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Effects of Different CO_2 Levels on Chicken Embryonic Development During Early Stage of Incubation

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Abstract: During the early stage of incubation of chicken eggs, different CO_2 concentrations play an important role in the embryonic and postnatal growth. However, the mechanism involved in this process is still not well clarified. In this study, chicken eggs were incubated under different CO_2 concentration levels (0.03-0.05, 2, 5 and 10%) during the 1st 4 days of incubation and measured of several embryo growth parameters. It was then found that higher CO_2 concentration significantly played down the albumen pH value after 2 days of incubation and the embryo weight and length, diameter of area vasculosa of yolk sac and number of somite all decreased with the increasing of CO_2 level. Compared with the mortality (3.23%) and abnormality (0%) in the control group, the corresponding percentages in three treatment groups were much higher with the lowest at 17 and 29%, respectively. Taking the two sides together, it might be well concluded that high CO_2 concentration during the 1st 4 days of egg incubation inhibited chicken embryonic development through the lowering of albumen pH value.

Key words: CO_2, albumen pH, chicken embryo, early incubation, egg, concentration

INTRODUCTION

It is becoming increasingly clear that different levels of CO_2 could play an important role in the embryonic development of chicken egg incubation (Walsberg, 1980; Dzialowski *et al*., 2002; De Smit *et al*., 2006). Many studies have shown that chicken embryos were extremely sensitive to CO_2 level during its early stages of incubation (Sadler *et al*., 1954; Taylor and Kreutziger, 1965) and recent reports argued that gradually increasing CO_2 level (1-1.5%) had some positive effects on embryo growth and hatching (Hogg, 1997; Bruggeman *et al*., 2007). However early researches, all showed that CO_2 concentration >1% depressed egg hatchability (Taylor *et al*., 1956; Taylor and Kreutziger, 1966) and the time of initiating CO_2 rising also played a determining part during early embryonic development (Bruggeman *et al*., 2007).

Upon the onset of incubation, the albumen pH increased rapidly as a result of sudden mounting up of internal temperature which in turn accelerated CO_2 loss from the albumen and soon reached its highest point at 2-3 days. The albumen pH was proved to be alkaline before the start of incubation yet it shifted gradually from alkalinity toward acidity during incubation there after (Romanoff and Romanoff, 1949). Bruggeman *et al*. (2007) described the effect of different external CO_2 concentrations on the albumen pH from 3-12 days of incubation. Changes in albumen pH could exert some substantial influences on the blastoderm which was adjacent to the albumen (Benton and Brake, 1996). At stage XII-XIII (Hamburger and Hamilton, 1951), the dorsoventral polarity of the single-cell thick epiblast was established because of its interposing between two very different environments with the albumen being alkaline (pH up to 9.5) while the yolk neutral or slightly acidic (pH around 6.5-7.0). Research of Benton and Brake was performed on the significance of the initial rise of albumen pH in liquefaction and formation subembryonic fluid (Benton and Brake, 1996). Rubin and Walter have shown that the rate of fibroblast migration varied with the pH of the culture medium (Rubin, 1971; Walter and Gaetano, 1985). In addition, a high pH appeard to be essential for the normal gastrulation movements and the activation of cell migration in amphibian embryo (Holtfreter, 1944; Kubota and Durston, 1978). However, less information is available on how the albumen pH affects embryonic development. The aim of this study was to investigate the latent effects of different during the early stages of

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incubation. By measuring during early incubation, the researchers were able to investigate the latent effects of different CO_2 level (normal 2, 5 and 10%) on chicken embryo development through changing albumen pH as measured by body mass and length, diameter of area vasculosa of yolk sac, number of somite and morphology parameters.

MATERIALS AND METHODS

Incubation protocol: White leghorn eggs were obtained from China Agriculture University Hatchery and used within 2 days. Eggs were incubated in one of four groups: 0.03-0.05% CO_2 for the duration of incubation [Control Group (CG)]; 2% CO_2 for the duration of incubation [2% CO_2 Group (2-G)]; 5% CO_2 for the duration of incubation [5% CO_2 Group (5-G)]; 10% CO_2 for the duration of incubation [10% CO_2 Group (10-G)]. Eggs for each treatment were randomly placed into four CO_2 incubators with automatic CO_2 injection. Four groups of eggs were incubated at 37.5°C, 65-75% RH and turned erery 4 h.

Design of experiment 1: In this experiment, 192 eggs were assigned at random to four groups before incubation. At different time points during incubation: 0, 2, 4, 8, 12, 24, 48 and 72 h, six eggs from each group were sampled to measure the albumen pH value immediately by means of a pH meter. At the beginning, rotated the temperature button by ambient temperature and selected buffers for a two-point calibration. pH 7 and 10 buffers were used for the albumen which had a pH >7. Rinsed the sensing bulb of glass with distilled water and gently shaked off any excess between immersion in buffers or albumen. Collect 1 mL albumen in centrifuge tube to test at the same time as the eggs were sampled. Put the sensing bulb of glass in the albumen and wait for the reading to stabilize. Recorded the pH reading on the screen.

Design of experiment 2: A total of 120 eggs were randomly assorted to four groups at the beginning of incubation. Then they were incubated, respectively in CG, 2, 5 and 10 G. On the 4th day of incubation, all the eggs were sampled for measuring the following embryonic parameters; heart rate, diameter of area vasculosa of yolk sac, embryonic weigh, embryonic length, numbers of pairs of somites (developmental stages), mortality and abnormality rates.

Egg was first uncovered at the blunt end for counting their heart beats the instant that it was taken out of the incubator. Then the diameter of the area vasculosa of yolk sac was measured by the vernier caliper. Thereafter, the embryonic length measurement was done from the encephalic vesicle to the tail. After these measurement, chicken embryo was separated from their extra embryonic membrances, rinsed with distilled water and gently shaked off any yolk. Then if was prepared on the glass slide. The number of somites examined under inverted phase contrast microscope by the method previously described by Hamburger and Hamilton (1951). Then, the body was dried on absorbent paper and weighed by an electronic balance. Meanwhile, dead and morphologically malformed embryos were recorded to count the mortality and malformation rates involved.

Statistical analysis: The data were processed using the statistical software packages SAS Version 8.0. A significance level of 0.05 was used. For the comparisons that included albumen pH, diameter of area vasculosa of yolk sac, embryonic weigh and embryonic length, statistical analysis was base on least squares procedures, using the General Linear Models Procedure (PROC GLM). Percentage mortality and malformation data was analyzed by 2×2 χ^2-test for independence procedure (PROC FREQ) for the comparison. All data are presented as means±SD.

RESULTS

Experiment 1: According to the researchers, the pH of the albumen might undergo some changing from 7.6-9.7 at different stages of incubation after (Becker *et al*., 1968; Tona *et al*., 2002) and the undulation of ambient CO_2 in the incubator will exert radical influence on the albumen pH value (Bruggeman *et al*., 2007). In the study, the pH of albumen was measured at 0, 2, 4, 8, 12, 24, 48, 72 and 96 h during incubation of four groups. Before the start of incubation, albumen pH was 8.94±0.07. Changes in albumen pH as a function of incubation treatment through development are shown in Fig. 1. Since, 8 h of incubation, eggs that had been incubated in either 2 or 5, 10 G group had lower albumen pH than the control eggs.

Before albumen pH decreased after 72 h to get an albumen pH of 8.76±0.07 at 96 h, it had an initial rise of albumen pH in the CG eggs which reached its highest peak of 9.33±0.02 at 48 h. Compared with control group, albumen pH in 2 G decreased from 2-24 h to obtain 8.27±0.04 and maintained the level around 8.2 until 96 h. Decrease for albumen pH in either 5 or 10 G was dropped faster than in 2 G from 2-24 h. Albumen pH stayed at around 8.0 and 7.8, respectively in 5 and 10 G utill 96 h. Taking all these into consideration, the rapid acidification in the albumen of the treatment groups was definitely due to higher CO_2 concentration in incubator.

Experiment 2

Albumen pH value: With gradient levels of CO_2 in the incubator, the pH values of albumen in the four groups

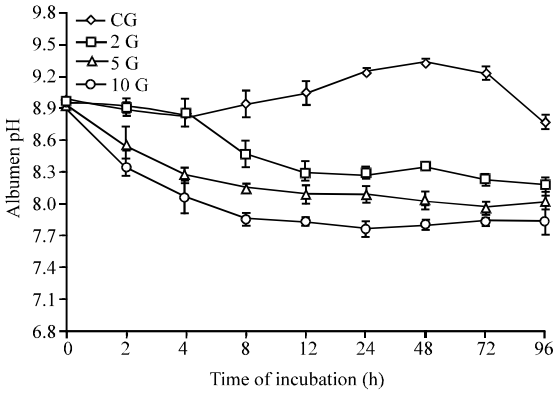


Fig. 1: Evolution of albumen pH in control and treatment groups during the 1st 4 days of incubation

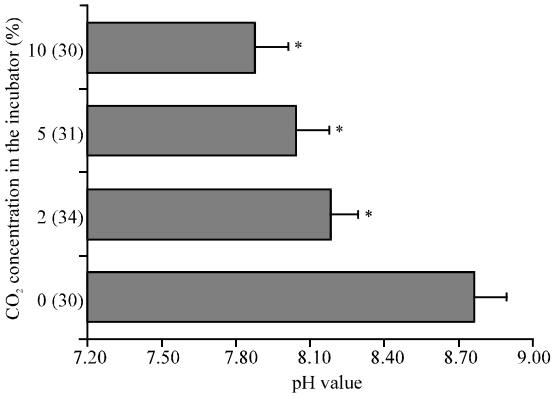


Fig. 2: Albumen pH value in control and treatment groups at the 4th day of incubation. Asterisks indicated significant differences ($p<0.05$) between control group and treatment group

(CG, 2, 5 and 10 G) showed some reverse correlation to their CO_2 concentration levels that is the higher the CO_2 concentration, the lower the albumen pH value. To further elucidate the data, the albumen pH values at day 4th of these four groups were pooled in Fig. 2. In CG, the pH (8.75±0.13) was significant higher than all other three treatment groups (2 G: 8.17±0.11, 5 G: 8.04±0.13, 10 G: 7.87±0.14) and distinctive differences could also be observed among any of the two treatment groups.

Numbers of pairs of somite: Undoubtedly, the researches of Hamburger and Hamioton described how to distinguish each stage with prominent characteristics concerned during chicken embryonic development. The numbers of pairs of somite could be chosen as a standard of reference to designate embryos progress after the 2nd day of incubation (Hamburger and Hamilton, 1951).

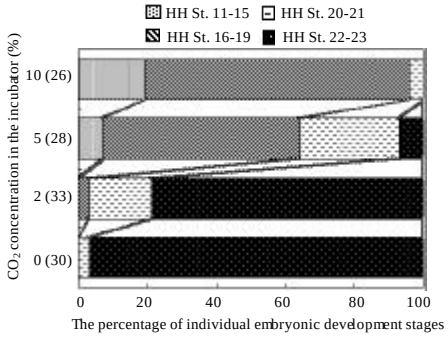


Fig. 3: The percentage of embryonic development stage at the 4th day of incubation. The embryos were staged according the HH (Hamburger and Hamilton, 1951) regime. HH St. 11-15 HH St. 16-19 HH St. 20-21 HH St. 22-23 represented the progress in development at 1.5-2, 2.5-3.5, 3.5 and 4 days separately

Table 1: Embryonic weight and length (mean±SD) at the 4th day of control 2, 5 and 10 G incubation groups

Treatments	N	Embryo weight (g)	N	Embryo length (mm)
CG	30	0.0294±0.0104[a]	30	6.9777±0.8438[a]
2 G	32	0.0193±0.0107[b]	33	6.6606±0.9295[a]
5 G	31	0.0123±0.0057[c]	32	5.4081±0.9874[b]
10 G	30	0.0072±0.0036[d]	31	4.8042±1.2097[c]

[a-d]Means without common superscript letters within a column are significantly different ($p<0.05$)

In this study, the numbers of somite were counted at 96 h. The embryonic developmental process was divided into HH stage 11-15, HH stage 16-19, HH stage 20-21 and HH stage 22-23 according the HH regime (Hamburger and Hamilton, 1951), corresponding to the period of incubation during 1.5-2, 2.5-3.5, 3.5 and 4 days, separately. Figure 3 shows the percentage of embryonic developmental stage.

In the control group, 96.67% embryos achieved full development (embryos at HH22-23) and the percentage of other three treatment groups dropped from 78.79-7.14% and then to 0% in rank.

As observed in these datum, the embryos showed a retarded growth in 2, 5 and 10 G (embryos at stages previous to HH 21). Most of the embryos developed at HH16-19 and HH20-21 in 5 G and at HH11-15 and HH16-19 in 10 G.

Embryonic weight and embryonic length: As shown in Table 1, the embryonic weight of the three treatment groups decreased significantly ($p<0.05$) in comparison with the control group (0.0294±0.0104) at the 4th day of incubation. Most notably, embryonic weight in 10 G showed the largest proportional decrease

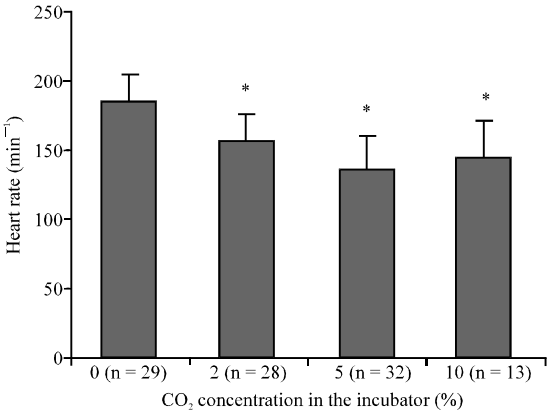


Fig. 4: Heart rate in chicken embryos at the 4th day of control, 2, 5 and 10 G incubation groups. Asterisks indicated significant differences ($p<0.05$) between control group and treatment group

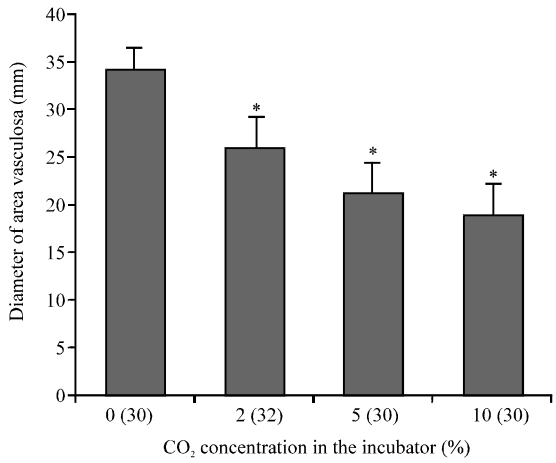


Fig. 5: Diameter of area vasculosa of yolk sac of control 2, 5 and 10 G incubation group. Asterisks indicated significant differences ($p<0.05$) between control group and treatment group

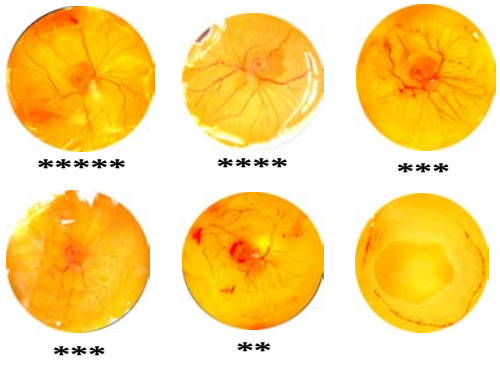
(0.0072±0.0036 g) as compared with control group and showed a smaller proportional decrease in 2 G (0.0193±0.0107) and 5 G (0.0123±0.0057). In all treatment groups, embryos measured smaller in size than the embryos in control groups. A trend towards shorter in embryonic length was similarly found in 2, 5 and 10 G (2 G: 6.6606±0.9295, 5 G: 5.4081±0.9874, 10 G: 4.8042±1.2097) contrasted with CG (6.9777±0.8438) being significant in 5 and 10 G. Both values, embryonic weight and embryonic length, decreased with the lower albumen pH due to increment of CO_2 concentration.

Heart rate: One of critical functions of cardiovascular system was nutrient delivery. So, the heart rate one of the most important indicators of this system would decrease when the growth rate decelerated and vice versa during early embryonic development (Birchard and Reiber, 1996). The effect of different CO_2 concentration on the heart rate of surviving embryos is shown in Fig. 4. Compared with the control group (182.41±20.64), eggs incubated in three treatment groups significantly decreased in the average heart rate ($p<0.05$). There was no significant differences among 2 G (154.29±19.71), 5 G (133.75±24.06) and 10 G (142.31±27.13). These results indicated that the embryonic heart rate slowed down with increase of CO_2 concentration during the first four day of incubation.

Diameter of area vasculosa of yolk sac: At HH stage 8, blood-islands were emerged in posterior half of blastoderm. With progressing of development, the blood-islands began to interconnect and formed the primary vascular plexus within the area opaca (ao) (Risau and Flamme, 1995). The formation of the yolk sac vascular system and its connection to the embryonic circulation was crucial for embryo survival in both mammals and birds (Le Noble *et al*., 2004). Diameter of area vasculosa (blood-islands) of yolk sac was measured at the 4th day during incubation. A significant effect of CO_2 concentration was observed for the diameter of area vasculosa. In all treatment groups, the diameter (2 G: 26.00±3.03; 5 G: 21.27±3.22; 10 G: 18.79±3.54) decreased obviously from that in control group (34.0980±2.45) (Fig. 5).

This rapid decrease occurred with the increase of CO_2 concentration among three treatment groups. In addition, some morphological changes of vascular systems manifested that albumen pH due to different CO_2 concentration had impact on vasculogenesis. In order to analyzed the relationship between vasculogenesis and albumen pH, six grades were classified and used to evaluate the eggs according to these judging indexes (Fig. 6). In 10 G, almost half of the eggs developed to the stage of HH8 with blood-island and no vascular plexus. In 5 and 2 G, the vascular systems presented small covered area, thin vessl and little blood volume.

Mortality and malformations: As shown in Table 2, the mortality rate in treatment groups was much higher than that in control group, most likely resulting from sharp decrease of albumen pH due to artificially imposed higher CO_2 concentration during incubation. In 10 G, the rate rose even more dramatically and >60% of embryos died before the 4th day of incubation. Athough, there was no significant effect on 2 and 5 G, their mortality, still appeared to be higher than that in control group due to an



Treatments	CG N = 30	2-G N = 33	5-G N = 30	10-G N = 28
******	86.7	56.70	3.3	-
*****	13.3	13.30	20.0	-
****	-	16.70	40.0	3.3
***	-	20.00	36.7	3.3
**	-	-	-	36.7
*	-	3.33	-	50.0

Fig. 6: The percentage of embryonic area vasculosa of yolk sac grades at the fourth day of incubation of control, 2-G, 5-G and 10-G incubation group. Six grades were classified according to the area vasculosa developmental condition; ******Normal area vasculosa of yolk sac with clear vitelline artery, dense vascular plexus and sufficient blood; *****Area vasculosa of yolk sac with slightly less of blood compared with that of Normal area vasculosa of yolk sac; ****Area vasculosa of yolk sac with less blood in the branching of vascular networks; ***Area vasculosa of yolk sac with thinner vessels and less blood in it; **Area vasculosa of yolk sac with less microvessels and extremely less blood and ood-islang with no area vasculosa of yolk

Table 2: Mortality and abnormality at the 4th day of control 2, 5 and 10 G incubation groups

Treatments	Mortality (%)	Abnormality (%)
CG	3.23 (1/31)[a]	0.00 (0/31)[a]
2 G	17.65 (6/34)[a]	29.41 (10/34)[b]
5 G	11.11 (4/36)[a]	33.33 (12/36)[b]
10 G	63.89 (23/36)[b]	72.22 (26/36)[c]

Mortality and abnormality are expressed in percentages; [a-c]Means without common superscript letters within a column are significant

initial drop of albumen pH. In addition, several kinds of malformations were observed in this study which include head and eye malformations, blood-island developmental inhibition and area vasculosa of yolk sac defects and all of them happen between ectoderm and mesoderm. A quantification analysis of pooled gross abnormalities of embryos was shown in Fig. 5. The malformations increased with augmentation of CO_2 concentration in the incubator being significant in 2 and 5 G and more critically significant in 10 G compared with the control group. The results indicated that changes of albumen pH during early incubation might disrupt the normal procedure of embryonic development.

DISCUSSION

This study showed that during early stage of embryonic development several parameters changed with external CO_2 concentrations. Albumen pH changed first as the result of high CO_2 level in the incubator. Thereafter, embryo parameters as embryonic length and weight, diameter of area vasculosa of yolk sac and number of somite, changed with the albumen pH due to high CO_2 level. The albumen pH was critical to chicken embryonic development during the whole hatching process (Mayes and Takeballi, 1987; Cotterill and Nordskog, 1954; Stern, 1991). As we know, the albumen pH undulated in pace with the transformation of incubation milieu resulting from the CO_2 exchange though the pores on the egg shell (Rahn, 1981; Rahn and Ar, 1980). In the CG, chicken eggs were incubated in natural environment with CO_2 concentration at 0.03% and the average albumen pH reached its peak about 9.3 at the 3rd day and then decreased gradually during incubation (Fig. 1). The continuous decreasing of albumen pH in the three treatment groups with high CO_2 concentration showed a similar pattern as the results of Bruggeman *et al.* (2007). However in 5 and 10 G, the albumen pH decreased significantly after only 2 h of incubation. Such a close correlation between the internal pH drop and the high CO_2 concentration clearly indicated that CO_2 concentration could directly effect the albumen pH (Fig. 1). Compared with the control group, the albumen pH among three treatment groups showed a similar trend of decrease during the 1st 3 days of incubation. During this period, chicken blastoderm will experience a series of complicated dramatic changes. The single layer of cells called epiblast, adhering to albumen, started to form the bilaminer embryo with hypoblast and endoblast and then it further developed to trilaminar embryo with ectoderm, endoderm and mesoderm. Meanwhile, the dorsoventral polarity of single cell establisheed due to the difference of pH value between albumen (9.5) and yolk (6.5). From the results observed in the study, the dramatic drop of albumen pH in treatment groups probably could disturb the whole process of bastoderm development.

The effect of CO_2 concentration on the chicken embryonic development was evident at the 4th day of high CO_2 incubation as shown by the somites formed thereof. The number of somites which was derived from mesoderm was a clear-cut indicator of designating the development stages of chicken eggs. Among the four groups, the rate of embryo achieved full development

were 97, 78, 7.1 and 0% in CG, 2, 5 and 10 G, respectively. It means that the development of chicken embryos had been retarded as a result of lower pH due to higher CO_2 concentration levels at least with 5 stages falling behind the control group in 5 and 10 G. And such retardation also displayed in the decrease of body weight and length in treatment groups (Table 1). However, some researchers (Hogg, 1997; De Smit *et al*., 2006; Bruggeman *et al*., 2007) showed that a gradual increase of CO_2 concentration to higher levels might have positive effect on embryonic growth and hatching. Sadler *et al*. (1954) reported that 4% CO_2 could stimulate the embryo growth during the 1st 48 h of incubation. In addition, Bruggeman indicated that the right time of initiating the CO_2 rise could induce higher embryonic weight and earlier hatching. The results of this research showed that higher CO_2 concentration during the 1st 4 days of incubation had some negative effects of chicken development and the higher the concentration, the more obvious the effect. Taking all results into account, it thus could be concluded that lower albumen pH probably might have delayed the embryonic development especially mesoderm development during the 1st 4 days due to high CO_2 concentration in the incubator.

Early studies showed that high CO_2 concentration could depress hatchability (Taylor *et al*., 1956; Taylor and Kreutziger, 1965, 1966). Another research recorded high mortality when the eggs incubated at a 10% CO_2 level for 24 h on any of the 1st 10 days of incubation (Haring *et al*., 1970) and the mortality was resulted from non-cardiac and cardiac malformations. Studies of Meuer *et al*. (1989) suggested that high environmental CO_2 level (3%) could lead to decrease in blood volume of the embryo which exerted substantial impact on cellular processes yet little was known about how the high CO_2 concentration could inhibit the chicken embryonic development. Similar findings were described about the development of embryonic cardiac system during the 1st 4 days when the eggs were incubated at high CO_2 level. In the three treatment groups, the embryos had smaller area vasculosa of yolk sac with less blood supply compared with the embryos that developed under standard CO_2 level (Fig. 5) and the heart rate decreased with the increase of CO_2 concentration at the range from 0-5%. Interestingly, the heart rate in 10% CO_2 is faster than that in 5% CO_2, most probably due to the fact that all embryos staying alive in 10 G are highly resistant against high CO_2 concentration. In addition both the mortality and malformation rate in 2, 5 and 10 G mounted higher than that in control group (Table 2). Most of dead chicken embryos in treatment group had some cardiovascular defect in terms of morphology and physiology such as shortage of blood supply and lower heart rate. The malformations of chicken embryos with head and eye malformations, blood-island developmental inhibition and area vasculosa of yolk sac defects were probably caused by high CO_2 concentration which caused lower pH value with affecting the formation of ectoderm and mesoderm.

CONCLUSION

In this study, all the results mentioned showed that high environmental CO_2 level (2-10%) administered during the 1st 4 days of incubation induced evident inhibition of embryonic development, manifested by reduced numbers of somites, smaller area vasculosa of yolk sac and slower heart rate. Considering the close correlation of albumen pH value and CO_2 level, albumen pH and ectoderm/mesoderm development, the external change of environment (mainly CO_2 level), most likely could depressed the embryonic development of during the 1st 4 days of incubationg through altered albumen pH.

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