical system in the kidney is the renin-angiotensin system (RAS). Normal RAS activity is important for the regulation of glomerular filtration rate as well as necessary for cellular differentiation and organ development (Lumbers, 1995; Guan et al., 2008). Studies examining the effect of low birth weight on the activity of the RAS found that renin and angiotensin activity are reduced in low birth weight animals as well as a reduction in renin gene expression, renin protein, and angiotensin II levels in newborn male offspring of modestly protein-restricted mothers (Woods et al., 2001). Pharmacological manipulation of the RAS also confirms the importance of RAS during nephrogenesis with low number of nephrons and salt sensitive
hypertension in adulthood resulting from angiotensin receptor blockade with Losartan during nephrogenesis in the rat (Woods et al., 1998).

Increased exposure to glucocorticoids has also been implicated as a possible mechanism for altering nephron development. Normally, the maternal circulation has a very high concentration of glucocorticoids compared to the fetal circulation. Under non-stressful conditions, the fetus is protected from these high levels by 11 β-hydroxysteroid dehydrogenase, which converts the active form of cortisol and corticosterone into the inactive form 11-keto forms (Murphy et al., 1974). However, under stressful situations, the activity level of placental 11 β-hydroxysteroid dehydrogenase decreases thereby allowing increased access of maternal glucocorticoids to the fetus. This increased exposure has been linked to low birth weight and incidences of hypertension and renal failure later in life (Edwards et al., 1993). In addition, glucocorticoid exposure during development may reduce angiotensin receptors increasing apoptosis in the metanephric mesenchyme leading a reduction in nephron numbers if this occurs during nephrogenesis (Glassberg, 2002; Moritz et al., 2003; Langley-Evans, 2008). A more recent proposed mechanism in programming adult disease is the role of epigenetics. Developmental outcomes are not strictly influenced by a single set of genes that code for one specific process. On the contrary, development is controlled by constant suppression and activation of multiple genes. Epigenetic changes have been observed in p53 in the kidney influencing renal apoptosis (Pham et al., 2003). Nephrogenesis is very complex with a variety of mechanisms potentially working together. Working with models that may allow for better manipulation, and monitoring capabilities of the fetal environment may help elicit some of these potential mechanisms.
III. Birds as an Animal Model for Renal Physiology

Many of the animal models used to study fetal programmings of adult disease have an obligate fetal-maternal interaction i.e. the fetus is in utero. This interaction can make it difficult when trying to distinguish a fetal response from one that is mainly maternal. The August Krogh principle states, “For such a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied” (Krogh, 1929). The embryonic chicken develops within a microhabitat that can be easily manipulated pharmacologically. To date, very few studies, have taken advantage of the embryonic chicken to examine renal function and the concept of renal fetal programming (Bolin, 2009).

A. Avian Osmoregulation

The avian kidney shares some morphological and physiological similarities and differences with its mammalian counterpart. Although there are slight differences in the arrangement of the nephrons the functional units of the kidney, both avian and mammalian species are able to produce hyperosmotic urine compared to blood plasma (Braun, 1985). The chorioallantoic membrane (CAM) and the developing kidneys work together to regulate ion transport and waste removal for the developing embryo. These systems are tightly regulated via reflexes, hormones, and behaviors (Guan et al., 2008). If the CAM or the kidneys are compromised, the embryo may become susceptible to imbalances in ions and insufficient blood filtration.

Interestingly, the kidneys of adult birds are made up of two types of nephrons: mammalian-type (MT) and reptilian-type (RT) nephrons. These two nephron types form a disorganized arrangement that is slightly different when compared to the highly organized
arrangement seen in mammalian kidneys (Wideman, 1989). The major distinction point between these two nephrons is either the absence or presence of a loop of Henle, with RT nephrons being considered as loop-less nephrons due to the lack of this structure (Wideman, 1989). In addition, RT nephrons can be classified as cortical nephrons because they do not project into the renal medulla (Wideman, 1989). Reptilian-type nephrons are the main nephron type in the avian kidney, accounting for roughly 70-90% of the developing nephrons (Braun, 1985; Casotti et al., 2000). In contrast, MT nephrons, which possess loops of Henle and extend deep within the medullary space of the developing kidney, account for the remaining 10-30% of the total number of nephrons in the developing embryonic avian kidney (Braun, 1985; Casotti et al., 2000). The MT nephrons are vitally important for avian osmoregulation because they allow for the generation of a countercurrent multiplication system as seen in mammalian kidneys. Briefly, a sodium chloride (NaCl) concentration gradient is established by the MT nephrons which allows for the concentration of urine (Braun, 1985). The concentrating ability of avian species is less compared to mammals because hyposmotic, or at best isosomotic filtrate from the RT nephrons drains into the same collecting duct as the MT nephrons. This dilutes the concentrating capabilities of the MT nephrons and dilutes the hyperosmotic fluid from the MT nephrons (Dantzler et al., 1980).

Although birds and mammals can produce hyperosmotic urine, the mechanism by which each species accomplishes this differs. In birds, the concentration gradient in the renal medulla is composed entirely of NaCl (Skadhauge et al., 1967). Filtration, secretion, and reabsorption occur at specific points along the length of the nephron. Filtration occurs at the glomerulus and is tightly regulated by hormones. The blood then moves into the
proximal convoluted tubule (PCT) where the majority of reabsorption of sodium and chloride is accomplished by active sodium transport and passive chloride transport in both birds and mammals (Laverty and Dantzler, 1982; and Braun and Dantzler, 1997).

In MT nephrons, once the filtrate has passed through the PCT, it enters the loop of Henle which consists of a thin descending limb and thick ascending limb. The thin descending limb is highly permeable to sodium and chloride and virtually impermeable to water (Nishimura et al., 1989). This property is different from parts of the mammalian descending limb, which can be highly permeable to water. The thick ascending limb, known as the diluting segment of the loop of Henle, actively pumps NaCl into the medullary interstitium without the osmotic accompaniment of water movement (Miwa et al., 1986). Thus, dilution in the ascending limb and concentration in the descending limb is achieved by active transport and passive diffusion of a single solute, NaCl. Due to the arrangement of the MT nephrons, and loops of Henle, this effect can be amplified creating an osmotic gradient along the medulla of the kidney (Nishimura et al., 1989).

The distal convoluted tubule follows the loop of Henle and acts as a diluting segment with low water permeability (Stoner, 1985). In addition to dilution of the filtrate, the distal convoluted tubule helps to regain sodium and potassium by a hydrogen ion exchange into the tubular lumen of the kidney, ultimately acidifying the urine (Stoner, 1985). Finally, the collecting ducts act primarily to reabsorb water from the filtrate. The developing kidneys, as well as extra embryonic structures, play vital roles in maintaining osmotic, ionic and waste homeostasis for the developing embryo. In situations with low nephron numbers, the remaining nephrons may have to hyper filtrate in order to maintain adequate blood filtration. If proper glomerular filtration is not achieved, it is likely the embryo may become
susceptible to osmotic stress and may not be able to achieve normal ionic composition of the blood and surrounding fluids.

B. Avian Kidney: Development

Avian kidney development is the result of intricate inductive signaling from the Wolffian duct, and in the absence of such signaling, kidney development ceases to continue. All kidney structures originate from the intermediate mesoderm, and development occurs in three stages: the pronephros, the mesonephros, and the metanephros. The pronephros is the first to appear, signifying the beginning of kidney development. The pronephros is believed to develop by self-differentiation, growing independently out of the surrounding environment (Gruenwald, 1937; Romanoff, 1960). Pronephros growth and development continues up until around the fourth day of incubation during which time the Wolffian duct also continues to differentiate and develop, a process crucial for mesonephros and metanephric development (Boyden, 1927; Gruenwald, 1937).

Early glomeruli begin to appear during pronephros degradation. These glomeruli are easily identifiable because they develop as external glomeruli. Pronephros external glomeruli degradation begins around the fourth day of incubation (Abdel-Malek, 1950), and the external glomeruli are no longer found on embryonic day eight.

Following the degradation of the pronephros, mesonephros development begins. Mesonephros development is not a complete self-differentiating process. Mesonephric function begins in the chick on approximately embryonic day 5 with maximum mesonephric function occurring between embryonic day 10 and 15 (Romanoff, 1960). Mesonephric degradation, to make way for the metanephros, has been shown to occur over a range of developmental days with degradation and cessation of function occurring on
embryonic day 16 (Mihalkovics, 1885), to between days 18-19 (Atwell and Hanan, 1926).

More than likely, mesonephric function begins to decline towards the end of incubation and the mesonephros still functions simultaneously with the last kidney developmental stage, the metanephros. Mesonephros glomeruli development follows pronephros external glomeruli formation around the time of mesonephros maximal functioning. Whereas the pronephros has only superficial external glomeruli, the mesonephric kidney develops internal glomeruli (Romanoff, 1960). The structure of the mesonephric glomerulus is similar to what will eventually become the main functioning metanephric glomerulus. Both the meso- and metanephric glomeruli function in tandem during the transition period between the meso- and metanephric kidneys (Mihalkovics, 1885; Abdel-Malek, 1950; and Romanoff, 1960).

The last stage of kidney development is metanephros development. The metanephros is the most highly developed and most complex kidney compared to the other two intermediate forms. Metanephric activity begins before the complete degradation of the mesonephros. Metanephros development appears on the 4th day of incubation as an extension from the ureteric bud. The ureteric bud functions similarly to the Wolffian duct by inducing metanephric development, and similarly, stimulation from the ureteric bud are necessary for metanephros differentiation (Boyden, 1932; Gruenwald, 1937). Overall, development of the metanephros is very similar to the mesonephros. Metanephros function begins during the middle of incubation and continues to develop until 30 days after hatching (Wideman, 1989). The glomerulus of the metanephros is similar in structure to that of the mesonephric glomerulus. The metanephric kidney will become the main
kidney in the adult; therefore, perturbations during kidney development can very well have lasting effects on the whole organisms physiology later in life.

IV. Importance of Retinoic Acid on Kidney Development

Retinoids and their active metabolites, such as tRA, have been demonstrated to be main regulatory signals during vertebrate organogenesis (Means et al., 1995) via regulation of transcription factors, as well as implicated in important roles for cell differentiation, and embryo homeostasis (De Luca, 1991). When tRA interacts with its nuclear receptor RAR, it alters gene expression at the level of transcription (Gudas et al., 1994; Mangelsdorf et al., 1995). There are two distinct nuclear receptors that make up the retinoic acid receptor family. These receptor subtypes include the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), and each receptor subtype has three isotypes (α, β and γ). The binding affinities for the many different active metabolites of retinoids are specific to each receptor. The RAR receptor family is activated by tRA as well as 9-cis retinoic acid (9cRA), whereas the RXR receptor family is only activated by 9cRA. Because tRA is a very important metabolite it was used in this study instead of the many other potentially active forms of retinoids.

During embryonic development, cell-cell communications are necessary to promote normal organ system development. This interaction between cells is mediated by availability of a multitude of growth factors and vitamins and deficiencies may have consequential effects due to communication breakdown (Wilson et al., 1948). For example, vitamin A deficiency has been shown to result in impaired kidney growth with many associated developmental abnormalities (Wilson and Warkany, 1948). Moreover,
administering vitamin A during the onset of renal organogenesis reversed these kidney abnormalities, suggesting that vitamin A is directly involved in kidney development (Wilson et al., 1953). Although these studies show the importance of vitamin A on kidney development, the question that needs to be addressed is; how does vitamin A induce renal organogenesis? Retinoic acid, an active metabolite of vitamin A, increases the capacity for branching from the ureteric bud, leading to more sites for developing nephrons (Vilar et al., 1996). The level of exogenous retinoids has been shown to modulate the nephron number in a dose dependent manner (Vilar et al., 1996). In addition, slight deviations from physiologically normal levels of vitamin A, may help explain the variation of total nephron numbers in the general population (Merlet-Benichou et al., 1999). The dose dependent increase in nephrons induced by vitamin A eventually will plateau and concentrations above this limit will have an inhibitory effect on developing nephron growth and differentiation (Vilar et al., 1996). It is important to note that retinoic acid is a promoting factor for nephrogenesis, and is not involved in any type of protective aspect of the developing nephrons. Finally, knockout mice deficient in many different RAR isotypes clearly demonstrate retinoic acid’s involvement in metanephric kidney formation through interactions with its nuclear receptor subtype RAR (Lohnes et al., 1994; Mendelsohn et al., 1994). Although the importance of retinoids on kidney development is clear, the mechanisms involved in this process are still unknown.

Because of the multiple receptor subtypes and isoforms of the retinoic acid receptor previously mentioned, retinoic acid is important for many other developmental processes in addition to kidney organogenesis. Retinoic acid and vitamin A have been implicated in early cardiovascular development and many teratogenic effects have been documented in
either overexposure or underexposure to retinoic acid during early heart development. Excess retinoic acid produces a host of developmental problems such as impaired heart looping (Osmond et al., 1991; Yutzet et al., 1994), while low levels of retinoic acid or missing RARs in mice lead to abnormal ventricular and cardiomyocyte pathologies (Mendelsohn et al., 1994; Sucov et al., 1994). As with any insult during embryonic development, the timing of such event dictates the effect the event will have on the embryo. Potentially, almost every organ system and tissue can be affected by retinoic acid if the timing of exposure corresponds with the critical period in development of that specific organ system or tissue. Fortunately, the majority of kidney development, specifically metanephros development, happens towards the last third of embryonic development. At this time, the heart will have reached its mature developmental stage allowing nephron manipulation through tRA dosage, avoiding heart abnormalities associated with retinoic acid when exposure occurs during critical periods of heart development. It is clear that retinoid derivatives such as vitamin A and tRA play important roles in developmental events that are mediated by the presence of such retinoids. In addition, the complexities of the nuclear receptors help to regulate many processes at the transcriptional level by activating and deactivating specific genes. tRA’s ability to modulate nephrogenesis in the developing kidney will enable us to expose embryos at specific critical periods in nephrogenesis to alter normal nephron numbers.

V. Research Objectives and Hypotheses

The kidney is vitally important for homeostasis in the developing embryo, and is very sensitive to changes during critical periods of nephrogenesis. Further, the kidney,
specifically nephrogenesis, has been implicated as either a primary or secondary factor of many adult onset diseases such as hypertension and renal failure (Barker, 1995; Moore et al., 1999; Ruta et al., 2010). Although, a wealth of information indicates the importance of the developing environment to the overall fitness of the embryo later in life, the mechanisms that cause predisposition to adult onset diseases remain unknown. The avian kidney could potentially become an important model to study not only the potential mechanisms that lead to adult onset diseases, but also to the study interacting systems during development due the similarities of the avian kidney to the mammalian kidney and the ease of embryonic manipulation. Although the broad goal of this research is focused on understanding the interactions between the cardiovascular and renal systems, this project is a necessary first step in that process.

The research presented in this study focuses mainly on functional and morphological assessments made on the developing kidney. These measurements were made in the chicken embryo on multiple days from embryonic day 12-18. Understanding how the kidney may alter its normal development when faced with a pharmacological manipulation will help us understand potentially when the avian kidney is most susceptible to insults. By utilizing a staining method, we are able to quantify not only the total number of nephrons in the kidney, but more importantly, the nephrons that are actively functioning at any given time. In addition to renal measurements, morphological measurements were made on embryo and heart mass to monitor overall development. Therefore, my first hypothesis is that exposure to excess retinoic acid during the critical period in nephrogenesis will alter the normal developmental trajectory of the kidney,
altering not only the developing nephrons but will also produce morphological differences when compared to embryos not treated with tRA.

Once renal disturbance with tRA was confirmed, I looked at the capability of the embryo to regulate its electrolyte balance during development. This will not only allowed me to verify renal disturbances, it also allowed me to identify what role the kidney may play in controlling and regulating the embryo’s electrolyte environment. The kidney, along with extra embryonic structures such as the allantois and the chorioallantoic membrane, work together to ensure that blood pH and osmolality are maintained throughout development. By making assessments of allantoic fluid, blood and blood plasma osmolality, blood pH, hematocrit, and ion concentrations of the different fluid compartments I can confirm that the treatment had the desired effect on kidney development. My second hypothesis is thus that embryos exposed to tRA, which will have previously altered the developmental trajectory of the kidney, will have altered internal electrolyte and fluid osmolality homeostasis.

In this current study, pharmacological manipulation with tRA was used to target critical periods in nephrogenesis aiming to disrupt normal kidney development. Understanding the critical periods for nephrogenesis in the chicken embryo may allow me to not only observe physiological or morphological changes in response to a specific drug, but also to begin to examine potential mechanisms that may be impossible to study in systems that have too many confounding variables due to the maternal influence seen in all mammalian species. During mammalian development, much of the stress that is imposed *in utero* is a direct result of stress placed upon the mother. Removing much of the maternal influence during development will allow us to directly target the embryo and ascertain that
any response that we see is largely an embryonic response. The embryonic chicken, therefore, should be considered as a useful model in the search for potential mechanisms that lead to many adult onset diseases that to this point remain unknown.
MATERIALS AND METHODS

I. Source and Incubation of Eggs

Fertilized white leghorn eggs (Gallus gallus domesticus, layer strain) were obtained from Texas A&M University (College Station, TX) and shipped to the University of North Texas (Denton, TX) Department of Biological Sciences. On arrival eggs were placed in incubation at 37.5 ± 0.5 °C and 55-60 % relative humidity and were turned automatically every three hours. Temperature and humidity in the incubator were monitored using wireless baro-thermo-hydrometers (model BTHR968, Oregon Scientific). On embryonic days ranging from 12-18 (stages 37-44; (Hamburger and Hamilton, 1951) eggs were removed for experimental analyses. The University of North Texas’ Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures.

II. Drug Preparation and Injection Protocol

A 10 mg/ml stock solution of all-trans-retinoic acid (Sigma-Aldrich) was prepared in dimethyl sulfoxide (Sigma-Aldrich). The stock solution was further diluted with chick Ringer solution to make 2 different concentrations of retinoic acid: 1 mg/ml and 3 mg/ml. Both drug concentrations were diluted to a final concentration volume of 5 % dimethyl sulfoxide (Sigma-Aldrich). Additionally, a sham solution was prepared with chick Ringer with a final volume of 5 % dimethyl sulfoxide. 1 ml aliquots were made from all solutions and stored at -20 °C until required. Solution was removed from cold storage, thawed, and briefly vortexed. Care was taken to prevent the retinoic acid from exposure to long periods of UV light prior to use in the experiments.
Eggs were removed from the incubator at day 8 (stage 33) of embryonic development. Briefly, eggs were candled to locate an injection site that would not interfere with any of the vasculature of the CAM. The eggs were then transferred to an incubator (HOVA-BATOR, model 1602N) set at 37.5 ± 0.5 °C and 60% relative humidity. A small hole was made in the egg shell, at the previously determined site, using an 18 ½ gauge needle. An injection of 100 µl of retinoic acid or sham solution was then administered into the allantoic fluid via a 25-gauge needle attached to a 1 ml syringe. After the injection, the hole was sealed with a 2 cm piece of electrical tape followed by application of JB Quickweld® to bind the tape to the shell. The eggs were then returned to the original incubator for continued incubation.

III. Mortality Assessment

Mortality of embryos receiving the injection of retinoic acid or sham solution was checked on embryonic day 10 by candling. Mortality percentage was calculated for 1 mg/ml retinoic acid, 3 mg/ml retinoic acid and sham solutions from the number of dead eggs divided by number of eggs injected X 100. Embryos from many different injection days were pooled together by treatment group.

IV. Embryonic Body Mass Measurement

Embryos were euthanized by 0.1 ml pentobarbital injection directly into the embryo on the selected study day then removed from the shell. Wet mass was determined (Denver Instrument Company XD-800) after removal of excess fluids with Kimwipes® (Kimtech Science). The embryo was then pinned to a surgical mat, where the abdominal cavity was opened, and with the assistance of a dissecting microscope, the heart, and each kidney was
removed, lightly dried with Kimwipes® and wet mass was determined. The tissue was then placed in a tissue oven (Fisher Isotemp® 100 series model 106 G) for 2 days. Following the 2 day drying time, dry mass was determined for embryo, heart and kidneys (Denver Instrument Company XD-800). The remains of the embryo were disposed of according to IACUC protocol.

V. Catheterization Protocol

At the selected developmental ages, eggs were removed from the incubator, briefly candled to locate a peripheral chorioallantoic membrane (CAM) vein and placed (less than 1/3 of egg buried) in a sand-filled chamber in a double-walled glass container thermostated 37 ± 0.5 °C through internal circulation of heated water, allowing maintenance of constant egg temperature during the procedure. The previously located CAM vein was exposed by removing a small portion of the eggshell and occlusively cannulated with a polyethylene catheter (PE-50, Clay Adams) with the tip heat-pulled to an outer diameter of ~0.5 mm. A ligature was placed downstream to eliminate retrograde flow, and a second ligature was placed upstream of the first to hold the catheter in place once in the vessel. The catheter was then glued to the eggshell with superglue (Loctite®) to ensure the catheter did not become dislodged from the vessel. Zipkicker (PACER®) was applied to the superglue to decrease drying time. After the procedure was completed, eggs were placed in a temperature and humidity controlled incubator (37.5 ± 0.5°C and relative humidity at 60%; HOVA-BATOR model 1602N) for the duration of the alcian blue staining procedure. (For surgical procedure reference see Crossley et al., 2003).
VI. Glomerular Distribution Analysis

After cannulation, the embryos were allowed 15 min to recover before being subjected to the following protocol adapted from Bolin (2009). A 2.5% Mannitol injection, which opened the perfused nephrons, was administered via a 1 ml syringe through the catheter in a bolus slowly over the course of 1 min. The volume of fluid injected into the animal was based on the estimation of 3% of the total blood volume (Romanoff, 1960), determined by the following calculations: Embryo wet weight (g) X 15% = total blood volume in grams; Total blood volume in grams X 3% = amount to be injected in ml. After a period of 10 min, an injection of 0.2% Alcian blue dye was injected into the embryo following the same protocol as with the Mannitol injection. This injection was allowed to circulate for 30 min. Following the 30-min period, an injection of 0.1 ml pentobarbital was injected into the embryo via the catheter to euthanize the embryo.

The right kidney was selected from each embryo for the purposes of glomerular distribution analysis. Upon dissection, the kidneys were placed in 50% ethanol at 4 °C overnight. The following day, the kidneys were moved into a Bluing mix (50:50 mixes of 50% ethanol and 1% ammonium hydroxide (H\textsubscript{4}NH\textsubscript{4}OH)) for 90 min at 4 °C. Next, each kidney was rinsed twice with cold deionized water and placed into a vial containing 20% hydrochloric acid (HCl) and incubated at 37 °C for 2-3 h. The acid was decanted and the kidneys were rinsed 3-4 times with cold deionized water. The kidneys were then left in a final water mixture of 2 ml for 12-24 h at 4 °C. Following the incubation, the final water mixture was brought up to 6 ml and a stir bar was added to the vial. The vial was placed on a stir plate (Fisher Scientific) and stir speed was set to medium for 10 min. A volume of 2 µl was removed via Gilson micropipette and spread across a microscope slide. The solution
was allowed to settle for 30 sec before visualization began. A total of 5 2 µl samples were used and the glomerular counts were averaged to get an estimate of total and perfused glomeruli per kidney. Kidney samples were viewed using Nikon Eclipse E200 binocular scope at 40X, 100X and 400X magnifications. Glomeruli were identified at 100X and verification of perfused nephrons was made at 400X magnification. Glomeruli were considered perfused if the Alcian blue dye accumulated within the glomerular tuft. The formula for estimating glomeruli numbers is as follows:

\[(\text{Kidney wet mass (mg)}/6.0 \text{ ml water}) = (x/.002\text{ml})\].

\*x = weight of tissue in sample. 0.002 was used because of the 5 2 µl samples the number of glomeruli counted in each sample was averaged to get a total number of glomeruli counted for each kidney.

\[\frac{\text{Total glomeruli counted}}{\text{Weight of tissue in sample (mg)}} = \frac{\text{glomeruli}}{\text{mg}}.\]

\[\frac{\text{Glomeruli}}{\text{mg}} \times \text{Total kidney wet weight (mg)} = \text{Estimate # glomeruli / kidney.}\]

VII. Fluid Osmolality

Approximately 100 µl of allantoic and amniotic fluids were removed from the embryo with a 25 gauge needle attached to a 1 ml syringe. Osmolality of whole blood that was collected was also measured as well as the blood plasma. Approximately 100 µl blood samples were centrifuged (Fisher micro-centrifuge model 235A) for 5 minutes at 5,000 g and the plasma was removed for analysis. Osmolality measurements for all fluids were obtained by injecting 10 µl of sample into a vapor pressure osmometer (Wescor® VAPRO, 5520).
VIII. Flame Photometry

Sodium (Na\(^{+}\)) and potassium (K\(^{+}\)) ion concentrations were measured in plasma and allantoic fluid from a separate set of embryos (control embryos, sham treated embryos, embryos exposed to 1 mg/ml tRA, and embryos exposed to 3 mg/ml tRA) from day 12 to day 18. Approximately 300 µl of blood was removed from the chorioallantoic vein with a 25 gauge needle attached to a heparinized 1 ml syringe. The blood was immediately centrifuged (Fisher micro-centrifuge model 235A) for 5 min at 5,000 g and the plasma was removed, placed in liquid nitrogen, and stored at -80 °C. For allantoic fluid, 300 µl was removed from the same embryo in which blood was removed with a different 25 gauge needle attached to a 1 ml syringe. Allantoic fluid was quickly placed in liquid nitrogen and stored at -80 °C. Samples were removed from storage and briefly vortexed. 50 µl samples of both plasma and allantoic fluid were diluted 400X in ultrapure water making a final volume of 20 ml. After dilution, each plasma and allantoic fluid sample was separated into 2 5 ml aliquots since only one ion can be measured at a single time. Standard concentrations for both Na\(^{+}\) and K\(^{+}\) were serially diluted from a pre-made 20 ppm standard (SCP Science) with the same ultrapure water used to dilute the samples. Assessments of Na\(^{+}\) and K\(^{+}\) ion plasma and allantoic fluid concentrations were made using an atomic absorption spectrometer (Perking Elmer Instruments Analyst 330).

IX. Hematology/Blood Chemistry

Different eggs were removed from incubation on days 12, 14, 16, and 18 and candled to locate the major CAM vein. Once the major CAM vein was found, approximately 300 µl of blood was removed via a 25 gauge needle attached to a heparinized 1 ml syringe.
For measurement of hematocrit, red blood cell count, and total hemoglobin 35 µl of blood was injected into a Coulter counter (COULTER® Ac.T). 125 µl of blood was used to measure pH and bicarbonate ion concentration (HCO₃⁻) using a Radiometer (Copenhagen Radiometer ABL 5).

X. Statistical Analysis

All data was first tested for normality of distributions (Shapiro-Wilks test for normality) before any other statistical test was performed. Two-way parametric ANOVA was used to test whether or not developmental day, treatment or the interaction between the two had an effect on the data. Post hoc Student-Newman Keuls (SNK) multiple range tests were run if no significant interaction occurred to separate data into distinct groups. If a significant interaction was seen between development and treatment concatenation was done to separate statistically distinct groups within each developmental stage. Treatment mortality was assessed by One-way parametric ANOVA followed by post hoc Student-Newman Keuls (SNK) multiple range test to separate treatments into statistically distinct groups. All statistical analyses were performed using SAS 9.2 software. Statistical decisions were made at an alpha level of 0.05. Mortality data are presented as mean ± SD while all other data are presented as mean ± SE.
RESULTS

I. Mortality Assessment

Mortality from the sham treated embryos was not different from mortality seen in control embryos when assessed on embryonic day 8. Mortality from a single injection of either sham or 1 mg/ml tRA on embryonic day 8 showed no significant difference when assessed on embryonic day 10. However, a significant increase in mortality was seen when embryos were exposed to a single injection of 3 mg/ml tRA (33 ± 8 %) compared to either control (8 ± 2 %), sham (10 ± 2 %), or 1 mg/ml tRA treatments (19 ± 12 %) (1-way parametric ANOVA, P < 0.05). Mortality increased an average of 23 % when comparing sham and 3 mg / ml tRA treatments (Fig. 1).

II. Mass Assessment

Wet and dry mass of all organs increased significantly (2-way parametric ANOVA, P < 0.001) during the incubation period. Predictably, wet body mass showed the largest proportional increase (about 9-fold) from day 12 (5.2 ± 0.1 g) to day 14 (9.8 ± 0.2 g), then showed a slightly smaller proportional increase in mass (about 4-fold) as growth continued from day 16 (17.5 ± 0.32 g) to day 18 (25.0 ± 0.44 g) (Fig. 2A). Moreover, embryo dry mass mirrored wet mass with its greatest proportional growth (about 20-fold) from day 12 (396 ± 9 mg) to day 14 (1,262 ± 47 mg), followed by a smaller proportional increase (about 7-fold) between day 16 (3,367 ± 150 mg) and day 18 (5,611 ± 581 mg) (Fig. 3A). During normal development, both heart and kidney mass (wet and dry) were significantly larger on day 18 compared to day 12. (2-way parametric ANOVA, P < 0.001, Fig. 2B and C and Fig. 3B and C, respectively). Taken together my results indicate that normal growth rate was
fastest early in development (from day 12 to day 14), and although continued to increase over development was not as fast. Late in development, after day 14, the embryo and its organs may be maturing, thus growing at a smaller proportional rate when compared to early in development.

A significant interaction was seen between treatment and development for embryo wet and dry mass measured from days 12-18 (2-way parametric ANOVA, \( P < 0.0001 \), Fig. 2A and Fig. 3A). No significant difference was seen between control and treatment embryos wet or dry mass on days 12 and 14. However, on day 16, control wet mass (17.5 ± 0.3 g) was significantly greater compared to both 1 mg/ml tRA (14.6 ± 0.3 g) and 3 mg/ml tRA (13.5 ± 0.5 g) \( P < 0.05 \); while no significant difference was seen between control and sham treatments (Fig. 2A). Embryo dry mass was not significantly different among control, sham, and 3 mg/ml tRA, but a significant decrease was seen on day 16 in embryos treated with 1 mg/ml tRA (Fig. 3A). Finally, on day 18, embryo wet mass was decreased 31 % and 79 % from control by treatment of 1 mg/ml tRA and 3 mg/ml tRA respectively. No difference was seen on day 18 between control and sham treatment. Concatenation produced three statistically distinct groupings signifying that control/sham, 1 mg/ml tRA, and 3 mg/ml tRA were different from one another. The same trend was not seen on day 18 for embryo dry mass. Although treatment with tRA decreased embryo dry mass (similarly to embryo wet mass), no statistical difference was observed between treatments with tRA (Fig. 3A). Again, no difference was observed in embryo dry mass between control and sham treatment. Interestingly, embryo wet and dry mass did not behave similarly on day 18 of development. Both wet and dry mass was reduced upon treatment with tRA on day 16 and
18; however, embryo wet mass seems more sensitive to changes in tRA compared to dry mass.

During normal development, heart wet mass increased throughout development (2-way parametric ANOVA, $P < 0.001$) with its largest mass increase, (11-fold), from day 12 (44.7 ± 2.8 mg) to day 14 (94.6 ± 2.9 mg) (Fig. 2B). In addition, heart dry mass increased significantly over development ($P > 0.001$) roughly doubling in size from day 12 (5.1 ± 0.2 mg) to day 14 (11.6 ± 0.4) (Fig. 3B). Both control and dry heart mass continued to proportionally increase in size towards the end of development, although at a smaller rate. The only difference seen in heart mass (wet or dry) between control and sham was seen on day 18 with sham treatment producing smaller hearts compared to control ($P < 0.05$). A significant interaction was seen between treatment and development for both wet and dry heart mass (2-way parametric ANOVA $P > 0.0001$, Fig. 2B and Fig. 3B). Neither heart wet mass nor dry mass was affected by either concentration of tRA or sham from day 12 to 14. Treatment with 3 mg/ml tRA produced a significant decrease in heart wet mass (138 ± 6 mg) compared to control (172 ± 5 mg) and sham treatment (163 ± 6 mg) ($P < 0.05$). However, no difference was observed between tRA concentrations (Fig. 2B). Heart dry mass was reduced with treatment of 1 mg/ml tRA (148 ± 6 mg) compared to other treatments, including control (Fig. 3B). On day 18, both 1 mg/ml tRA (162 ± 7 mg) and 3 mg/ml tRA (149 ± 5 mg) treatment, although not different from each other, produced a significant decrease in heart wet mass that was 34% lower than control (217 ± 9 mg), and sham (207 ± 6 mg) ($P < 0.05$, Fig. 2B). Finally, day 18 heart dry mass produced similar results compared to wet mass showing decreased heart mass from both control and sham with either concentration of tRA (Fig. 3B). Neither concentration of tRA produced apparent
developmental affects on heart wet and dry mass until day 18 of development. Neither heart wet nor dry mass behaved like embryo wet mass on day 18 indicating that heart mass is not as sensitive compared to embryo wet mass.

Kidney wet mass showed the largest proportional increase, roughly doubling in mass, from day 12 (52 ± 3 mg) to day 14 (109 ± 6 mg). Similarly, kidney dry mass showed the largest increase, almost tripling in mass from day 12 (6.9 ± 0.3 mg) to day 14 (17 ± 1 mg) (Fig. 2C and Fig. 3C respectively). A significant interaction was seen between treatment and development for both wet and dry kidney mass (2-way parametric ANOVA, \( P < 0.0001 \)). No difference was seen in kidney wet and dry mass between control and sham treatment over development (\( P > 0.05 \)). Kidney wet mass showed no difference from day 12 to 14. On day 16, both kidney wet and dry mass was reduced upon exposure to 1 mg/ml tRA when compared to control; but kidney wet mass was not different from 3 mg/ml tRA (Fig. 2C). Kidney wet mass showed a dose-dependent response to tRA on day 18 reducing kidney wet mass by 26 % and 69 % from control upon exposure to 1 mg/ml tRA and 3 mg/ml tRA respectively (\( P > 0.05 \), Fig. 2C). Kidney dry mass however did not show a dose-dependent decrease upon exposure to tRA. Kidney dry mass was reduced from control upon exposure to 1 mg/ml tRA, but no difference was seen either between the two concentrations of tRA or control and 3 mg/ml tRA (Fig. 3C). Kidney wet mass mirrored embryo wet mass on day 18 showing a dose-dependent decrease when exposed to tRA. Kidney dry mass was not affected in the same way compared to kidney wet mass. Both embryo and kidney development seemed most responsive and sensitive to tRA compared to heart mass. Moreover, wet mass was affected differently compared to dry mass indicating that tRA may not be as responsive to the difference between tRA used in this
study. Regardless, tRA produced developmental differences only over the last 2 days of development studied (day 16 to day 18), while no affect was observed early in the developmental timeframe studied (day 12 to day 14).

When standardizing organ wet mass (heart and kidney) to embryo body wet mass, no difference was observed between control and sham treated embryos (Fig. 4A and B). Moreover, the only difference seen in heart / body mass ratio was on day 14 between sham (not different from control) and both treatments of tRA ($P < 0.05$). Finally, no differences were observed at any developmental day between control and treatments for kidney / mass ratio (Fig. 4B). The data show that the organ differences noted above where due to an overall decrease in embryo development (identified by mass), and not differences in individual organ growth or development.

III. Analysis of Total and Perfused Nephrons

There was a significant interaction between treatment and development for total nephrons per milligram kidney tissue (2-way parametric ANOVA, $P < 0.05$, Fig. 5A). No statistical difference was observed between control and sham treatments at any day during development. On day 12, treatment with 3 mg/ml tRA produced a significant ($P < 0.05$) and large decrease in nephron number per milligram kidney tissue (342 ± 34) compared to control (582 ± 74), while no differences were seen between control and 1 mg/ml tRA (Fig. 5A). No other differences were observed between treatments and control at any day over development. Concatenation separated concentrations of tRA on embryonic day 16 ($P < 0.05$); however, this difference did not continue past day 16 (Fig. 5A).
No significant difference was seen in perfused nephrons / milligram kidney tissue between control and sham treatment at any point during development (SNK multiple range test, $P > 0.05$). In addition, there was no difference between control and either concentration of tRA at any point during development (SNK range test, $P > 0.05$). Perfused nephrons / milligram kidney tissue was influenced from day 14 to day 16 with difference seen between concentrations of tRA (Fig. 5B). The biggest difference seen between treatments (51 %) was observed on day 16 (SNK multiple range test, $P < 0.05$, Fig. 5B). No difference was seen on day 18 between any treatment compared to control (Fig. 5B). Collectively, nephron number and perfused nephrons were largely unaffected after treatment with tRA. Any differences seen early in development did not persist over any other developmental day.

IV. Fluid Osmolality

Blood osmolality (mmol/kg) remained constant over development with an average blood osmolality of 274 mmol / kg (Fig. 6A). A significant interaction was seen between treatment and development for blood osmolality (2-way parametric ANOVA, $P < 0.0001$). Blood plasma osmolality (mmol / kg) also remained constant over development with an average osmolality of 271 mmol / kg (Fig. 6B). A significant interaction was seen between treatment and development for blood plasma osmolality (2-way parametric ANOVA, $P < 0.0001$). No difference was observed between control and sham for either blood or blood plasma osmolality at any point during development ($P > 0.05$). Also, 1 mg/ml tRA did not alter blood osmolality significantly at any developmental day when compared to control (Fig. 6A). On the other hand, 3 mg/ml tRA treatment consistently reduced blood osmolality.
compared to control over development with an average decrease of 5 % (274 ± 2 mmol/kg vs. 260 ± 2 mmol/kg) (Fig. 6A). Blood plasma osmolality was significantly reduced on day 12 upon treatment with both 1 mg/ml tRA (259 ± 1 mmol/kg) and 3 mg/ml tRA (255 ± 1 mmol/kg) compared to control (268 ±1 mmol/kg); however, the concentrations did not differ. 1 mg/ml tRA’s affect was transient as no differences were exhibited from day 14 to day 16 compared to control. Interestingly, on day 18, 1 mg/ml tRA (265 ± 2 mmol/kg) again produced a significant decrease in blood plasma osmolality compared to control (275 ± 2 mmol/kg) ($P > 0.05$, Fig. 6B). In contrast to 1 mg/ml tRA, 3 mg/ml tRA produced significant decreases over the entire length of development with an average blood plasma osmolality decrease of 5 % compared to control (271 ± 3 mmol/kg vs. 259 ± 5 mmol/kg). Neither concentration was different from one another on day 18, although both were different from control (Fig. 6B). Both blood and blood plasma osmolality was reduced upon treatment with tRA. Although treatment did reduce blood and blood plasma osmolality, it may not be directly resulted from the tRA injection itself.

Allantoic fluid osmolality (mmol/kg) decreased over development with a significant decrease in osmolality between day 12 (229 ± 3 mmol/kg) and day 18 (175 ± 4 mmol/kg) ($P < 0.05$, Fig. 7A). A significant interaction was seen between treatment and development for allantoic fluid osmolality (2-way parametric ANOVA, $P < 0.0001$). No difference was seen between control and sham treatment allantoic fluid osmolality over any point in development. From day 12 to day 14 no difference was observed between control and treatments. However, on day 16, embryos treated with 1 mg/ml tRA and 3 mg/ml tRA showed marked increases in allantoic fluid osmolality (209 ± 6 mmol/kg, and 227 ± 3 mmol/kg respectively) compared to control (186 ± 8 mmol/kg) ($P < 0.05$), although they
were not different from each other (Fig. 7A). On day 18 allantoic fluid osmolality increased on average 25 % between tRA treated (220 ± 6 mmol/kg) and control (176 ± 4 mmol/kg) embryos, with no difference between tRA concentrations (Fig. 7A). Amniotic fluid osmolality remained relatively constant over development (2-way parametric ANOVA, $P > 0.05$). The only difference observed occurred on day 16 with amniotic fluid osmolality decreased from control after treatment with tRA; however, this difference was short lived since no difference existed on day 18 (Fig. 7B). The data indicate that allantoic fluid osmolality was affected by treatment with tRA. Interestingly, while blood plasma osmolality was decreased upon treatment, allantoic fluid osmolality was elevated compared to control. Again it is unclear if the elevated allantoic fluid osmolality is a direct result of the tRA itself, or if the drug interacts with other mechanisms that control fluid volume and/or electrolyte balance.

V. Fluid Compartment Ion Concentrations

Control embryo blood plasma sodium concentration $[\text{Na}^+]$ remained markedly steady over development averaging a $[\text{Na}^+]$ of 194 ± 3 mmol/L from day 12 to day 18 (2-way parametric ANOVA, $P > 0.05$, Fig. 8A). No significant difference was seen on any developmental day between control and sham treated embryos. On day 12, embryos treated with 1 mg/ml tRA (181 ±3 mmol/L) and 3 mg/ml tRA (168 ± 1 mmol/L) had reduced blood plasma $[\text{Na}^+]$ compared to control embryos (197 ±2 mmol/L) ($P < 0.05$, Fig. 8A). This difference represents a change from control of 9 % and 17 % respectively. From day 14 to day 18 embryos treated with 1 mg/ml tRA showed reduced blood plasma $[\text{Na}^+]$ compared to control, however, these values did not differ significantly from sham treated
embryos. On the other hand, embryos treated with 3 mg/ml tRA showed markedly reduced blood plasma [Na⁺] from control not only on day 12, but also over the entire developmental timeframe of the current study. Average blood plasma [Na⁺] was 194 ± 3 mmol/L for control embryos, while embryos treated with 3 mg/ml tRA averaged 165 ± 2 mmol/L blood plasma [Na⁺]. That represents a 19 % change over development between the two groups (Fig. 8A).

Control embryo allantoic fluid [Na⁺] decreased significantly over development with day 18 (69 ±4 mmol/L) being significantly lower compared to day 12 (154 ± 5 mmol/L) (P < 0.05, Fig. 8B). Sham treated embryos showed no difference from control embryos with the exception of day 18 when sham treated embryos showed a reduced allantoic fluid [Na⁺] (45 ± 10 mmol/L) compared to control embryos (69 ± 4 mmol/L). A significant interaction was seen between treatment and development for allantoic fluid [Na⁺] (2-way parametric ANOVA, P < 0.05, Fig. 8B). No difference was seen from day 12 to day 14 among any groups. Day 16 embryos treated with 1 mg/ml tRA (156 ± 3 mmol/L) showed an increased allantoic fluid [Na⁺] compared to control embryos (94 ± 16 mmol/L, P < 0.05); while no difference was seen between concentrations of tRA (Fig. 8B). On day 18, embryos treated with 1 mg/ml tRA (141 ± 5 mmol/L) and 3 mg/ml tRA (124 ± 5 mmol/L), while not different from one another, showed elevated allantoic fluid [Na⁺] compared to control (69 ± 4 mmol/L) (P < 0.05, Fig. 8B). From the data it is clear that sodium ion is normally tightly regulated over the last part of development given that control embryos maintained constant blood plasma [Na⁺] from day 12 to day 18. In addition, it appears that [Na⁺] in the different fluid compartments within the egg may greatly influence the final osmolality of the fluid within those compartments.
Blood plasma potassium concentration \([K^+]\) remained constant over development (2-way parametric ANOVA, \(P > 0.05\), Fig. 9A). No difference was seen at any point in development between control and treatment groups. Control embryos showed an average blood plasma \([K^+]\) of 2.5 ± 0.2 mmol/L from day 12 to day 18 (Fig. 9A).

Allantoic fluid potassium concentration \([K^+]\) increased significantly over development with day 18 (23.8 ± 2.3 mmol/L) being higher compared to day 12 (6.6 ± 1.9 mmol/L) in control embryos \((P < 0.05\), Fig. 9B). A significant interaction was seen between treatment and development for allantoic fluid \([K^+]\) (2-way parametric ANOVA, \(P < 0.05\), Fig. 9B). No difference was seen between control and sham treated embryos at any point during the study period. Allantoic fluid \([K^+]\) was not altered with tRA from day 12 to day 14.

On day 16, embryos dosed with 1 mg/ml tRA (13.8 ± 2.9 mmol/L) and 3 mg/ml tRA (6.1 ± 1.3 mmol/L) showed markedly lower allantoic fluid \([K^+]\) compared to control (23.1 ± 1.9 mmol/L) \((P < 0.05\), Fig. 9B). These differences were not sustained as no difference was seen among any treatment on day 18 \((P > 0.05\), Fig. 9B). It appears that blood plasma potassium is tightly regulated over the last part of development, as levels remained constant from day 12 to day 18. In addition, allantoic fluid \([K^+]\) was largely unaffected upon treatment with tRA. Taken together, the flame photometry data suggests that sodium is likely a more important ion when it comes to influencing fluid osmolality during avian embryonic development.

VI. Hematology

Hematocrit (Hct (%)) in control embryos increased significantly over development from 21 ± 6 % on day 12 to 36 ± 1 % on day 18 (2-way parametric ANOVA, \(P < 0.05\), Fig. 9A).
10A). Red blood cell count (10^6/µL) increased significantly (2-way parametric ANOVA, \( P < 0.05 \)) over development from 1.32 ± 0.03 10^6/µL on day 12, to 2.28 ± 0.6 10^6 /µL on day 18 (Fig. 10B). Neither red blood cell count nor Hct differed between control and sham treatment at any point during development. Red blood cell count as well as Hct was elevated above control in embryos treated with 1 mg/ml tRA on day 12, while 1 mg/ml tRA produced no significant differences when compared to control after day 12 for both red blood cell count (Fig. 10B) and Hct (SNK multiple range test, \( P > 0.05 \), Fig. 10A). Treatment with 3 mg/ml consistently elevated red blood cell count over the course of development compared to control, with a mean average difference of 27 % (1.80 ± 0.04 10^6/µL vs. 2.26 ± 0.08 10^6/µL) (Fig. 10B). Similarly, Hct was elevated in embryos treated with 3 mg/ml tRA from days 12 to 16 (26 ± 2 % vs. 34 ± 2 %); however, these differences did not continue to day 18 (Fig. 10A).

Control hemoglobin (Hb (g/dL)) concentration increased slightly over development with day 18 (10.6 ± 0.2 g/dL) being significantly higher compared to day 12 (6.5 ± 0.2 g/dL) (\( P > 0.05 \), Fig. 10C). A significant interaction was seen between treatment and development (2-way parametric ANOVA, \( P < 0.0001 \)). No difference was observed between control and sham treatment Hb concentration at any point during development (\( P > 0.05 \)). On day 12, both tRA treatments produced an elevated Hb concentration compared to control, although no difference was seen between the two tRA concentrations. From day 14 to day 18, treatment with 3 mg/ml tRA produced elevated Hb concentrations averaging 30 %, with the largest difference between control (8.7 ± 0.2 g/dL) and 3 mg/ml tRA (12.1 ± 0.4 g/dL) on day 16 (Fig. 10C). Finally, an increased Hb concentration was observed on day 18 when embryos were treated with 1 mg/ml tRA (11.8 ± 0.7 g/dL) compared to control (10.6
Embryos exposed to tRA had altered hematocrit, red blood cell count, and hemoglobin over the developmental timeframe studied, especially from day 12 to day 16. It appears that the developing mechanisms that regulate these various parameters are sensitive to the changing retinoic acid environment.

VII. Blood Chemistry

Blood pH decreased slightly over development with day 18 (7.52 ± 0.02) being significantly lower compared to day 12 (7.72 ± 0.01) (2-way parametric ANOVA, \( P < 0.05 \), Fig. 11A). In contrast to blood pH, bicarbonate ion concentration \([\text{HCO}_3^-]\) slightly increased over development with day 18 (25 ± 1) significantly higher compared to day 12 (13 ± 2) \( (P < 0.05, \text{Fig. 11B}) \). No significant differences in blood pH or \([\text{HCO}_3^-]\) were observed between control and sham treatment at any point in development (Fig. 11A and B respectively). Blood pH was significantly lower on day 12 in embryos treated with 3 mg/ml tRA (7.52 ± 0.01) compared to control (7.72 ± 0.01), sham (7.73 ± 0.1), and 1 mg/ml tRA (7.69 ± 0.01) \( (P < 0.05) \). On day 16, 1 mg/ml tRA produced a significant decrease in blood pH when compared to control. No differences in blood pH were observed on day 18 between any groups \( (P > 0.05, \text{Fig. 11A}) \). \([\text{HCO}_3^-]\) only differed on day 12 with embryos treated with 3 mg/ml tRA (25.2 ± 1.3) exhibiting higher \([\text{HCO}_3^-]\) compared to control (12.7 ± 1.1) \( (P < 0.05, \text{Fig. 11B}) \). The acid base balance environment of the developing embryo was largely unaffected by treatment with tRA. Small differences seen on day 12 were transient and did not continue past day 12.
Fig. 1. Percent mortality assessed on embryonic day 10 in the chicken exposed to a single dose of either sham or drug treatment on embryonic day 8. Different letters indicate statistically distinct groups (1-way parametric ANOVA, p < 0.05). Embryos from multiple injection days were pooled together for the purposes of analysis. n = 80 for control and each treatment. Data are presented as mean ± SD.
Fig. 2. (A) Embryo wet mass (g), (B) Heart wet mass (mg), and (C) Kidney wet mass (mg) measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way parametric ANOVA, p < 0.05). Sample sizes are given in parentheses (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3 mg/ml tRA). Data are presented as mean ± SE.
Fig. 3. (A) Embryo dry mass (mg), (B) Heart dry mass (mg), and (C) Kidney dry mass (mg) measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way parametric ANOVA, p < 0.05). Sample sizes are given in parentheses (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3 mg/ml tRA). Data are presented as mean ± SE.
Fig. 4. (A) Ratios of Heart mass (g), and (B) Kidney mass (g) to embryo body mass (g) measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way Parametric ANOVA, p < 0.05). Sample sizes are given in parentheses (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3 mg/ml tRA). Data are presented as mean ± SE.
Fig. 5. (A) Total nephrons and (B) Perfused nephrons per mg kidney tissue measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way parametric ANOVA, p < 0.05). Sample sizes are given in parentheses (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3 mg/ml tRA). Data are presented as mean ± SE.
Fig. 6. (A) Blood, and (B) Blood plasma osmolality (mmol / kg) measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way parametric ANOVA, p < 0.05). Sample sizes are given in parenthesis (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3 mg/ml tRA). Data are presented as mean ± SE.
Fig. 7. (A) Allantoic and (B) Amniotic fluid osmolality (mmol / kg) measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way parametric ANOVA, p < 0.05). Sample sizes are given in parentheses (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3mg/ml tRA). Data are presented as mean ± SE.
Fig. 8. (A) Blood Plasma [Na⁺] (mmol / L) and Allantoic Fluid [Na⁺] (mmol / L) measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way parametric ANOVA, p < 0.05). Sample sizes are given in parentheses (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3mg/ml tRA). Data are presented as mean ± SE.
Fig. 9. (A) Blood Plasma [K⁺] (mmol / L) and Allantoic Fluid [K⁺] (mmol / L) measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way parametric ANOVA, p < 0.05). Sample sizes are given in parentheses (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3mg/ml tRA). Data are presented as mean ± SE.
Fig. 10. (A) Hematocrit (%), (B) Red blood cell count (10^6/ µL), and (C) Blood hemoglobin (Hb) (g/dL) measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way parametric ANOVA, p < 0.05). Sample sizes are given in parentheses (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3 mg/ml tRA). Data are presented as mean ± SE.
Fig. 11. (A) Blood pH, and (B) Bicarbonate ion (HCO$_3^-$) concentration (unit) measured from days 12-18 in the embryonic chicken. Means that are not significantly different are grouped within boxes (2-way parametric ANOVA, p < 0.05). Sample sizes are given in parentheses (control embryos, embryos dosed with sham treatment, embryos dosed with 1 mg/ml tRA, embryos dosed with 3 mg/ml tRA). Data are presented as mean ± SE.
DISCUSSION

This study examined the effects of tRA on the developing embryo, kidney, and osmoregulatory system in the embryonic chicken. The findings demonstrate that this potent regulator of developmental processes can modify embryonic development, kidney development specifically, and the functions of the kidney.

I. Retinoid Effect on Early Embryonic Chickens

A. Embryo Mortality

Mortality of control and sham treated embryos was approximately 10% compared to the 30% mortality in embryos injected with 3 mg/ml tRA (Fig. 1). Thus, it appears that all-trans retinoic acid (tRA) has specific effects on embryo mortality. During normal development the intrinsic levels of retinoic acid to which embryos are exposed to over the course of development are likely tightly regulated both spatially and temporally. In the current study, mortality was significantly increased in embryos exposed to the highest dose of tRA (3 mg/ml tRA) when compared to sham treated embryos, while no difference was seen in mortality of embryos receiving the lowest dose of tRA (1 mg/ml tRA). No such studies have looked at the mortality associated with tRA over the last third of embryonic chicken development.

B. Retinoid Effect on Organ Growth

Retinoids, and their active metabolites, such as tRA, are important regulatory signaling molecules during development. Early in development, tRA acts as a morphogen influencing early vertebrate organogenesis and cell differentiation (Means et al., 1995). Numerous studies have shown the importance of retinoic acid during early embryogenesis.
(De Luca, 1991). However, no such studies have examined how retinoic acid influences growth and development later in the incubation period for avian embryos.

In the current study, as anticipated, both embryo wet and dry masses increased throughout development with all organ masses studied being significantly larger later in development (day 18) compared to earlier in development (day 12) (Fig. 2 and 3). Moreover, the greatest increase in mass for all organs, including embryo mass, occurred from day 12 to day 14. During the early period of study (from day 12 to day 14) the large increases in organ mass could be a function of increased organogenesis (organ mass increase) and cell proliferation. Later in development, after day 14, organ mass increases, although not as quickly as previous days, suggesting that organ maturation could be occurring compared to early in development when organ growth, potentially through organogenesis, was taking place.

Embryos exposed to tRA showed altered growth at the end of the developmental timeframe studied (Figures 2 and 3). Early in development, embryos exposed to a single injection of tRA on day 8 showed no significant differences compared with control and sham embryos. Moreover, when organ mass was standardized for the initial decrease in embryo wet mass, there was no difference observed in the organ growth over any time point studied (Fig. 4A and B). This suggests that although organ masses were reduced in tRA treated embryos, they experienced proportional growth over development. The data indicate that embryo growth was depressed/reduced over the developmental timeframe studied, while no specific organ measured was affected more or less upon exposure to tRA. Interestingly, it appears that embryo and kidney wet mass were more sensitive to the changing retinoic acid environment compared to dry mass as evident by the dose –
dependent decrease in both embryo and kidney wet mass on day 18 (Fig. 2A and C). Heart
wet and dry mass seemed equally affected with either treatment of tRA (Fig. 2B and 3).

Injection of tRA on embryonic day 8 may correspond to a critical period in embryo
growth or development that does not become apparent until later in the incubation period.
The idea of critical periods in development indicates that over certain periods of time, or
“windows”, the developmental path of the organ or organ system may be susceptible to
changes in the surrounding environment (e.g. Spicer and Burggren, 2003). Thus, if
embryonic day 8 corresponds to a period of rapid organogenesis and cell proliferation
(organ growth), and given the fact that tRA has been implicated in altering these processes
(Means et al., 1995), it is possible that differences in mass may not become apparent until
later in development. This would further support the idea that overall embryo growth
appeared to be depressed in the current study given the fact that organ masses, although
smaller in treated embryos, grew proportionally when standardized for embryo wet mass.
Altering the injection day could hit other critical windows effectively changing the timing
when mass differences would become apparent.

C. Retinoids, Growth Hormone, and Avian Development

Retinoids, including tRA, are classified as steroid hormones and belong to a group of
steroid hormones known as the steroid/thyroid hormone receptor superfamily (Evans,
1988). When tRA interacts with its nuclear receptor, it alters gene expression at the level of
transcription (Gudas et al., 1994; Mangelsdorf et al., 1995). Interactions between tRA and
its hormone response element within the nucleus of the cell have been shown to influence
the rate of transcription, and effectively the level of gene expression (Evans, 1988).
Retinoic acid’s impact on pituitary growth hormone production may result from a common
responsive element that could facilitate the regulation of growth hormone gene expression by retinoic acid (Bedo et al., 1989). In addition, retinoic acid increased the amount of growth hormone mRNA by the retinoic acid receptor complexes interacting with DNA sequences in the growth hormone gene, effectively producing direct control of growth hormone mRNA transcription (Bedo et al., 1989). Bedo et al.’s (1989) study did not examine the effect of higher levels of retinoic acid on the level of growth hormone mRNA. Potentially, there may be a plateau in which high levels of retinoic acid may inhibit growth hormone mRNA production. In the current study, higher levels of retinoic acid were used compared to Bedo et al.’s (1989) study. Therefore, if these higher levels of tRA alter gene expression at the level of transcription, it is likely that these changes would not become apparent until later in development, thus the mass differences that were seen in the current study (decreased proportional growth from day 16 to day 18) suggest that high levels of retinoids (tRA) may alter normal gene transcription.

Growth hormone appears in the chick embryo pituitary as early as day 4.5 of development (Thommes et al., 1987) and is secreted by at least day 14 or 16 (Porter et al., 1995). Growth hormone was not detectable (<4 ng/ml) in the embryonic chicken blood plasma until after 17 days of incubation (6 ng/ml) (Harvey et al., 1979). If embryonic development were under control of growth hormone, it would most likely be affected later in development when measurable levels are in the blood plasma. Thus the decreased overall growth in embryos dosed with tRA during late incubation (day 16 and 18) occurred at a time when growth hormone is increasing. Although growth hormone concentration or pituitary expression was not directly measured in the current study, it is a potential
mechanism that could support the suggestion that growth hormone (influenced by tRA) affected mass late in development.

D. Possible Link Between the Pituitary and Embryo Growth

Hypophysectomy (removal of the pituitary gland) at day 9 of development resulted in embryos that were markedly smaller compared to controls, in addition to multiple other abnormalities (Betz, 1967). Moreover, hypophysectomy on day 9 resulted in less retention of the yolk sac and elimination of the metabolic capacity to utilize yolk lipids (Betz, 1967). This inability to metabolize yolk lipids may be due to a deficiency of thyroid hormones, estrogens, and adrenal corticoids that would normally be secreted from the pituitary gland, allowing for normal metabolism and growth (Betz, 1967). In the current study, retinoic acid treatment may have altered the normal development of the pituitary gland, resulting in lower metabolism and overall smaller embryos. If high levels of tRA influence the amount of pituitary growth hormone mRNA, or disrupt normal pituitary growth, the reduction in mass seen in this study might be explained by an inhibition of pituitary development upon exposure to retinoic acid. Further analysis is necessary to confirm the possible effect tRA may have on the developing pituitary gland.

E. tRA Effects on Nephron Development

Both kidney wet and dry mass was affected in embryos dosed with tRA. tRA–treated embryos had smaller kidneys compared to control embryos. However, this difference was a function of embryo size since no difference was seen in the ratio of kidney to body mass between tRA–treated embryos and control embryos. tRA’s influence on the developing nephrons was assessed by a staining technique that allows for identification of actively perfused nephrons as well as an estimate of total nephrons per kidney.
Initially, exposure to 3 mg/ml tRA significantly decreased in total nephrons per milligram kidney tissue when compared to control. However, this difference did not persist over development and returned to normal for the remainder of the study period (Fig. 5A). The functional capacity of the kidneys of embryos dosed with tRA seemed to be largely unaffected since no difference was seen compared to control in perfused nephrons per milligram kidney tissue at any point in the developmental timeframe of the current study (Fig. 5B). This result is surprising considering the impact vitamin A, and its active metabolites, have on the developing kidney (Lohnes et al., 1994; Mendelsohn et al., 1994; Vilar et al., 1996). Retinoic acid increases the capacity for branching from the ureteric bud, leading to more sites for developing nephrons (Vilar et al., 1996). In addition, the levels of exogenous retinoids regulate the nephron number of the metanephric kidney in a dose-dependent manner (Vilar et al., 1996).

Examining the nephron data in the current study, it appears that the developing chicken embryonic kidney (specifically nephrogenesis) is not permanently altered when given a single dose of tRA on embryonic day 8. While the highest concentration of tRA (3 mg/ml tRA) did significantly reduce the number of total nephrons per millgram of kidney tissue on day 12, this result was only transient and did not persist over the other developmental days. Two distinct possibilities may help explain the nephron results observed in this study. Day 8 is not the critical window in nephrogenesis for the developing metanephric kidney. To that point, intrauterine growth restriction (IUGR) due to twinning leads to a reduced nephron endowment, where as late gestational IUGR does not, suggesting that reduced nephron endowment is dependent of the timing of the growth
restriction (Mitchell et al., 2004). If this is true, altering the timing of injection may elicit differences in nephrogenesis later in development.

On the other hand, day 8 may have been within the critical window of metanephric nephrogenesis, but the embryo was not exposed long enough to the retinoic acid. Recent studies focusing on the impact of tRA have primarily used metanephric organ culture (Vilar et al., 1996). In the current study, tRA was injected into the allantoic fluid so it is not clear of the exact dosing to the kidneys, nor how long tRA persisted. Potentially, if the embryo were exposed to the drug longer, total nephron number and perfused nephron number would be altered late in development (from day 16 on), when the metanephric kidney becomes the sole functioning kidney (Romanoff, 1960). Further exploration is necessary to being to sort out the critical windows in avian nephrogenesis, and how different concentrations of tRA affect the developing kidney.

II. tRA Effect on the Developing Osmoregulatory System

A. Fluid Osmolality

Whole blood and plasma osmolality remained relatively constant over development (Fig. 6A and B). Interestingly, both whole blood and plasma osmolality was significantly reduced in embryos treated with the highest dose of tRA (3 mg/ml tRA) compared to both control and sham treatment. While whole blood osmolality remained unaffected in embryos treated with 1 mg/ml tRA over development, plasma osmolality was significantly reduced on day 12 as well as day 18 (Fig. 6B). Embryos treated with 1 mg/ml tRA showed no statistical difference in blood plasma osmolality from day 14 to 16. Allantoic fluid osmolality decreased over development in both the control and sham treated embryos.
with the lowest osmolality observed on day 18 (Fig. 7A). This decrease in allantoic fluid osmolality across normal development is a result of sodium and chloride movement across the allantois into the blood (Stewart and Terepka, 1969). Consequently, the allantoic fluid osmolality becomes progressively more hyposmotic over development compared to whole blood. On the other hand, embryos treated with tRA exhibited elevated allantoic fluid osmolality towards the end of incubation at day 16 and day 18 (Fig. 7A).

Because the allantoic fluid composition is determined by the action of three major osmoregulatory systems, - kidneys, gut, and chorioallantoic membrane - it is necessary to understand how this fluid compartment changes over development (e.g. osmolality, volume, electrolyte composition) and with tRA treatment. Plasma sodium concentration remained constant over development in both the control and sham treatment averaging \( \approx 190 \text{ mmol/L} \) (Fig. 8A). Allantoic fluid sodium concentration decreased over development in both the control and sham treatments (Fig. 8B). The volume of the allantoic fluid reaches its maximum on days 12-13 of incubation, and thereafter declines in addition to a decrease in sodium and chloride concentrations in the allantoic fluid (Stewart and Terepka, 1969). This reabsorption of sodium and chloride occurs against large concentration gradients for each ion (Stewart and Terepka, 1969; Murphy et al., 1982) as well as against an electrical driving force for sodium (Graves et al., 1984).

Interestingly, allantoic fluid sodium concentration in embryos treated with tRA was significantly elevated from control on days 16 and 18, while no difference was seen between tRA treatments (Fig. 8B). Between days 12 and 19 of embryonic development, the allantoic epithelium appears to transport sodium actively, generating the short circuit current (SSC) source across the epithelium (Graves et al., 1986). Furthermore, as the
allantoic epithelium matures, the SCC (i.e., active sodium transport) increases substantially, thus serving as an important function of the allantoic sac in the late-stage embryo, i.e., the reabsorption of a hyperosmotic fluid into the embryo’s circulation (Stewart and Terepka, 1969; Murphy et al., 1982; Helms et al., 1983). It is possible that embryos dosed with tRA are unable to actively pump sodium out of the allantoic fluid. If this is the case, the driving force that normally drives a net absorption from the allantoic sac into the embryonic circulation may be altered.

Fluid in the amniotic compartment accumulates as a result of chloride being actively pumped into this compartment with water passively following (Murphy et al., 1991). Amniotic fluid remained largely constant over development in control and sham treated embryos (Fig. 7B). Embryos treated with 1 mg/ml tRA also maintained relatively stable amniotic fluid osmolality over development (not different from control at any day). However, 3 mg/ml tRA injection produced a significant decrease in amniotic fluid osmolality on day 16. This was the only day during the study in which amniotic fluid was affected by treatment with tRA (Fig. 7B). At the present time it is unclear as to why a transient decrease in amniotic fluid osmolality was observed in embryos treated with 3 mg/ml tRA.

Blood plasma potassium concentration was largely unaffected by tRA treatment, or development (Fig. 9A). Allantoic fluid potassium concentration increased from day 14 to day 16 and then plateaued for the remainder of the study period (Fig. 9B). Treatment with tRA decreased allantoic fluid potassium concentration on day 16 when compared to control. However no difference was seen between tRA doses. On day 18, no difference existed between control and treatments.
Collectively, it appears that sodium is the major ion contributing to both blood and allantoic fluid osmolality. Elevated allantoic fluid sodium concentrations in tRA treated embryos, as well as decreased blood and blood plasma osmolality indicates that sodium may be unable to move out of the allantoic fluid into the blood over development. Although ion contents of the amniotic fluid was not determined, it is likely that the mechanisms that regulated ion and fluid movement were not affected in embryos treated with tRA, thus not altering the amniotic osmolality much over development.

B. Endocrine control of Avian Osmoregulation

Endocrine control over the developing kidney and extraembryonic structures plays an important role in the osmoregulation system of the developing chicken embryo. Of the many hormones released from the pituitary, arginine vasotocin (AVT), prolactin, and growth hormone in particular exert profound effects on electrolyte and water balance in the developing embryo. Exogenous prolactin injected into chicken embryos on day 6 markedly reduces the sodium and chloride concentrations in the allantoic fluid (Murphy et al., 1986). When considering the current osmolality as well as flame photometry data in the context of the pituitary and its hormonal regulation, it might be that tRA injection on day 8 disrupts normal pituitary function or development. Considering that under normal conditions, AVT and prolactin are not present in measurable quantities until late in development (day 16 or later) (Harvey et al., 1979; Nouwen et al., 1984; Klempt et al., 1992; Muhlbauer et al., 1993) it is likely that these hormones will not have significant influence over the embryonic structures that are important for osmolality and electrolyte control until, at the earliest, day 16. Interestingly, allantoic fluid osmolality and sodium ion concentration in embryos treated with tRA showed no difference until day 16 and after of
development (presumably when under normal conditions the pituitary would assume regulatory roles of the allantois and metanephric kidney).

Further support for the pituitary hormonal influence on the hydromineral state in the developing embryo has come from extensive studies examining the effect of hypophysectomy (removal of the pituitary gland) in embryonic chickens (Fugo, 1940; Betz, 1975; Doneen et al., 1981a). Hypophysectomized embryos were unable to maintain a high sodium and chloride gradient between the allantoic fluid and blood, and showed elevated sodium ion concentration in the allantoic fluid (Doneen and Smith, 1982a). This further supports the suggestion that tRA dosed embryos may be lacking normal pituitary “input” late in development; especially to the allantoic epithelium and metanephric kidney.

While measuring pituitary growth or function, as well as hormones released from the pituitary are outside the scope of this project, further study is necessary to assess what role tRA may have on the developing chicken pituitary gland.

Although there is convincing evidence for the role of the pituitary in regulating the allantoic epithelium (Doneen and Smith, 1982a, b) what direct influence, if any, do the pituitary hormones have on the kidney. To this end, it is a reasonable assumption that the concentrations of sodium and chloride in the allantoic fluid might be inversely related to the activity of the metanephric sodium – potassium – ATPase since the kidney filtrate accumulates in the allantois. Better sodium reabsorption from the filtrate (via active transport) by the kidney, will result in less sodium movement into the allantoic fluid.

Prolactin injection into embryonic chickens from day 6 stimulates metanephric sodium – potassium – ATPase activity (Doneen and Smith, 1982a, b). On the other hand, if prolactin is not present (due to decreased pituitary activity), the metanephric sodium – potassium –
ATPase’s may not be able to recover as much sodium from the filtrate compared to normal embryos (where pituitary function is normal). Further, the elevated allantoic sodium ion concentration and osmolality seen from day 16 to day 18 in tRA treated embryos suggests that tRA may influence the metanephric kidney’s ability (either directly, or indirectly through the pituitary) to regain sodium from the filtrate as it passes through the kidney.

It cannot be determined directly if the elevated sodium ion concentration in the allantoic fluid of embryos treated with tRA is a function of reduced metanephric sodium – potassium – ATPase activity, disruption of the active transport properties of the allantoic epithelium, or a change in the volume of the allantoic fluid compartment. Results from the current study suggest that tRA may influence the development or function of the pituitary gland in developing chicken embryos. Future studies are necessary to clarify the possible impact tRA may have on the pituitary gland and its hormonal regulation of the hydromineral balance in the developing chick embryo.

III. tRA’s Influence on Hematology and Blood Chemistry

A. Hematology

Hematocrit and red blood cell count were significantly elevated from day 12 to day 16 in embryos treated with 3 mg/ml tRA (Fig. 10A and B). Additionally, 1 mg/ml tRA elevated both red blood cell count and hematocrit on day 12 but not on any other day studied. The exact mechanism by which tRA influenced both the hematocrit and red blood cell count was not assessed in the current study. Oral administration of retinoic acid to vitamin-A depleted rats elevated the erythropoietin serum concentration (Okano et al., 1994). Moreover, this increase in erythropoietin was due to the accumulation of
erythropoietin mRNA (Okano et al., 1994). Thus, it is likely that tRA has some erythrocyte-stimulating properties and could produce increased hematocrit and the number of red blood cells in tRA treated embryos.

Embryos treated with 3 mg/ml tRA had elevated hemoglobin concentration over development (Fig. 10C). Elevated hemoglobin concentration would also lend support for the rise in hematocrit, in addition to the increased red blood cell count. Further studies need to be completed to tease apart the possible mechanisms that would cause the results seen in this current study.

B. Blood Chemistry

Embryonic acid base balance was maintained for the duration of development (Fig. 11A and B). The kidney maintains alkaline pH through the conservation of base (bicarbonate) and excretion of acid (Hydrogen ion (H\(^+\))). Secreted H\(^+\) ions combine in the tubular lumen with filtered bicarbonate to form CO\(_2\) that diffuses into the tubule cells (Laverty, 1989). Bicarbonate reabsorption in the avian kidney likely occurs in the distal part of the nephron, the area in which activity of carbonic anhydrase is high (Laverty, 1989). In addition, avian renal H\(^+\) secretion takes place in part by sodium/hydrogen ion exchange (Laverty, 1989). More than likely the many different parts of the nephron contribute to different physiological processes for the developing embryo.

IV. Summary

The current study was undertaken to evaluate the effects of tRA on the developing embryo and the developing osmoregulatory system. Embryo development was depressed by treatment with tRA while total nephron number and perfused nephrons were largely unaffected. While nephron numbers were not reduced as was anticipated upon treatment
with tRA, the osmoregulatory consequences may not be a function simply of nephron numbers. This is evident by increased allantoic fluid osmolality in all embryos treated with tRA, and a reduction in whole blood and plasma osmolality in embryos exposed to the higher dose (3 mg/ml) of tRA. Although beyond the reach of this study, it is possible that tRA altered the developmental trajectory of the developing pituitary gland, supported by depressed overall embryonic growth, fluid compartment osmolality differences, and electrolyte imbalances seen in tRA treated embryos. In addition, it is also possible that tRA affected the metanephric kidney sodium – potassium – ATPase activity. Since the metanephric kidney continues to develop post-hatching, and eventually becomes the adult kidney, this study could be easily extended into the post hatching period. This would allow us to expand on the idea of critical periods in development and fetal programming. The embryonic environment is very dynamic and changes during that environment may have long lasting consequences on the organism well into adult hood.
FUTURE DIRECTIONS

Since it has been established that all-trans retinoic acid (tRA) influences development (growth) of the embryonic chicken, a number of questions arise. For example, would tRA injections either before or after embryonic day 8 produce smaller embryos towards the end of incubation (day 16-18)? This is an interesting question regarding potential critical windows in embryonic development. While most of the literature has focused on retinoids, and tRA’s influence early in development (De Luca, 1991; Spron et al., 1994; Means et al., 1995), this is the first to look at tRA’s effect on embryo growth later in development. Interestingly, all of the differences in embryo and organ mass did not become apparent until 8 days after injection of tRA. At present, it is unclear if tRA influenced normal organogenesis and cell proliferation, or if the maturation of the embryo and organs was affected. Identifying how exactly tRA altered the normal developmental trajectory of the embryo would lend tremendous insight into the possible consequences on early fetal exposure to high levels of vitamin A, and its active metabolites, especially tRA.

Surprisingly, in the current study, tRA treated embryos did not show altered nephrogenesis. Considering that tRA has influenced metanephric kidney nephrogenesis (Lohnes et al., 1994; Mendelsohn et al., 1994; Vilar et al., 1996), it was expected that metanephric kidney nephrogenesis would have been changed. It is possible that the developing kidney is not sensitive to a changing retinoic acid environment on embryonic day 8. Thus, it is necessary to vary the day of injection and assess the developing kidney (specifically nephrogenesis). tRA is a good therapeutic approach to begin assessing fetal programming because the developing metanephric kidney continues to develop 30 days post hatch (Wideman, 1989), and will become the adult kidney. Studies that include post
hatch measurements are necessary to see how changes early in kidney development present themselves post-hatch. In addition, is the hatchling able to “correct” the reduced nephron endowment at birth and regain normal levels of nephrons once nephrogenesis is complete? Examining the effect of an early insult to nephrogenesis post-hatch not only allows us to see what consequences, if any, result from such an insult, but whether the hatchling is able to overcome such a deficiency. If the hatchling is able to overcome the nephron deficit, it may give insight into developmental plasticity and how it may relate to the developing kidney.

Although no changes were observed in metanephric kidney nephrogenesis, the developing osmoregulatory system in tRA treated embryos was significantly influenced. While the kidney is an important player in the osmoregulatory capabilities of the embryo, the extraembryonic structures, especially the CAM and the allantois work in combination with the kidney to maintain internal volume, osmolality and electrolyte homeostasis. Interestingly, much of the research focusing on the endocrine control of the developing osmoregulatory system point to the pituitary gland and its secreted hormones as a key regulator of the hydromineral state of the embryo (Betz, 1967; Doneen et al., 1981; and Thommes and Woods, 1993). Hypophysectomized embryos (removal of the pituitary), exhibit a number morphological and physiological differences from chicken embryos that have a functional pituitary gland. For instance, hypophysectomized embryos exhibit elevated allantoic fluid sodium concentrations (Doneen and Smith, 1982(a)) and reduced embryonic growth (Betz, 1967). In the current study, results indicate that embryos injected with tRA may have non-functional or a functionally depressed pituitary gland since these embryos exhibited elevated allantoic fluid osmolalities, increased allantoic fluid sodium
concentration, and depressed growth. In addition, none of these changes become apparent until the end of the study period (16-18), presumably when the pituitary gland would assume humoral control over the osmoregulatory system. Further study is needed to assess what affect tRA may have on the developing embryonic chicken pituitary.

Finally, this study has largely focused on understanding renal physiology through a series of morphological and physiological measurements during embryonic development. The majority of this work focuses solely on the kidney with little analysis of the developing heart. Incorporating heart rate and blood pressure measurements will help expand this study beyond the scope of just renal development. However, the heart and or the kidney may not only change structurally or functionally, it may also change on a molecular basis. Looking at kidney and heart development through a molecular lens would complement the work presented here. Monitoring protein expression changes throughout development in both the heart and kidney, and comparing the global protein expression between control and experimental kidneys. These types of analyses are useful because it may help to place the morphology and physiology that is presented here in more of a complete developmental frame-work by integrating not only physiology, but also molecular techniques.
REFERENCES


**Atwell, W. J. and Hanan, E. B.** (1926). The time during which the mesonephros and the metanephros of the developing chick are able to store trypan blue. *Anat. Rec. 32*, 228.


on a 10-mm. human embryo exhibiting unilateral renal agenesis. The Anatomical Record. 52, 325-349.


(2003). Glomerular number and size in autopsy kidneys: The relationship to birth


really an inverse association between birthweight and subsequent blood pressure?

Press.

arginine vasotocin secretory responses to osmotic stimulation in the chick


Osmoregulation* (ed. M.R. Hughes and A. Chadwick), Leeds Philosophical and
Literary Society, Leeds, United Kingdom.


Physiol. 285, R962-R970.


