Changes in Acid-Base Balance and Related Physiological Responses as a Result of External Hypercapnia During the Second Half of Incubation in the Chicken Embryo

N. Everaert,*1 L. De Smit,* M. Debonne,* A. Witters,* B. Kamers,† E. Decuypere,* and V. Bruggeman*

*Department of Biosystems, Division Livestock-Nutrition-Quality, Katholieke Universiteit Leuven, Kasteelpark Arenberg 30, 3001 Heverlee, Belgium; and †Department of Biosystems, Division Mechatronics, Biostatistics and Sensors, Katholieke Universiteit Leuven, Kasteelpark Arenberg 30, 3001 Heverlee, Belgium

ABSTRACT This study investigated the effect of high CO2 (4%) from embryonic day (ED)10 until ED16 on the acid-base balance and related parameters in the chicken embryo. From ED10 to ED16, blood was taken from a vein from the chorioallantois membrane and was analyzed for pH, partial pressure of CO2, partial pressure of O2 (pO2), [HCO3−], [K+], and [Ca2+]. Allantoic fluid was taken for measurement of pH, NH3-N, phosphate, and calcium concentration. The right tibia was ashed, and calcium was measured with atomic absorption spectroscopy. Embryos exposed to high CO2 showed a consistent higher blood pH than control embryos. Notwithstanding this alkalosis, bicarbonate concentration was significantly higher in the CO2 group from ED12 until ED16. Potassium concentration in the blood was significantly higher in the CO2 group from ED11 until ED16. The pH of the allantois was significantly higher on ED14 and ED15. Ammonia N concentration was significantly higher in the CO2-incubated embryos on ED12 and ED13, whereas phosphate did not differ between groups. Calcium per tibia dry weight did not differ between incubation conditions. We can conclude that embryos adapt to high CO2 during the second half of incubation by increasing blood HCO3−. It appears that this increase in HCO3− is mainly the result of the stimulated intracellular exchange of H+ with K+, although temporary reabsorption of HCO3− by the kidney cannot be excluded.

Key words: high-carbon dioxide incubation, acid-base balance, potassium, calcium, allantois

INTRODUCTION

In a previous study, the effect of 4% CO2 during the second half of incubation on embryonic development, the hatching process, and posthatch performance in a modern commercial broiler line was determined (Everaert et al., 2007). This tolerance to high CO2 suggests that the chicken embryo has adaptation mechanisms to counteract the imposed hypercapnia. Earlier studies showed already that there is an important rise in bicarbonate ions ([HCO3−]) when chicken embryos are incubated under high CO2 levels. Tazawa et al. (1971a) coated eggs at the beginning of incubation with epoxy cement, hereby creating an accumulation of CO2 and an O2 deficit in the egg. From incubation d 10 until incubation d 18, the increase in blood partial pressure of CO2 (pCO2) of the coated group resulted in an increased concentration of HCO3− together with a decrease in blood pH. Dawes and Simkiss (1971) exposed White Leghorn eggs to 9% CO2 from the ninth incubation day and compared the acid-base blood parameters with normal incubated eggs. Blood pCO2 and HCO3− levels increased during development and were higher in the CO2 group compared with the controls. Blood pH remained relatively stable during normal development; exposure to high CO2 resulted only in a small decrease (0.1 units) in blood pH. The CO2 embryos showed less excretion of protons into the allantoic fluid than did the control group. Because there was no evidence that the high amount of bicarbonate ions was the result of some renal mechanism, it was postulated that the increased concentration of HCO3− would be the result of an increased resorption of eggshell minerals (CaCO3), thereby releasing more Ca2+ and HCO3− into the blood. Measurements of calcium of the whole embryo could, however, not confirm their hypothesis (Dawes and Simkiss, 1971). Until now, this reason of rise in bicarbonate ions as a consequence of high CO2 remains to be elucidated.

The aim of this study was therefore to investigate further the embryonic adaptations to extra hypercapnia above the normal respiratory acidosis that chicken embryos develop during ontogeny. The physiological reactions and adaptations of the chick embryo itself on external hypercapnia (4% CO2 from the 10th until the 18th day of incubation)
were studied by measuring blood parameters related to acid-base balance. Moreover, measurements of allantoic pH (H\(^+\)), NH\(_4\) and phosphate concentrations were included, because changes of these parameters are an indicator of renal mechanisms.

**MATERIALS AND METHODS**

**Incubation and Experimental Design**

Seven hundred fifty Cobb eggs were incubated under standard incubation conditions (temperature of 37.8\(^\circ\)C, wet bulb temperature of 29\(^\circ\)C, turning of 90\(^\circ\)/h; incubator Pas Reform, Zeddam, the Netherlands) during the first 9 d. On embryonic day (ED)10, the experimental group (300 eggs) was put in a closed incubator, and CO\(_2\) in the incubator was controlled (input and output) to gradually rise until 2% CO\(_2\) on the 11th day. The CO\(_2\) levels continued to rise to reach 4% at ED12, and this high CO\(_2\) level was sustained until ED18. The incubation of the control eggs (300) was done in the normal ventilated incubator. The humidity in both incubators was matched based on wet bulb temperature to prevent differences in egg weight loss. Temperature, humidity, O\(_2\), and CO\(_2\) levels in the CO\(_2\) incubator were continuously measured and controlled by a computer with 2 data acquisition boards (PCI-6023E, National Instruments, Zaventem, Belgium). Software was written in the real-time module of Labview 8 PDS (National Instruments, Spring 2006). Also in the control incubator, continuous measurements were recorded through this computerized system by using the same specialized sensors as in the CO\(_2\) incubator [CO\(_2\): GMM221, Vaisala (Bonn, Germany); RH sensor: Hygrosmart S7000.1, Gefran (Olen, Belgium); temperature: Pt-100 direct 1/3 DIN, Gefran (Olen, Belgium); O\(_2\): only used in the CO\(_2\) incubator, SST Sensing, MF 010-0-LC, Honeywell (Brussels, Belgium). Oxygen in the CO\(_2\) incubator did not drop below 19.7%.

**Sampling**

From ED10 until ED16, blood was taken daily from 15 living embryos per group from the allantoic vein (O\(_2\)-rich blood) of the chorioallantois membrane. Blood sampling and blood gas analysis were done as described in Bruggeman et al. (2007). Eggs were candled to search for a living embryo per group from the allantoic vein (O\(_2\)-rich blood) of the chorioallantois membrane. Blood sampling and blood gas analysis were done as described in Bruggeman et al. (2007). Eggs were candled to search for a living embryo per group from the allantoic vein (O\(_2\)-rich blood) of the chorioallantois membrane. Blood sampling and blood gas analysis were done as described in Bruggeman et al. (2007). Eggs were candled to search for a living embryo per group from the allantoic vein (O\(_2\)-rich blood) of the chorioallantois membrane. Blood sampling and blood gas analysis were done as described in Bruggeman et al. (2007). Eggs were candled to search for a living embryo per group from the allantoic vein (O\(_2\)-rich blood) of the chorioallantois membrane.

**Statistical Analysis**

The data were processed using the statistical software package SAS version 8.2 (SAS Institute Inc., Cary, NC). A GLM was used to analyze the effect of embryonic age and incubation condition (normal or CO\(_2\)-incubated) on pH, pCO\(_2\), pO\(_2\), [HCO\(_3\)]\(^-\), [K\(^+\)], and [Ca\(^{2+}\)] of the blood and on pH, ammonium, phosphate, and calcium concentration of the allantoic fluid from ED11 to ED16. Calcium per dry tibia weight was measured by atomic absorption spectrophotometry (Solaar 969, atomic absorption spectrometer, Thermo Optek, Bornem, Belgium) within a range of 0 to 25 mg/L. The amount of calcium present in the tibia was expressed relative to dry tibia weight (mg of Ca\(^{2+}\)/g of dry tibia weight).

**RESULTS**

From ED10 until ED16, pH of the blood of the allantoic vein from the chorioallantois membrane did not change...
Figure 1. The course of pH and concentration of bicarbonate ions (mmol/L) from embryonic day (ED)10 until ED16 from blood from the allantoic vein of the CO2 group and the control group. Filled symbols: CO2 group; open symbols: control group; dashed line: pH; solid line: $[\text{HCO}_3^-]$. Asterisk: means between experimental groups differ ($P < 0.05$) per embryonic day. All values are expressed as mean ± SEM.

Figure 2. The course of the partial pressure of CO2 ($p\text{CO}_2$) and partial pressure of O2 ($p\text{O}_2$; mmHg) from embryonic day (ED)10 until ED16 from blood from the allantoic vein of the CO2 group and the control group. Filled symbols: CO2 group; open symbols: control group; dashed line: $p\text{O}_2$; solid line: $p\text{CO}_2$. Asterisk: means between experimental groups differ ($P < 0.05$) per embryonic day. All values are expressed as mean ± SEM.

significantly with embryonic age in both groups (Figure 1). The average pH was 7.69 and 7.61, respectively, for CO2 and control embryos. The blood pH was significantly higher in the CO2 group than in the control group from ED11 until ED16 ($P < 0.0001$). The concentration of bicarbonate ions increased during embryonic development and reached a plateau from ED15 onwards in the CO2 group (38 mmol/L), whereas the bicarbonate levels of the control group further increased until ED16 (34 mmol/L; Figure 1). The $\text{HCO}_3^-$ levels were significantly higher in the CO2 group from ED12 until ED16 ($P < 0.0001$). The $p\text{CO}_2$ increased in both groups with embryonic age, reaching maximum levels at ED15 to ED16 (32 to 34 mmHg; Figure 2). The $p\text{CO}_2$ did not differ during embryonic development between the control and CO2 group ($P = 0.7664$). The $p\text{O}_2$ decreased from 104.55 mmHg on ED10 to 64.16 to 66.07 mmHg on ED16 (Figure 2). Incubation treatment had a significant effect on the $p\text{O}_2$ in the blood from ED11 to ED17 ($P = 0.0080$); $p\text{O}_2$ was significantly lower only at ED13 in the CO2 group compared with the control group. The potassium concentration increased with embryonic age until ED16 (Table 1). From ED11 until ED16, embryos of the CO2 group had significantly higher blood concentrations of potassium.

From ED10 until ED14, the blood of the normal incubated embryos showed respiratory acidosis, which was seen in the Davenport diagram by a shift to the upper left of the graph (Figure 3). From ED14 until ED16, a compensation in the blood was seen by a shift right upwards in the curve. From ED10 until ED15, the curve of the CO2 embryos went straight up due to an increase in $p\text{CO}_2$ and $\text{HCO}_3^-$ ions but a rather stable pH. Because there was no acidification during development, the compensation (shift right upwards) did not occur. An initial slight increase in blood pH in the CO2 group was observed together with

Table 1. Average potassium concentration in the blood from the allantoic vein of the chorioallantois membrane from embryonic day (ED)10 until ED16

<table>
<thead>
<tr>
<th>ED</th>
<th>Control</th>
<th>CO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.28 ± 0.1$^a$</td>
<td>3.28 ± 0.1$^a$</td>
</tr>
<tr>
<td>11</td>
<td>2.92 ± 0.06$^b$</td>
<td>3.41 ± 0.16$^a$</td>
</tr>
<tr>
<td>12</td>
<td>3.08 ± 0.092$^b$</td>
<td>3.61 ± 0.13$^a$</td>
</tr>
<tr>
<td>13</td>
<td>3.04 ± 0.051$^b$</td>
<td>3.72 ± 0.08$^a$</td>
</tr>
<tr>
<td>14</td>
<td>3.15 ± 0.088$^b$</td>
<td>3.72 ± 0.14$^a$</td>
</tr>
<tr>
<td>15</td>
<td>3.46 ± 0.093$^b$</td>
<td>4.1 ± 0.13$^a$</td>
</tr>
<tr>
<td>16</td>
<td>3.73 ± 0.11$^b$</td>
<td>4.45 ± 0.21$^a$</td>
</tr>
</tbody>
</table>

$^a,b$Means between experimental groups differ ($P < 0.05$) per embryonic day.

Figure 3. Davenport diagram of the blood from the allantoic vein of the CO2 group and the control group per embryonic day (ED). Filled symbols: CO2 group; open symbols: control group. Solid and dashed isobars of partial pressure of CO2 with indication level of partial pressure of CO2.
a much higher increase in bicarbonate ions compared with the control group. The blood pCO2 of CO2-incubated embryos on the other hand shifted to the same isopleths with embryonic age as the control group, except for ED15 and ED16.

From ED10 to ED16, pH of the allantoic fluid only slightly decreased from ED10 to ED14 but showed a steep decrease from ED14 onwards until ED16 (Figure 4). The CO2 treatment during incubation had a general effect on pH of the allantoic fluid from ED11 to ED16 (P = 0.0026). On ED14 and ED15, the pH of the allantoic fluid from the CO2 group was significantly higher than the pH of the control group. The concentration of NH3 increased with embryonic age and showed a steep increase from ED14 to ED15 to reach 27.7 mg/L of N (CO2 group) and 29.8 mg/L of N (control group) on ED16. The NH3 concentration was significantly higher in the CO2 group on ED12 and ED13, but this group difference disappeared at ED14. Phosphate concentrations increased with embryonic age in both groups from 3.81 to 8.57 and 12.56 mg/L on ED16 in the CO2 and control group, respectively (Figure 5). Phosphate concentrations did not differ between the 2 groups from ED11 until ED16. There was, however, a trend to a higher phosphate concentration in the control group on ED15 (P = 0.08) and ED16 (P = 0.06).

During embryonic development, the calcium concentration in the blood from the allantoic vein decreased until ED16 (P < 0.0001; Figure 6). There was no general group effect for blood Ca2+ (P = 0.4277). There was no age or group effect for the calcium concentration in the allantoic fluid (P = 0.0880; Figure 6). The Ca2+ per dry tibia weight (mg/g) increased with embryonic age (P < 0.0001), whereas no group effect was found (P = 0.6790; Figure 6).

**DISCUSSION**

Our previous study showed the remarkable tolerance of chicken embryos of a modern commercial broiler line (Cobb) to high levels of CO2 (4%) from ED10 until ED18 (Everaert et al., 2007). Complementary to the hypercapnia studies of Dawes and Simkiss (1971) and Crooks and Simkiss (1974) on a layer line, the underlying adaptation mechanisms to this tolerance of broiler embryos were investigated in this study.

The age-observed patterns of the measured blood parameters are in general in accordance with literature (Dawes and Simkiss, 1969; Erasmus et al., 1970–1971; Freeman and Misson, 1970; Girard, 1971; Boutilier et al., 1977). The pCO2 from the blood increased due to increased metabolism and the limited diffusion rate, due to eggshell resistance. The pO2 in the blood on the other hand decreased during embryonic development. During normal development, the blood pH stayed relatively constant in the allantoic vein, in accordance with results of Dawes and Simkiss (1969). As in their study, a small drop of the blood pH can be seen from ED13 to ED14. Tazawa et al. (1971b), Freeman and Misson (1970), and Erasmus et al. (1970–1971) on the other hand found a decrease with time of blood pH during the second week of incubation. Differences in absolute values between our experiment and other studies might be due to strain or flock differences, which are known to affect embryonic growth and eggshell permeability (Tazawa et al., 1971b). The concentration of bicarbonate ions increased during development, which probably stabilized the pH, hereby buffering for the respiratory acidosis that arises during the second half of development (Erasmus et al., 1970–1971; Freeman and Misson, 1970). The maximal level of HCO3− concentration was reached at ED16 in the control group or possibly even later, because no blood samples were taken thereafter. The attainment of the highest levels of HCO3−, however, was accelerated in the CO2 group. Girard (1971) suggested that the plateau phase reached around ED15 indicates a steady state of acid-base balance when a maximal degree of respiratory
Figure 6. The course of calcium concentration (mmol/L) from the blood and the allantoic fluid from embryonic day (ED)10 until ED16. Calcium per dry tibia weight (mg/g) at ED12, ED14, and ED16. Filled symbols: CO2 group; open symbols: control group. All values are expressed as mean ± SEM.

acidosis is reached. Dawes and Simkiss (1969), Erasmus et al. (1970–1971), and Freeman and Misson (1970) suggested that bicarbonate ions are provided from the shell, are conserved by the kidney when created by the activity of carbonic anhydrase, or both. The protons emerging from the interaction of CO2 and H2O, catalyzed by carbonic anhydrase, could then be excreted into the allantoic fluid (Boutillier et al., 1977). As a result, a decrease in the allantoic pH from 7.71 on the tenth incubation day to 6.82 and 7.13 on ED16 in the control and CO2 group, respectively, occurred as in the study of Dawes and Simkiss (1971). The Davenport diagram showed a relative respiratory acidosis occurring during development, seen by a shift to the upper left in the control group. From ED14 to ED16, compensation by increased HCO3− can be seen by a shift to the upper right.

Exposure of embryos to high CO2 did surprisingly not increase blood pCO2 nor air cell pCO2 (Everaert et al., 2007; experiment 2) and contradict previous findings of similar experimental design (Everaert et al., 2007; experiment 1), although an additional rise in HCO3− was observed. Differences in eggshell permeability between eggs of different experiments might explain the differences observed in partial pressure of gases in the air cell and blood between the experiments. Still, the bicarbonate concentration was significantly higher in the CO2-incubated group. Three different mechanisms could explain the higher bicarbonates in the blood; renal compensations, increased eggshell resorption, and changes at the cellular level.

From our results, it is difficult to conclude whether renal compensations played a major role in increasing HCO3−. If more HCO3− would have been produced due to conversion by carbonic anhydrase, the concomitantly formed H+ are normally buffered in the allantois by NH3 or phosphate. Allantoic pH was slightly more acid in the CO2 group from ED11 to ED13, and in this period, NH4+ concentration was also higher in the CO2 group, suggesting that the extra created protons are buffered as NH3 in the allantoic fluid. No changes in inorganic phosphate concentration were observed after CO2 exposure. It is, however, not necessary that absolute concentrations of these buffers increase for neutralizing protons. Therefore, buffer capacity by measuring titratable acid should be performed in future research to draw a firm conclusion on the renal buffering in CO2-exposed embryos. The pH increased to a higher pH from ED14 onwards compared with control embryos, possibly due to a simultaneous escape of HCO3− from the blood into the allantois. This was supported by a study of Carter et al. (1959), in which adult rats were chronically exposed to high CO2 causing respiratory acidosis. Surprisingly, a more alkaline pH in the urine was observed, suggesting that a portion of the filtered bicarbonate escaped into the urine to buffer for high protons, especially when the serum bicarbonate concentration was maximal. This could also be the case in our study, because the concentration of bicarbonate ions in the blood of the CO2 group was higher and had reached a plateau from ED15. Also, Rowlett and Simkiss (1989) suggested that the increase in HCO3− and base excess seen during normal development of shell-less embryos was the result of metabolic compensation acting via the embryonic kidney driven by the pCO2 of the blood. Still, a note of caution is needed when interpreting data on allantoic fluid, because the allantois would be a difficult index of renal compensation, which is reflected in a large
scatter (large SE) among individual eggs, probably due to the influx of uric acid (Dawes, 1974).

Besides the possible renal adaptations, eggshell compensations might have occurred. The reaction of more H⁺, resulting from hypercapnia, with calcium carbonate from the shell would lead to a higher release of calcium and bicarbonate ions. However, calcium concentrations in the blood and in the allantoic fluid and calcium per dry tibia weight were not different between treatments. Compensation by an increased release of calcium and bicarbonate ions from the shell is therefore not considered to occur as a result of hypercapnia. Crooks and Simkiss (1974) even suggested that high CO₂ would inhibit calcium resorption, when embryos are exposed to 9% CO₂.

A third possibility to explain the absence of protons together with higher HCO₃⁻ seen in the blood of the CO₂-incubated group might be due to an exchange with intracellular electrolytes. In general, respiratory acidosis is accompanied with normal or elevated concentrations of potassium (Emmett and Seldin, 1989), as seen in the increase in potassium concentration during embryonic development in our study. During the period in which embryos were exposed to CO₂ exchange of protons with intracellular potassium occurred, resulting in the significantly higher blood potassium concentrations in the CO₂ group, generating HCO₃⁻ in the extracellular fluid (Emmett and Seldin, 1989). This adaptation could contribute to the high bicarbonate concentration in the blood of CO₂ embryos. Moreover, these bicarbonate ions might be excreted by the kidney into the allantoic fluid, causing an increased pH.

Besides the bicarbonate buffering system, hemoglobin acts as the most important nonbicarbonate buffer in blood. During normal development, hematocrit (and hemoglobin) increased, causing an increase in buffering capacity of the blood (Erasmus et al., 1970–1971; Tazawa and Piiper., 1984). Hassanzadeh et al. (2002) showed that CO₂ (0.4% from ED15 until ED20)-incubated embryos had higher hematocrit values at external pipping. In our CO₂ studies, however, an additional increase of hematocrit due to CO₂ incubation was not observed (N. Everaert, unpublished data). Therefore, it is unlikely that hemoglobin buffering would contribute to buffer protons originating from CO₂ hydration. In conclusion, embryos exposed to 4% CO₂ during the second half of incubation adjusted to the induced acidosis, hereby creating a blood alkalosis, which was illustrated by higher blood pH combined with a sharp increase of HCO₃⁻ but without changes in blood pCO₂. The main mechanism to explain the extra amount of bicarbonate ions in the blood is most probably the stimulated intracellular exchange of H⁺ with K⁺, although temporary contributions of renal metabolism cannot be excluded.

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