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Metanephric kidney development in the chicken embryo: Glomerular numbers, characteristics and perfusion

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ABSTRACT

The developing metanephric kidneys and chorioallantoic membrane (CAM) work in unison to ensure ion and water homeostasis in the avian embryo within its egg. This study focused on how avian renal structure and glomerular perfusion change in concert during development, as well as on changes in body fluid compartment osmolalities. White leghorn chicken eggs were incubated at 37.5 °C and 55–60% relative humidity and were examined during days (D) 10–18 of development. Alcian blue, a stain that forms solid aggregations in actively perfused glomeruli of the metanephric kidney, was used to identify the proportion of glomeruli actually perfused. Total nephron number increased from 4705 ± 1599 nephrons/kidney on day 12 to 39,825 ± 3051 nephrons/kidney on day 18. Actively perfused nephrons increased ~23-fold from 761 ± 481 nephrons/kidney on day 12 (~16% of total nephrons) to 17,313 ± 2750 nephrons/kidney on 18 (~43% of total nephrons). Glomerular volume increased from days 12 to 14, remaining constant thereafter. Blood and cloacal fluid osmolality ranged from 270 to 280 mOsm/L. Amniotic fluid osmolality changed in a complex fashion during development but was comparable to blood on days 10 and 18. Allantoic fluid had the lowest osmolalities (175–215 mOsm/L) across development. Uric acid increased steadily within the allantoic fluid compartment, from 36 ± 1 mmol/L to 63 ± 4 mmol/L. The avian metanephric kidney thus shows a dramatic increase in both recruitment of nephrons and potential filtering capacity during the last half of incubation, in preparation for the degeneration of the allantoic membranes prior to internal piping and subsequent hatching.

1. Introduction

The regulation of water and ion balance in avian embryos involves a balance between water loss occurring through evaporation from the shell and water gain occurring from the accelerating rate of metabolism of yolk lipids as development progresses. The net effect of these two water fluxes in chicken embryos is in an overall loss in egg mass of approximately 12% during the incubation period (Lundy, 1969). Eggs losing too much or too little water exhibit lower hatchability (Davis et al., 1988). Early in development, the embryo depends on the chorioallantoic membrane (CAM) to regulate ion and water balance, but the mesonephric kidney, progressively replaced by the metanephric kidney, assumes increasingly important roles in water balance, ion regulation and nitrogenous waste excretion as development proceeds (for a review, see Gabrielli and Accili, 2010).

The CAM comprises amniotic and allantoic compartments. Amniotic fluid is contained within the amnion, made up of ectoderm and avascular mesoderm lying adjacent to the embryo (Baggott, 2001). By embryonic day 5, the amniotic sac has formed with a contractile outer layer of mesoderm that fuses to the vascular mesoderm of the allantois. The sero-amniotic connection forms on day 12 as a duct between the amniotic sac and albumen to allow the movement of proteins to the embryo (Baggott, 2001). The allantoic sac begins to expand on day 3.5, reflecting the early onset of mesonephric kidney function. This early filtrate only aids water regulation and the removal of waste and also contributes to the rapid expansion of the allantoic sac (Romanoff, 1967). Between days 5 and 7, the volume of the allantoic sac increases 6-fold, corresponding with both differentiation and growth in size of newly forming nephrons of the metanephros combined with retained mesonephros function (Friebova-Zemanova et al., 1982). Allantoic fluid, in addition to being a repository for waste materials, also serves the embryo as a water reservoir for embryonic hydration. Collectively, the allantoic and amniotic compartments of the CAM serve as sources/sinks for both fluid and electrolytes (Graves et al., 1986; Hoyt, 1979).

As the avian embryo continues to develop, the kidneys begin to develop in a process similar to that occurring in mammals and reptiles. The pronephros emerges first out of surrounding mesoderm but is then eclipsed during embryonic growth by the growth of mesonephros, which begins differentiating around embryonic day 3 (Romanoff, 1960). The mesonephros appears morphologically on days 3–4, functions from days 5 to 11 and degenerates about day 15 as it is replaced by the metanephros, or "definitive kidney," which further differentiates into the permanent functional kidney. Ultrastructural and biochemical observations of the metanephric kidney on embryonic day 12 reveal an active fluid resorptive process by

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way of fully differentiated renal corpuscles and proximal convoluted tubules (Narbaitz and Kacew, 1978). The so-called "mammalian-type" and "reptilian-type" nephrons evident in adult birds are found in the embryonic metanephros. Interestingly, mammalian-type nephrons, characterized by more extended tubular loops and larger glomeruli, are the first to form (Narbaitz and Kacew, 1978; Wideman, 1989). These are followed by reptilian-type nephrons, characterized by typically shorter loops and smaller glomeruli, which do not replace but rather add to the adult configuration of nephron types evident at hatching.

In addition to a long-standing appreciation of the changing nephron types during avian development, the gross morphology of the developing avian embryonic kidney has been well documented (Klusonová and Zemanová, 2007). However, very limited information is available regarding developmental changes in actual nephron numbers in embryonic birds. Do all nephrons of each type occur concurrently and then grow by hypertrophy, or are nephrons added as development proceeds? Are all nephrons perfused soon after their first appearance, or might they be intermittently perfused (and thus intermittently functional) as in adults of other vertebrates (Yokota et al., 1985)? Uncertainty also surrounds how nephron numbers correlate with potential changes in embryonic regulation of water and salt balance and how changes in nephrons numbers might actually influence embryonic kidney performance. This study then explores metanephric kidney development during the second half of development of the chicken embryo and correlates morphological and physiological renal changes with osmotic characteristics of the embryo's main fluid compartments. We hypothesize that the nephron number will parallel metanephric kidney growth, and that all nephrons are potentially active in plasma filtration.

2. Materials and methods

2.1. Source and Incubation of Eggs

Fertilized white leghorn eggs (*Gallus gallus domesticus*) were obtained from Texas A&M University (College Station, TX, USA) and shipped to University of North Texas (Denton, TX, USA). Upon arrival eggs were weighed, individually marked and then randomly chosen for placement within one of three Hova–Bator incubators, which were ventilated with fresh air at 55–60% relative humidity (RH) and temperature of 37.5 °C ± 0.5 °C. Humidity was measured using wireless Baro-Thermo-Hydrometers (model BTHR968, Oregon Scientific).

Eggs were weighed on incubation days 10–18, corresponding to stages 36–44 (Hamburger and Hamilton, 1951). Loss in mass during development was calculated as the difference between pre-incubation and experimental day whole egg mass. On embryonic days 10, 12, 14, 16 and 18, eggs were removed from each incubator for experimental analyses (*n* values are provided in Results section).

All experimental procedures were approved by The University of North Texas' Institutional Animal Care and Use Committee (IACUC).

2.2. Kidney Harvesting

All embryos were sacrificed by injection of 100 μL pentobarbital sodium (50 mg/mL) into a CAM vein exposed through removal of a small portion of eggshell. The embryo was removed from the shell and separated from the extra-embryonic membranes, after which body wet weight was measured. The abdominal cavity was opened, and all organs on the ventral-side were removed first, revealing the mesonephros and metanephros, which were separately identified. Subsequently, the mesonephros with the gonads were removed. The extracted metanephric kidneys (hereafter termed simply "kidneys") were separated from the gonads, and their wet weight was measured after careful removal of surface fluid through blotting with Kimwipes® (Kimtech Science). Kidneys were then placed on aluminum foil squares and dried for 24 h at 70 °C prior to determining dry weight.

Kidneys to be used for light microscopy analysis were placed into 10% neutral buffered formalin with a pH of 7.2 at 4 °C for ~12 h. Kidneys to be used for glomerular distribution analysis were placed in 50% ethanol at 4 °C overnight.

2.3. Glomerular perfusion assessment

One objective of this study was to identify the maximum number of potentially functional glomeruli at any given point in chicken embryo development. While we did not assess actual glomerular filtration rate in the embryos in this study, glomeruli that were not perfused were *ipso facto* not creating urine, so the ratio of maximum possible perfused to total glomeruli was a key index of glomerular functionality.

2.3.1. Dye injection and staining of perfused glomeruli

Eggs were candled to locate a CAM vein, the location of which was marked on the shell with a pencil. To maintain temperature of the embryo during the experiment, the pointed end of the egg was buried to a depth of ~3 cm into clean quartz sand maintained at 37 °C. A 1-cm square piece of egg shell was then removed over the previously located CAM vein, which was then cannulated with a bent 30 gauge needle connected to PE-50 tubing. The cannula was secured to the surface of the eggshell with putty, to eliminate vessel movement during the injections.

To stain individual glomeruli that were actually being perfused following mannitol stimulation, we employed the Alcian blue staining technique previously established as suitable for assessing glomerular perfusion in chickens (Wideman et al., 1987). Alcian blue is a large, positively conjugated dye molecule (Scott et al., 1964). Cationic isothiouronium groups in Alcian blue are thought to bind via electrostatic interactions to the anionic sulfate and carboxylate groups located within the carbohydrate moieties of mucin. Alcian differentially accumulates and stains the glomerular tuft, an aggregation of glomerular capillaries sitting within Bowman's capsule (Fig. 1A). Key to the experiments, such staining, will occur in perfused nephrons potentially capable of ultrafiltration, but not in non-perfused and therefore non-functional nephrons (Wideman et al., 1987). Importantly, Alcian blue not only visibly accumulates within the glomerular tuft of each perfused nephron, but given sufficient injected concentration and time for ultrafiltration, the aggregation actually forms a stable "micro-pellet" within the glomerular tuft of each nephron that is not disrupted by homogenation of the kidney tissue. The concentration of such micro-pellets can be used as a quantitative tissue perfusion marker, analogous to methodologies of using entrapped microspheres in the microcirculation (for a review, see Robertson and Hlastala, 2007). The advantage of this technique over the visual examination of individual histological slides is that with Alcian blue staining the average concentration of perfused glomeruli can be easily determined for the entire kidney from a single procedure. This is in contrast to histological examination, which would have required separate examination of large numbers of individual specific kidney regions within the field of view of a microscope, with the tenuous assumption that such spatial sampling represents the characteristics of the kidney overall.

Mannitol was first administered as an osmotic diuretic, acting indirectly to temporarily maximize nephron perfusion, after established protocols for avian kidney assessment (Bankir and Hollenberg, 1983; Unflat et al., 1985). An injection of 2.5% mannitol in saline comprising 3% of total blood volume (embryo wet weight ×0.15, after Rychter et al., 1955) was injected over a 1-min period via a glass Hamilton syringe through the cannula. Ten minutes after mannitol treatment, a bolus of 0.2% Alcian blue dye in saline (3% blood volume) was injected at the same rate as the mannitol injection. After 30 min of dye circulation, shown by pilot experiments to be sufficient for Alcian blue