Effects of Oxygen Gas Injection on the Subsequent Development of Chick Embryos in a Shell-Less Culture System 

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The effects of oxygen gas injection starting on day 17 of incubation (D17) in a chick shell-less culture system (cSLC) on the subsequent embryo development were examined on day 19 of incubation (D19). On D19 of cSLC, the plasma phosphorus and total cholesterol concentrations of the embryos were significantly higher ($P<0.05$), while the plasma calcium concentrations were significantly lower ($P<0.05$) than those in the intact control (IC) group. However, no significant differences in embryo viability and other major blood component levels were observed among the experimental groups ($P>0.05$). The percutaneous oxygen saturation was lower in D17-cSLC embryos before oxygen gas supplementation than in the IC ($P<0.05$) embryos. Severe renal tubular degeneration of the metanephros was observed in D19-cSLC embryos despite oxygen gas injection starting from D17. These results indicate that D19-cSLC embryos are hypoxia even after injecting oxygen gas starting on D17.

**Key words:** chicken embryo, hatchability, metanephros, oxygen gas injection, shell-less culture, $SpO_2$

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**Introduction**

The development of a chick shell-less culture (cSLC; Tahara and Obara, 2014; Tahara et al., 2021) system has enabled uninterrupted omnidirectional visual observation of the development of chick embryos through transparent poly-methylpentene film. This novel research tool is potentially valuable in multiple research areas, such as embryo manipulation (Boulland et al., 2010), bioimaging (Funahashi et al., 2014), and basic regenerative medicine research (Chiba et al., 2010).

One of the main factors contributing to the successful hatching of normal chicks in a cSLC is thought to be oxygen gas injection (OGI) into the cSLC starting on day 17 of embryo development (D17) (Tahara et al., 2021). Here, the duration of embryo incubation in the cSLC is expressed in days, using the notation $D_i$ where $i=3, ... 19$.

The need for OGI for cSLC was recognized when most dead embryos showed renal tubular necrosis, a typical pathological finding of renal hypoxia (unpublished data). Indeed, experimentally induced hypoxia in chick embryos resulted in retarded growth or increased mortality (Lourens et al., 2007; Zhang and Burggren, 2012). Furthermore, hypoxia has adverse effects on mammalian fetal kidney development (Wilkinson et al., 2015). However, the effects of OGI on chick embryo development during cSLC have not been critically evaluated. Therefore, this study validated the effects of OGI during cSLC starting on D17.

**Materials and Methods**

**Chicken Eggs**

The experiments used fertilized White Leghorn eggs from
the MB line (National Livestock Breeding Center, Okazaki, Japan) maintained at the Tsukuba Plant Innovation Research Center farm, University of Tsukuba, Japan.

**Culture Vessel**

The embryo culture vessel used in the cSLC was identical to that in our previous study (Obara et al., 2022) with slight modifications: a 2-cm-diameter hole was made in the side of the plastic cup, and a 2-mm-diameter tube (Atom Multipurpose Tube; Atom Medical, Tokyo, Japan) was inserted through this hole to supply oxygen (Tahara and Obara, 2014).

**Embryo Culture**

Embryos were cultured in a cSLC using an incubator (P-008; Showa Furanki, Saitama, Japan), as reported previously (Tahara and Obara, 2014). Briefly, 3-day-old chick embryos at stages 15–16 (Hamburger and Hamilton, 1951) were transferred to a cSLC containing sterilized distilled water (2.5 mL) with 250 mg of powdered calcium lactate (Cat #:031-00675; Fujifilm Wako Pure Chemical Co., Osaka, Japan) on a plastic film. On D17, the embryos were randomly assigned to one of the following treatments: treatment1 (Trmt-1), cSLC under atmospheric air; treatment 2 (Trmt-2), cSLC with OGI at a rate of approximately 500 mL/h; and intact control (IC), fertilized shelled eggs incubated under conventional conditions (37.8°C, 70% relative humidity).

On day 19 of embryo development (D19), approximately 0.5 mL of blood was collected from a major blood vessel in the chorioallantoic membrane of the embryo. The concentrations of plasma calcium (Ca), inorganic phosphorus (IP), lactate dehydrogenase (LDH), glucose (GLU), aspartate aminotransferase (AST), triglyceride (TG), and total cholesterol (TCHO) were measured using an automated clinical chemical analyzer (DRI-CHEM V4000; Fujifilm Co., Tokyo, Japan). The Ca concentration in the Trmt-1 and Trmt-2 groups was significantly lower than that in the IC group (P<0.05). The average Ca concentration in the Trmt-1, Trmt-2, and IC groups was 10.9, 10.5, and 12.8 mg/dL, respectively. The Ca concentration in the Trmt-1 and Trmt-2 groups was significantly lower than that in the IC group (P<0.05; Fig. 1A). Among the viable embryos on D17, the proportion of embryos that remain viable on D19 in the Trmt-1 and -2 groups were 81.0% and 94.3%, respectively. No significant differences were observed among the treatment groups (P>0.05).

On D19, the average plasma Ca concentration in the Trmt-1, Trmt-2, and IC groups was 10.9, 10.5, and 12.8 mg/dL, respectively. The Ca concentration in the Trmt-1 and Trmt-2 groups was significantly lower than that in the IC group (P<0.05; Fig. 1B). The average plasma IP concentration in the Trmt-1, Trmt-2, and IC groups was 12.0, 13.5, and 6.0 mg/dL, respectively, and the IP concentrations in both treatment groups were significantly higher than that in the IC group (P<0.05; Fig. 1C). In the Trmt-1, Trmt-2, and IC groups, the average plasma LDH concentration was 771.6, 744.5, and 528.3 U/L, respectively; the average plasma GLU concentration was 229.0, 268.3, and 206.2 mg/dL, respectively; the average plasma AST concentration was 528.3 U/L, respectively; and the average plasma TG concentration was 280.9, 262.4, and 217.0 mg/dL, respectively. The average plasma LDH, GLU, AST, and TG concentrations did not differ significantly among the treatment groups (all P>0.05; Fig. 1D-G). The average plasma TCHO concentration in the Trmt-1, Trmt-2, and IC groups was 514.9, 531.9, and 261.5 mg/dL, respectively, and the TCHO concentration in both treatment groups were significantly higher than that in the IC group (P<0.05; Fig. 1H).

**Histopathological Examination**

After blood collection, three embryos per treatment were randomly selected and fixed using 10% formalin in a neutral buffer solution (Cat #:062-01661; Fujifilm Wako Pure Chemical Co., Osaka, Japan). The kidney tissue was embedded in paraffin wax, and 5-µm-thick tissue sections were stained with hematoxylin and eosin and observed under a light microscope (Nikon Eclipse Ni; Nikon, Tokyo, Japan). The renal tubule lesions in the sections were categorized into one of three grades based on the proportion of renal tubules with pathological degeneration (pRTD): grade 1 (slight renal tubule degeneration), pRTD<25%; grade 2 (moderate renal tubule degeneration), 25%<pRTD<49%; and grade 3 (severe renal tubule degeneration), 50%<pRTD.

**Statistical Analysis**

Statistical analyses were performed using Easy-R (EZR ver. 1.37; Saitama Medical Center, Jichi Medical University, Saitama, Japan; Kanda, 2013) on a graphical user interface for R ver. 3.4.1 (The R Foundation for Statistical Computing, Vienna, Austria). Viability data were analyzed using Fisher’s exact probability test. Data were tested for homogeneity using Bartlett’s test. Plasma components and SpO2 data with homogenous variance were statistically analyzed using a one-way analysis of variance followed by Tukey’s test, and those with heterogeneous variance were analyzed using the non-parametric Kruskal-Wallis test. Pearson’s correlation coefficient was calculated between the plasma IP and TCHO concentrations. Differences with a value of P<0.05 were considered significant.

**Institutional Approval**

All animals received humane care as outlined in the University of Tsukuba Guide for the Care and Use of Experimental Animals, and the experiment was approved by the University of Tsukuba Animal Experiment Committee (approval no. 210-435).

**Results**

Among the viable embryos on D17, the proportion of embryos that remain viable on D19 in the Trmt-1 and -2 groups were 81.0% and 94.3%, respectively. No significant differences were observed among the treatment groups (P>0.05).

The average SpO2 in the cSLC and IC groups was 76.6% and 93.4% on D17, respectively, and 87.8%, 90.2%, and 96.0% in the Trmt-1, Trmt-2, and IC groups on D19, respectively (Fig. 1A). No significant differences were observed among the treatment groups (P>0.05).

On D19, the average plasma Ca concentration in the Trmt-1, Trmt-2, and IC groups was 10.9, 10.5, and 12.8 mg/dL, respectively. The Ca concentration in the Trmt-1 and Trmt-2 groups was significantly lower than that in the IC group (P<0.05; Fig. 1B). The average plasma IP concentration in the Trmt-1, Trmt-2, and IC groups was 12.0, 13.5, and 6.0 mg/dL, respectively, and the IP concentrations in both treatment groups were significantly higher than that in the IC group (P<0.05; Fig. 1C). In the Trmt-1, Trmt-2, and IC groups, the average plasma LDH concentration was 771.6, 744.5, and 528.3 U/L, respectively; the average plasma GLU concentration was 229.0, 268.3, and 206.2 mg/dL, respectively; the average plasma AST concentration was 53.6, 51.8, and 43.7 U/L, respectively; and the average plasma TG concentration was 280.9, 262.4, and 217.0 mg/dL, respectively. The average plasma LDH, GLU, AST, and TG concentrations did not differ significantly among the treatment groups (all P>0.05; Fig. 1D-G). The average plasma TCHO concentration in the Trmt-1, Trmt-2, and IC groups was 514.9, 531.9, and 261.5 mg/dL, respectively, and the TCHO concentration in both treatment groups were significantly higher than that in the IC group (P<0.05; Fig. 1H).

Fig. 2 shows the intensity of renal tubule lesions, as determined via pRTD. In the IC group, no major renal tubule lesions were observed (Fig. 2Aa). The renal tubular epithelial cells with some vacuolation (arrowheads in Fig. 2Ab) were considered to be normal. Furthermore, tubular lumen dilatation (asterisk in Fig. 2Ac), cytoplasmic granule formation (arrowheads in Fig. 2Ad), and pyknotic nuclei (arrowheads in...
Fig. 1. **Plasma SpO₂ and biochemical concentrations.** A. Two different methods were used to describe the SpO₂ values for each treatment group: box plots (left) and the mean ± standard error (right). In the box plots, the top and bottom of the box indicate the 75th and 25th percentiles, respectively; the horizontal bar in the box denotes the median value; and the top and bottom whiskers are the maximum and minimum values, respectively. B–H. The average plasma concentrations of Ca, IP, LDH, GLU, AST, TG, and TCHO at D19, respectively. Abbreviations: SLC, shell-less culture; IC, intact control; Trmt-1, SLC without oxygen supply after D17; Trmt-2, SLC with oxygen supply after D17; Ca, calcium; IP, inorganic phosphorus; LDH, lactate dehydrogenase; GLU, glucose; AST, aspartate aminotransferase; TG, triglyceride; TCHO, total cholesterol.
Fig. 2Ae) were observed in the Trmt-1 and -2 groups. Based on three representative sections, the pRTD tended to be higher in the Trmt-1 and -2 groups than in the IC group (Fig. 2B).

Fig. 3 shows the relationship between the plasma IP and TCHO concentrations. The plasma IP and TCHO concentrations of six embryos in the IC were $\leq 7.8$ and $\leq 394$ mg/dL, respectively (Fig. 3C). The dotted lines added in Fig. 3 denote that the highest plasma IP and TCHO observed in Fig. 3C are
the upper limit concentrations of the respective blood components in normal embryos. The correlation coefficient between IP and TCHO in the Trmt-1, Trmt-2, and IC groups was 0.741, 0.829, and −0.26, respectively. The IP and TCHO concentrations in the Trmt-1 and -2 groups were significantly correlated ($P < 0.05$) but not those in the IC group ($P > 0.05$).

Discussion

OGI starting on D17 is needed to enable normal chicks in cSLC to hatch (Tahara and Obara, 2014). However, the physiological effects of OGI during cSLC on the subsequent embryo development have not been critically evaluated. Therefore, we examined the effects of OGI starting on D17 on the subsequent chick embryo development in cSLC.

We found no significant difference in the average embryo viability and the blood component levels between D17 and D19 for the Trmt-1 and -2 groups ($P > 0.05$). However, compared with the IC groups, the plasma IP and TCHO concentrations were significantly increased ($P < 0.05$), while the plasma Ca concentration was significantly decreased ($P < 0.05$) in the Trmt-1 and -2 groups. In our previous study, we found no significant differences in the plasma Ca, IP, and TCHO concentrations between the IC and D17-cSLC groups (Obara et al., 2022). Therefore, we speculate that the plasma IP, Ca, and TCHO concentrations shifted between D17 and D19 in the cSLC groups.

In humans, renal dysfunction results in increased blood IP concentrations associated with hypocalcemia since elevated blood IP binds with free blood Ca (Lumeij, 2008). Furthermore, hypercholesterolemia is observed under various pathological conditions, including chronic renal dysfunction (Vaziri, 2016). To the best of our knowledge, there is no es-
established method for assessing renal function in developing avian embryos. Therefore, an evaluation of the renal function of embryos in cSLC based on histopathological analysis associated with blood tests is necessary.

In normal chick embryos, the metanephros starts to develop on day 12 of embryonic development, whereas the mesonephros starts to degenerate on day 15 (Narbaiz and Kacew, 1978; Bolon and Burggren, 2013). The effects of OGI starting on D17 on the kidney were evaluated by preparing metanephros tissue samples on D19. Histopathologically, the tissue samples revealed renal convoluted tubule degeneration on D19 of cSLC in both Trmt-1 (Fig. 2Bf–h) and Trmt-2 (Fig. 2Bi–k) groups, suggesting impaired renal function. The relationship between the plasma IP and Ca concentrations during renal dysfunction in avian species is not well understood. If the relationship between serological responses and renal dysfunction in humans can be extrapolated to that in avian species, the shift in IP and Ca concentrations between D17 and D19 may also reflect renal dysfunction in avian species.

Notably, a significant correlation between the plasma IP and TCHO concentrations was observed in both the Trmt-1 (Fig. 3A) and Trmt-2 (Fig. 3B) groups. Using the maximum IP (7.8 mg/dL) and TCHO (394 mg/dL) concentrations to plot the IC group in the third quadrant (Fig. 3C), 56% (5 of 9) of the embryos in the Trmt-1 (Fig. 3A) group and 73% (8 of 11) in the Trmt-2 (Fig. 3B) group were in the first quadrant, respectively.

Of the three representative embryos in each treatment group whose histological grading was performed, the plasma IP and TCHO concentrations of two embryos in the Trmt-1 (Fig. 2Bh, g) group and one embryo in the Trmt-2 group (Fig. 2Bk) were in the first quadrant, and the histological evaluations of these samples determined them to be grade 3 (Fig. 2B). Furthermore, two embryos in the Trmt-2 group (Fig. 2Bi, j) were in the fourth quadrant, and histologically these samples were also graded as grade 3 (Fig. 2B). These data support the use of plasma IP and TCHO concentrations as indicators of renal dysfunction in developing chick embryos. Future studies should elucidate the relationship between serological parameters and the physiological status of the developing chick embryos.

In the D17-cSLC group, SpO2 levels tended to be lower than those in the IC group, suggesting that embryos were already oxygen-deprived. After OGI for 3.5 h, the blood vessel network in the chorioallantoic membrane became brightly colored (Fig. S2), indicative of oxidized hemoglobin in embryonic erythrocytes.

The SpO2 on D19 tended to be higher than that on D17, even in the Trmt-1 group without OGI. The SpO2 on D19 was measured only in the surviving embryos; this most likely reflects the elimination of dead embryos with a low SpO2 on D17–D19.

Although the average SpO2 was higher in the Trmt-2 group, they exhibited large standard errors (SEs; Fig. 1A). Although the reason for such large SEs is unclear, we speculate that the efficiency of O2 absorption by each embryo might differ for various reasons, such as slight differences in the fine structure of culture vessels, minute wrinkles in the plastic film, the position and location of embryos in the culture vessel, and differences in embryo size.

The transition from allantoic gas exchange to pulmonary ventilation in avian species starts when the embryo beak pierces the chorioallantoic membrane before hatching and reaching the air cell. In intact shelled chicken eggs, this transition to pulmonary ventilation starts approximately on D19 (Burton and Tullett, 1985). In cSLC, the embryo survival rate starts to decline approximately on D17 (Tahara and Obara, 2014), which is before the start of the transition to pulmonary ventilation. In this study, the mean embryo SpO2 concentration had a large SE in the D17-cSLC group compared with the IC group. The same was observed between the Trmt-1 and -2 groups and the IC group on D19. From these observations, we hypothesized that embryos with a high SpO2 on D17 can survive until D19, whereas those with a low SpO2 on D17 in cSLC develop hypoxia associated with renal tubule lesions. A method to measure SpO2 without removing the embryo from the cSLC needs to be devised to verify this hypothesis.

Future research should be directed towards optimizing the timing, methods, and quantity of the oxygen supply to improve the survival rate and hatchability of embryos in cSLC.

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Author Contributions

Katsuya Obara designed and conducted the experiments and wrote the paper; Chizuka Obara (Henmi), Ikki Mitsui and Yumi Une prepared and examined tissue samples; Mitsuru Naito, Atsushi Asano and Atsushi Tajima discussed the results and contributed to the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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