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Hematocrit and blood osmolality in developing chicken embryos (*Gallus gallus*): *In vivo* and *in vitro* regulation

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ABSTRACT

Hematocrit (Hct) regulation is a complex process involving potentially many factors. How such regulation develops in vertebrate embryos is still poorly understood. Thus, we investigated the role of blood pH in the regulation of Hct across developmental time in chicken embryos. We hypothesized that blood pH alterations *in vitro* (i.e., in a test tube) would affect Hct far more than *in vivo* because of *in vivo* compensatory regulatory processes for Hct. Large changes in Hct (through mean corpuscular volume (MCV)) and blood osmolality (Osm) occur when the blood was exposed to varying ambient temperatures (T_a's) and P_{CO_2} *in vitro* alongside an experimentally induced blood pH change from ~7.3 to 8.2. However, homeostatic regulatory mechanisms apparently limited these alterations *in vivo*. Changes in blood pH *in vitro* were accompanied by hydration or dehydration of red blood cells depending on embryonic age, resulting in changes in Hct that also were specific to developmental stage, due likely to initial blood gas and $[HCO_3^-]_v$ values. Significant linear relationships between Hct and pH ($Hct/\Delta pH = -21.4\%/(pH\ unit)$), Hct and $[HCO_3^-]$ ($\Delta Hct/\Delta[HCO_3^-] = 1.6\%/(mEq\,L^{-1})$) and the mean buffer value ($\Delta[HCO_3^-]/\Delta pH = -13.4\ (mEq\,L^{-1})/(pH\ unit)$) demonstrate that both pH and $[HCO_3^-]$ likely play a role in the regulation of Hct through MCV at least *in vitro*. Low T_a (24 °C) resulted in relatively large changes in pH with small changes in Hct and Osm *in vitro* with increased T_a (42 °C) conversely resulting in larger changes in both Hct and Osm. *In vivo* exposure to altered T_a caused age-dependent changes in Hct, demonstrating a trend towards increased Hct at higher T_a. Further, exposing embryos to a gas mixture where $P_{CO_2} = 5.1$ kPa for >4 h period at T_a of 37 or 42 °C also did not elicit a change in Hct or Osm. Presumably, homeostatic mechanisms ensured that *in vivo* Hct was stable during a 4–6 h temperature and/or hypercapnic stress. Thus, although blood pH decreases (induced by acute T_a increase and exposure to CO_2) increase MCV and, consequently, Hct *in vitro*, homeostatic mechanisms operating *in vivo* are adequate to ensure that such environmental perturbations have little effect *in vivo*.

1. Introduction

Hematocrit (Hct) is a key factor influencing blood rheology and blood distribution within the various vascular beds of the circulation. Thus, regulation of Hct is of critical importance in the ultimate effectiveness of the perfusion and oxygenation of tissues. How Hct changes in response to *in vivo* blood chemistry perturbations is currently poorly understood in adult vertebrates. Further, how Hct regulation first appears and matures in the embryo, particularly during ontogeny when other interrelated systems (e.g., blood acid–base, metabolic, respiratory) are still developing, is largely unknown.

Hct is a product of mean corpuscular volume (MCV) and red blood cell concentration ([RBC]). Changes in blood pH and $[HCO_3^-]$ can alter MCV (and hence, Hct) by influencing Donnan equilibrium mechanisms and Na^+, K^+-ATPase activity (see Hoffmann et al., 2009; Nikinmaa, 1992 for review). In the ectothermic bird embryo, blood pH can be altered indirectly via changes in ambient temperature (T_a) or P_{CO_2} (Tazawa, 1973, 1982; Tazawa et al., 1981; Tazawa and Ono, 1974; Wangensteen et al., 1970/1971; Wangensteen and Weibel, 1982). With decreased blood pH (respiratory acidosis) Hct (measured at 24 h) increased in day 17 (d17) chicken embryos through increased MCV rather than increased erythropoiesis, demonstrating that embryonic Hct potentially responds to changes in P_{CO_2} (and thus pH) through changes in MCV (Tazawa et al., 1988). Day 19 chicken embryos additionally increase erythropoiesis concomitant with respiratory acidosis and deficiency of oxygen (Tazawa et al., 1988) suggesting that the response of Hct to altered P_{CO_2} (pH) in chicken embryos differs according to developmental stage, an observation driving the current investigation.

Accordingly, we hypothesize that *in vivo* Hct is regulated acutely by altered T_a and P_{CO_2} (and consequently pH) due to changes in MCV. Additionally, we hypothesize that the response of Hct to altered T_a may be age-dependent and associated with changes in

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blood osmotic pressure or osmolality (Osm). This study is designed to elucidate both the *in vivo* and *in vitro* effects and underlying mechanisms of altered P_{CO_2} and T_a on pH, Osm, and Hct of blood collected from developing chicken embryos. Homeostatic mechanisms *in vivo* should tightly regulate MCV so that changes in Hct are minimal as RBC's are circulated around the body, whereas outside the body the RBC's are directly susceptible to alterations in pH. It is thus expected that inducing pH changes via CO_2 exposure *in vitro* will have a greater effect on Hct than *in vivo*.

2. Materials and methods

2.1. Incubation of eggs and blood collection

Fertile eggs of the domestic fowl (*Gallus gallus domesticus*, layer strain) were obtained from Texas A & M University (College Station, TX, USA). Eggs were weighed (±0.01 g), then incubated at a temperature of 37.5 ± 0.1 °C and relative humidity of ~55% (Incubator: 1502, G.Q.F. Manuf. Co., USA). Individual embryos were removed from the incubator on d11 through 18, and their hematological variables measured as described below. On the target developmental day (one of d11, 13, 15, 17, 18), blood was collected from the allantoic artery, which had been located by candling one day previously. A 6–8 mm diameter region of the eggshell was removed and the underlying allantoic artery gently lifted by forceps through the hole in the eggshell. The blood vessel was punctured for sampling by a 25 gauge needle mounted on a 1 mL sampling syringe that had been flushed with heparinized saline (100 mg in 100 mL saline). A mean volume of approximately 0.27, 0.47 and 0.70 mL of blood could be collected from d11, d13 and d15–18 embryos, respectively. Blood from two d11 embryos and in some cases two d13 embryos was pooled to meet the required volume of at least 0.5 mL of blood necessary for two blood analyses with intervening tonometry. Sampled eggs were then euthanized via exposure to a cold and anoxic environment for 2 h and the embryos were removed from their eggshell, following confirmation of cardiac arrest. The yolk and extra-embryonic membranes were then removed, and the embryo's body mass measured (±0.01 g) with an electronic balance. When blood from two individuals was pooled, the egg and body masses of the same embryos were similarly averaged and treated as a single datapoint.

2.2. In vitro effects of altered P_{CO_2} and T_a on Hct

Each hermetically sealed syringe containing sampled blood was inverted several times to ensure sample mixing. Then 0.12 mL of blood was immediately measured for pH, P_{CO_2} and $[HCO_3^-]$ (calculated by the analyzer from pH and P_{CO_2}) with a blood gas analyzer (ABL5, Radiometer Medical A/S) at 37 °C. Duplicate preparations of 0.06 mL of blood were transferred into sealed hematocrit tubes and centrifuged for 4 min at 10,000 rpm and the mean % Hct determined (±0.1%, READACRIT Centrifuge, Becton Dickinson). The pH, P_{CO_2}, $[HCO_3^-]$ and Hct determined at 37 °C immediately after blood collection from the allantoic artery were identified as "venous values" of individual parameters; i.e., pH_v, Pv_{CO_2}, $[HCO_3^-]_v$ and Hct_v (Note that allantoic arterial values represent mixed venous values, since an allantoic artery is analogous to an artery in the pulmonary arterial circulation) (Piiper et al., 1980).

After ensuring the blood sample was well mixed, the remainder of each sample was then transferred into a tonometer consisting of a glass, concave-ended vial (5.5 cm height, 2.8 cm diameter) with inlet and outlet conduits to allow gas mixtures to pass through the sample. The tonometer was sealed with a rubber lid, and placed on the lab bench at ~24 °C until the blood of 5–6 embryos had been collected and the initial Hct and blood gas variables determined, a process requiring ~1 h. The blood in 5–6 separate tonometers was then equilibrated with one of the following gas mixtures: air (P_{CO_2} = 0.7 kPa), P_{CO_2} = 2.9 kPa or P_{CO_2} = 5.1 kPa (with 20.6 kPa O_2, N_2 balance) at 24 °C, 37 °C or 42 °C. For equilibration with P_{CO_2} = 2.9 and 5.1 kPa gas mixtures (supplied by pre-mixed gas cylinders; Air Liquide), tonometers were vented with the gas mixtures for 5 min in the waterbath. They were then sealed to avoid water evaporation from the blood and the samples were shaken within the tonometers ensuring that a thin layer of blood coated the walls allowing for equilibration for the remaining 85 min. Preliminary data collected using blood samples from embryos on d11, 13, 15, 17 and 18 of incubation (N = 10–12 for each group) demonstrated that changes in Hct occurred after 30 min to 1 h of equilibration and reached plateau after 1 h at each measured T_a (24 °C, 37 °C and 42 °C), thus, values are reported after an equilibration time of 1.5 h. After 1.5 h, the tonometer was removed from the waterbath and the blood immediately assessed for pH, P_{CO_2} and $[HCO_3^-]$ at 37 °C; referred to as pH_{eq}, $P_{CO_2(eq)}$ and $[HCO_3^-]_{eq}$ and Hct_{eq} (again measured in duplicate and the mean determined). Further, values were expressed as ΔpH ($= pH_{eq} - pH_v$) and $\Delta[HCO_3^-]$ ($= [HCO_3^-]_{eq} - [HCO_3^-]_v$). In presenting changes in Hct (ΔHct), the *in vitro* change from *in vivo* arterial value ($Hct_{eq} - Hct_v$) was normalized by individual Hct_v and expressed by percent change, $\Delta Hct = 100 \times (Hct_{eq} - Hct_v)/Hct_v$), to emphasize even small changes in Hct. The absolute changes in Hct can be obtained by multiplying the fractional Hct_v value. The blood equilibrated at 24 °C or 42 °C was corrected for pH determined at 37 °C by the factor of −0.016 pH unit/°C (Dejours, 1981; Howell et al., 1970); i.e., +0.21 for 24 °C blood and −0.08 for 42 °C blood. Blood samples from 12 embryos were measured at each T_a (24 °C, 37 °C and 42 °C), at gas mixture (P_{CO_2} = 0.7, 2.9 and 5.1 kPa) and developmental stage (d11, 13, 15, 17 and 18). This protocol resulted in 9 different treatment groups on 5 development days with a total of 540 embryos.

Osm was measured for the blood samples of an additional 148 embryos in total on d13, 15 and 17 of incubation. Collected blood was measured for blood gas variables and equilibrated with one of three gas mixtures (P_{CO_2} = 0.7, 2.9 and 5.1 kPa) at one of three T_a's (24 °C, 37 °C and 42 °C) with the tonometer. Following subsequent determination of blood gas variables and Hct, Osm was determined on 0.01 mL of blood using a vapor pressure osmometer (Vapro 5520, Wescor).

2.3. In vivo responses of Hct to altered T_a and P_{CO_2}

The *in vivo* responses of Hct to altered T_a were examined in d11, 13, 15, 17 and 18 embryos. Preliminary experiments indicated that egg temperature equilibrated with T_a after approximately 3–5 h and the protocol was designed ensuring that embryo body temperature had reached steady state. Thus, embryos were exposed to the experimental conditions for at least 4 h with no exposure time exceeding 6 h. On the experimental day, embryos were divided into 4 treatment groups:

- group 1: control embryos incubated continuously at T_a of 37.5 °C,
- group 2: embryos exposed to T_a of 24 °C for >4 h,
- group 3: embryos exposed to T_a of 42 °C for >4 h, and
- group 4: embryos exposed to T_a of 42 °C for 4-h with subsequent exposure to 37.5 °C for another >4 h.

Eggs in group 2 were transferred from a 37.5 °C-incubator to room temperature of ~24 °C. Eggs in groups 3 and 4 were placed into an incubator at 42 °C and half of them (group 4 eggs) were returned to the 37.5 °C-incubator (after 4 h of 42 °C exposure) for a further >4 h. After exposure to altered or control T_a, approximately 0.2 mL of blood was collected and Hct determined as outlined for