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Influence of *In vitro* Culture System on Development of Quail Embryo

By

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Summary：An *in vitro* quail embryo culture system was developed using an artificial vessel, which comprised a polytetrafluoroethylene membrane and a polypropylene tube. *In vitro* culture was set up either directly from the blastoderm stage or after 60 hours incubation from the blastoderm stage, in the presence or absence of airspace. When embryos were cultured from the blastoderm stage, embryo viability gradually decreased in the presence of airspace from the initial stage of culture until stage 45, just before hatching. When embryos were preincubated for 60 hours before *in vitro* culture, viability initially remained high although mortality was high at 9 days and beyond, and all embryos died before reaching stage 45 in the absence of airspace. Using an artificial vessel under this embryo culture system, developmental stage of dead embryos was delayed, and body weight and 3rd toe length were lower than in controls (intact egg embryos). In addition, several types of malformations were observed at the blastoderm stage, and we conjectured that early stage ectodermal damage had contributed to teratogenesis and early mortality. Moreover, the absence of airspace contributed to mortality in the later developmental stages.

Key words：*in vitro* culture system, avian embryo culture, malformations, Japanese quail

Introduction

Most embryonic growth in avian species occurs within the egg. In addition, 80% of minerals required for embryonic growth are provided by the eggshell[1]. Therefore, embryonic development is difficult within abnormal or cracked eggshells and outside the eggshell. This can be resolved by using an embryo culture system termed the surrogate eggshell system[2], which involves a mechanism peculiar to avian species. PERRY[2] devised an *in vitro* culture system for developing chick embryos from the unicellular stage. This system comprised 3 systems (Systems I, II, and III). In System I, unicellular embryos were collected from the oviduct and incubated in a glass vessel for 24 hours to attain the blastoderm stage. In System II, the blastoderm stage was incubated for 2-3 days in the absence of airspace in a surrogate eggshell. In System III, after initial incubation for 2-3 days under System II, the embryo was cultured in a large surrogate eggshell in the presence of airspace until hatching. Perry's method was also applied to Japanese quail[3]. If this embryo culture system could be successfully applied to embryos of rare or endangered avian species within abnormal or cracked eggshells, it would greatly enhance their protection and breeding management. Although a surrogate eggshell of suitable size for avian species was not available to us, additionally, there was a high possibility of surrogate eggshells being infected, e.g., by pathogenic bacteria. Therefore, avian *in vitro* embryo culturing using artificial vessels was performed, mainly using Japanese quail. Embryo viability achieved for Japanese quail in Systems I[3], II[4], and III (hatching)[5] was 80%, 65%, and 43%, respectively. Therefore, an *in vitro* culture system using artificial vessels seems to be consummative in theory.

However, artificial vessels were used only for a part of the incubation period in these experiments, and a successful example of shell-less culture system using artifi-

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cial vessels for embryonic development from Systems I or II to System III has not been reported to date. In addition, studies on artificial vessel culture were mainly conducted in System III, while there are few data on embryo culture using artificial vessels in System II.

The influence of an *in vitro* culture system was examined, which began from System II or III on Japanese quail embryonic development.

Materials and Methods

(1) Eggs

Fertilized Japanese quail eggs were obtained from Tokaiyuki Co. (Aichi, Japan).

(2) Culture Vessels

The artificial vessels and calcium lactate weights used were as described by Kamihira *et al.*[5]. Fig. 1 depicts the artificial culture vessel developed in the present study. In addition, 35 ml distilled water was added to a 50 ml polypropylene tube (BD Falcon Conical Tube ; Nihon BD Co., Tokyo, Japan). A round-bottomed sac was constructed from a gas-permeable porous polytetrafluoroethylene (PTFE) membrane (Milliwrap ; Nihon Millipore Co., Tokyo, Japan) and a polypropylene tube (artificial vessel). Quail egg contents were transferred to the artificial vessel in the presence (Fig. 1A) or absence (Fig. 1B) of airspace, and sealed with Milliwrap and polyvinylidene chloride film (Saran Wrap ; Asahi Kasei Life and Living Co., Tokyo, Japan). All materials were sterilized by autoclaving before culture vessel assembly.

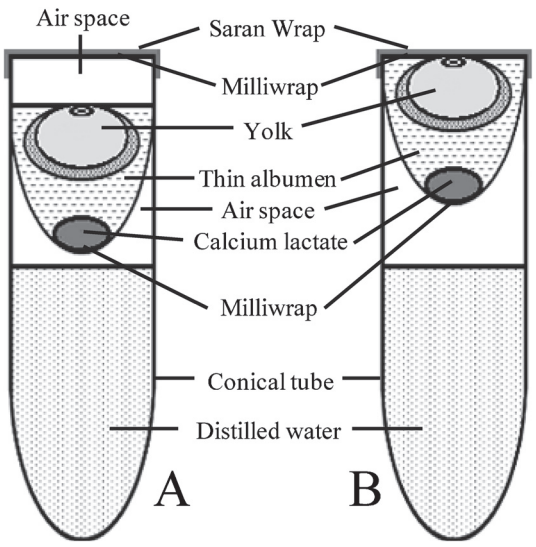

Fig. 1 Quail embryo culture vessels in the presence (A) and absence (B) of airspace ; the vessels were sealed with Saran Wrap and Milliwrap.

(3) Embryo Culture

Embryo cultures were initiated by addition of either blastoderm stage embryos or embryos that have undergone 60 hours preincubation from the blastoderm stage. Intact fertilized eggs, which were used as controls were incubated in their own eggshells at 38.0℃ and relative humidity of 60% while being rocked at 60°/hour.

Thin albumen (1.5 ml, derived from White Leghorn eggs) supplemented with 35 mg calcium lactate (Sigma-Aldrich Co., Tokyo, Japan) was added to the culture tubes. The embryos were cultured in an incubator equipped with an automatic rocking device (Type P-008 ; 60°/hour ; Showa Furanki Co., Tokyo, Japan). The culture was maintained at 38.0℃ and 60% relative humidity. Rocking was stopped 2 days before the expected hatching day. Milliwrap and Saran Wrap were punctured to facilitate pulmonary respiration 1 day before expected hatching day.

(4) Identification of Embryonic Developmental Stage

Embryo viability was confirmed daily, and dead embryos were removed. The criteria for embryonic death were cessation of blood flow, fetal activity, or cardiac arrest. Dead embryos were removed with the vitelline sac and egg yolk. The body weight and 3rd toe length of the embryos were measured. Embryonic developmental stage was determined according to the report by Hamburger and Hamilton[6].

(5) Statistical analysis

A significant difference ($p<0.05$) in viability was observed between the presence and absence of airspace in both the blastoderm stage and 60 hours preincubation, after analyses using the chi-square test. Statistical analyses of body weight and 3rd toe length were performed by Scheffé's multiple comparison test ($p<0.05$).

Results

(1) Viability and Developmental Stage of *In vitro* Cultures Set up from the Blastoderm Stage

Fig. 2 depicts the results for viability and hatchability of Japanese quail embryos. In the presence of airspace, embryonic viability gradually decreased from 1 day (100%, 52/52) to 15 days (19.2%, 10/52), and decreased further at 16 days (15.4%, 8/52). All embryos died by 17 days, while 8 embryos attained stage 45 (15.4%). In contrast, in the absence of airspace, embryonic viability gradually decreased from 1 day (100%, 58/58) to 8 days (65.5%, 38/58) and then decreased sharply on the 9th day (46.6%, 27/58). All embryos were dead by 17 days, and no embryo reached stage 45. Fig. 3A depicts the distribution of developmental stages attained by the dead

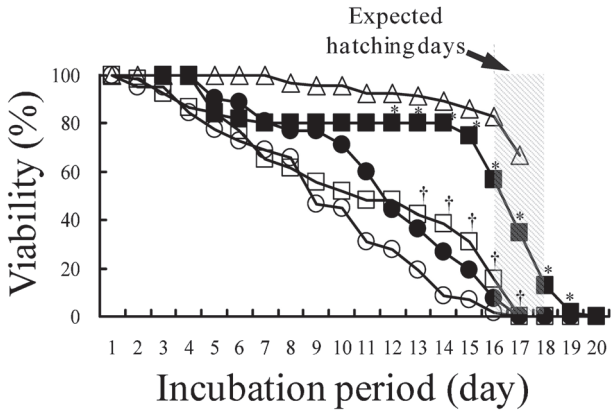

Fig. 2 Viability of quail embryos
In vitro culture was set up from the blastoderm stage in the presence (□ : $n=52$) or absence (○ : $n=58$) of airspace, and after 60 hours preincubation in the presence (■ : $n=55$) or absence (● : $n=52$) of airspace; controls (△ : $n=93$).
A significant difference ($p<0.05$) in viability was observed between the presence and absence of airspace in cultures from the *blastoderm stage or †60 hours preincubation, after analyses using the chi-square test.

embryos. In *in vitro* culture and the presence of airspace, the most developed stage attained was stage 45 on 16th and 17th days (15.4%, 8/52). In contrast, in *in vitro* culture in the absence of airspace, the most developed stage attained was stage 42 on the 14th day (1.7%, 1/58).

(2) Viability and Developmental Stage of *In vitro* Cultures Set up after 60 hours Preincubation

All embryos cultured in the presence of airspace survived up to 4 days. Embryonic viability was >80% from 6–14 days, and sharply decreased from 15 days (74.5%, 41/55) to 17 days (34.5%, 19/55). Almost all embryos had died by 20 days, and 10 embryos attained stage 45 (18.2%). In contrast, all embryos cultured in the absence of airspace survived up to 4 days, but mortality rapidly increased from 10 days (71.2%, 37/52) to 12 days (44.2%, 23/52). All embryos had died by 17 days without attaining stage 45. Fig. 3B depicts the distribution of developmental stage of dead embryos. The developmental stage attained under *in vitro* culture in the presence of airspace was stage 46 at 16 days (1.8%, 1/55). In contrast, under *in vitro* culture in the absence of airspace, the most developed stage attained by the dead embryos was stage 40 at 16 days (1.9%, 1/52).

(3) Viability and Developmental Stage of Intact Eggs

All embryos survived up to 7 days, embryonic viability gradually decreased from 8 days (96.8%, 90/93) to 15 days (86.7%, 80/93). Embryos hatched from 16 days to

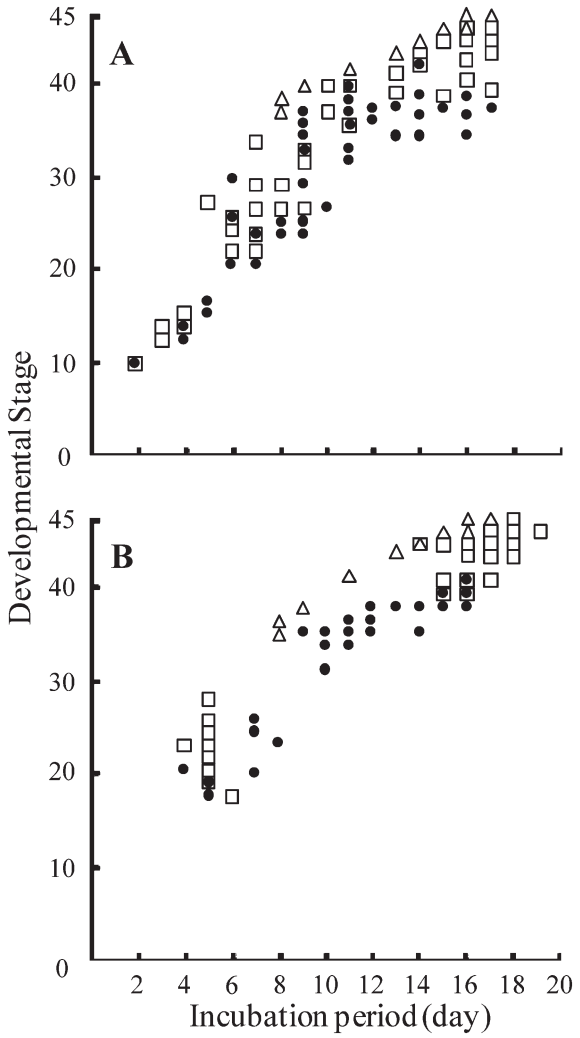

Fig. 3 Developmental stages attained by dead quail embryos
Embryonic culture set up from (A) the blastoderm stage or (B) 60 hours preincubation. In vitro culture in the presence (□) or absence (●) of airspace ; controls (△).

17 days (66.7%, 62/93), and 4 embryos attained stage 45 (4.3%, 4/93). Fig. 3 depicts the distribution of developmental stage for dead embryos. The most developed stage attained by the dead embryos was stage 46 at 16 days and 17 days (11.8%, 11/93).

(4) Body Weight and 3rd Toe Length of Dead Stage 45 Embryos

Fig. 4 reveals that embryos that died at stage 45 had lower body weight and shorter 3rd toe length ($p<0.05$) than the control, and that embryos cultured from the blastoderm stage had lower body weight and shorter 3rd toe length than 60 hours preincubation embryos ($p<0.05$).

(5) Malformations

Table 1 summarizes data regarding the rate of malfor-

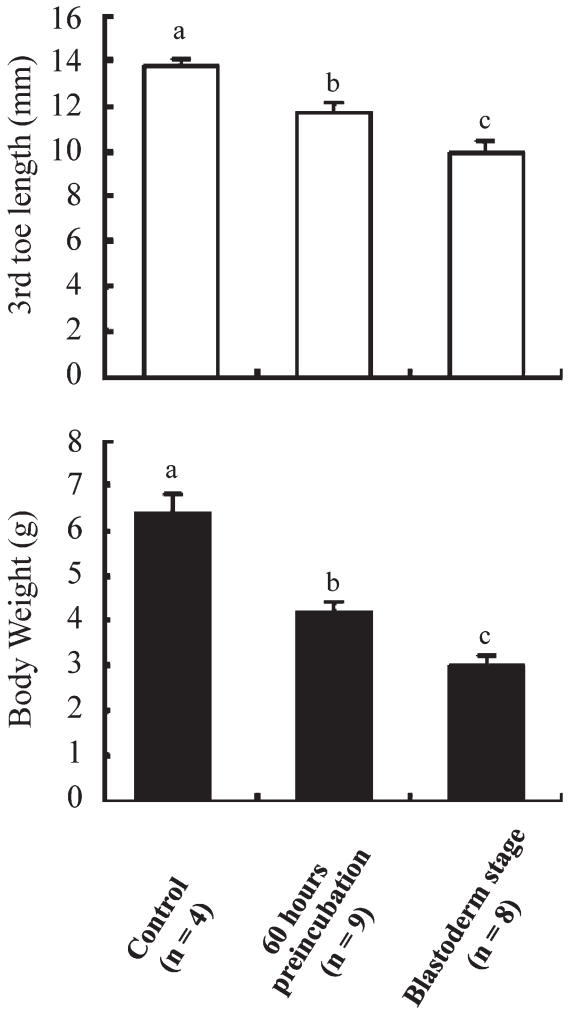

Fig. 4 Average body weight and 3rd toe length of dead embryos at stage 45
White columns represent 3rd toe length ; black columns represent body weight. Columns and vertical bars represent the mean and standard error of mean values, respectively. Different letters represent significant differences ($p<0.05$) as assessed by Scheffé's test.

mation in embryos cultured from the blastoderm stage in the presence (17.3%, 9/52) or absence (22.4%, 13/58) of airspace. Malformations were not observed in 60 hours preincubation embryos or intact fertilized eggs. Table 2 and Fig. 5 show the types of malformations observed, including anophthalmia and microphthalmos [69.2%, 9/13 (presence of airspace) ; 64.3%, 9/14 (absence of airspace)] in artificial vessel culture, with these two conditions accounting for the majority of malformations.

Discussion

KAWASHIMA *et al.*[4] reported 65% embryonic viability after 55 hours incubation from the blastoderm stage using a PTFE membrane and a plastic case in the absence of airspace, and with calcium addition in Japanese quail. In the present study, embryonic viability after 55 hours incubation start from the blastoderm stage with calcium addition was higher (>90%) than that reported

Table 1 Incidence of embryonic malformations

Experimental Group	Airspace	No. of embryos	Incidence of malformations
Blastoderm stage	+	52	9 (17.3%)
	−	58	13 (22.4%)
60 hours preincubation	+	55	0 (0%)
	−	52	0 (0%)
Control		93	0 (0%)

+: Presence of airspace; −: Absence of airspace

Table 2 Types of malformations observed in cultures using blastoderm stage embryos

Type of malformation	Airspace	
	+	−
Anophthalmia and/or Microphthalmos	9 (69.2%)	9 (64.3%)
Acrania	2 (15.4%)	3 (21.4%)
Hypoplastic breastbone	2 (15.4%)	1 (7.1%)
Digit hypoplasia	0 (0%)	1 (7.1%)
Total	13	14

+: Presence of airspace; −: Absence of airspace

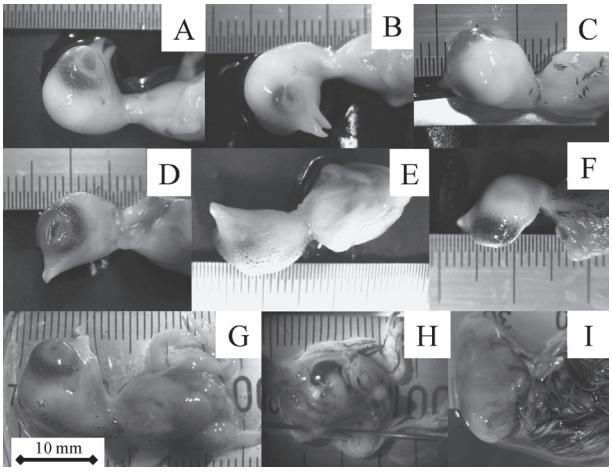

Fig. 5 Types of observed embryonic malformation
A, B and C : microphthalmos of left eye (same embryo) ; D, E and F : anophthalmia of right eye ; G : normal embryo ; H : evisceration caused by hypoplastic breastbone ; I : brain exposure caused by acrania.

by KAWASHIMA *et al.*[4] regardless of the presence or absence of airspace. KAMIHIRA *et al.*[5] reported that hatchability was improved by the addition of calcium lactate or eggshell powder to the culture medium in artificial vessel culture. The addition of calcium appears to enhance embryonic viability at the early stage of development under *in vitro* culture using artificial vessels.

Embryonic viability was >80% up to 14 days, but decreased sharply by 20 days when embryo culture was set up using 60 hours preincubated embryos in the presence of airspace. This culture system was referred to by KAMIHIRA *et al.*[3], and the change in viability is similar to that reported by KAMIHIRA *et al.*[3]. In the present study, viability was higher after 9 days in the presence of airspace than in its absence, regardless of the blastoderm stage or 60 hours preincubation, and embryonic viability in the absence of airspace gradually decreased between 10–17 days. PERRY[2] observed that an excess of medium above the embryo was not detrimental in System III until 8 days, when the embryos died unless the vascular extraembryonic membranes were exposed to the atmosphere, as in System III. Therefore, the absence of airspace may contribute to low viability and high mortality of embryos after 9 days.

Embryonic viability in the presence of airspace was sharply decreased from between 15–16 days, regardless of the blastoderm stage or 60 hours preincubation. Decreased viability in the later stages of Japanese quail embryo culture was reported by ONO *et al.*[3] and KAMIHIRA *et al.*[5] using artificial vessels, and by NIRASAWA *et al.*[7] using chick eggshells. Furthermore, decreased viability in the later stages of chick embryo culture was reported by DUNN[8] and FUJITA *et al.*[9] using artificial vessels, and by KAWASHIMA *et al.*[10] and FUJITA *et al.*[11] using surrogate chick eggshells. Because the allantoic membrane circulation transitions to the lungs post 19 days incubation in the chick[10,12,13], decreased viability might be responsible for the failure of the artificial vessel to compensate for increased embryonic oxygen requirement during transition within the circulatory system.

In contrast, lower hatchability has been reported when carbon dioxide concentration is high or oxygen concentration is low during the culture period[14–18]. Therefore, the low hatchability of embryo culture systems may be due to either the low oxygen or high carbon dioxide concentration.

The developmental stage of dead embryos was delayed in all embryo culture system groups, and it was slower in artificial vessel culture set up from the blastoderm stage than for 60 hours preincubation. It was shown that the longer the embryo was cultured *in vitro*, the slower the embryo developmental stage. FUJITA *et al.*[8] reported that the embryonic developmental stage was delayed when chick embryos were cultured in artificial vessels. However, on the basis of only the observations during embryonic developmental stage it cannot be concluded that delayed death at the developmental stage was due to culture in artificial vessels.

JOHNSTON and COMAR[1] suggested that approximately 80% of the calcium that is necessary for avian embryonic growth during the developmental stage is provided by the eggshell. In the present study, we added calcium lactate to the artificial vessel culture medium (with thin albumen). However, embryos were smallest when cultured *in vitro* from the blastoderm stage in terms of body weight and 3rd toe length, followed by 60 hours preincubation and controls. It was demonstrated that the longer the embryo was cultured *in vitro*, the smaller the embryo size. ONO and WAKASUGI[19] reported that the embryonic body weight in Japanese quail was high and 3rd toe length was long in the controls (intact embryos) under both mineral rich culture using chick eggshell and mineral free culture using Saran Wrap. KAMIHIRA *et al.*[5] also reported that body weights of embryos dying at 17 days were higher than those of controls under artificial vessel culture, regardless of calcium addition. In contrast, FUJITA *et al.*[20] reported that embryonic mineral content significantly ($p<0.05$) increased between 7–21 days in chicks. NAKANE and TSUDZUKI[21] reported that the calcification of the 3rd toe was observed on the 9th day post incubation in Japanese quail. Therefore, it was considered that minerals were not absorbed by embryos even if enough minerals were added to the culture medium in artificial vessel culture.

In contrast, RUIJTENBEEK *et al.*[22] reported that hypoxic embryonic body weight and visceral weight were lower for normoxic chick embryos incubated under normoxic (21% O_2) or hypoxic (15% O_2) conditions. Therefore, we conjectured that low body weight, short 3rd toe length, and delay in embryo development were due to the hypoxic status used in artificial vessel culture. It was considered that mineral absorption and aeration of culture were necessary in future embryo studies.

In this study, malformations were observed in artificial vessel culture set up from the blastoderm stage embryos. NIRASAWA *et al.*[22] reported that although malformations were observed in the cultured embryos (23.5%) in the presence of airspace in System II (surrogate chick eggshells), malformations were not observed when the surrogate eggshell was filled with thin albumen. KAWASHIMA *et al.*[13] concluded that early stage embryos with incomplete amnion are adversely affected by direct exposure to the atmosphere in System III (chick embryos cultured in surrogate eggshells). Amnion formation was found to be

complete after 65–69 hours or 55–63 hours incubation, in chick[10] and Japanese quail, respectively[23]. In the present study, because embryonic malformations were observed when the blastoderm stage embryos were developed *in vitro*, drying by the airspace or contact stimulus by the PTFE membrane in early embryogenesis may have contributed to teratogenesis. Furthermore, we observed numerous ocular malformations, similar to that reported by Nirasawa *et al.*[6] (82%, 18/22 versus 88%, 22/25). Ectodermal defects may be responsible for the high frequency of ophthalmic teratogenesis, as the eye develops from the ectoderm[24].

On the basis of the results, artificial vessel culture appears to contribute significantly to the increase in mortality and incidence of teratogenesis when quail embryos were cultured from the blastoderm stage to hatching. Consequently, environmental improvement in embryo culture at an early stage is considered to contribute to the reduction in both mortality and incidence of teratogenesis.

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人工容器培養がウズラ胚の発生におよぼす影響

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要約：ポリテトラフルオロエチレン（PTFE）膜とポリプロピレン製のコニカルチューブを用いて人工容器を作製し，ニホンウズラ胚を培養した。ニホンウズラ胚は，胚盤葉期または孵卵 60 時間後から人工容器に移し，空気層の設置区・未設置区を設けた後 PTFE 膜とサランラップで封をした。また，人工容器に移す際には乳酸カルシウムを 35 mg 添加した。人工容器を用いて培養をした結果，胚盤葉期から培養した胚は気層の有無にかかわらず孵化直前である発生ステージ 45 まで徐々に生存率を下げていった。一方で，孵卵 60 時間後から培養した胚は高い生存率を維持していたが，9 日目以降に空気層の未設置区では生存率が下がったのに対し設置区では高い生存率を維持していた。空気層を設置した区では，人工容器での培養開始時期に関係なく孵化直前であるステージ 45 まで到達し端打ちを行う胚が見られた。一方，空気層の未設置区では，人工容器での培養開始時期に関係なく全ての胚がステージ 45 に到達する前に死亡した。

人工容器で培養し，死亡した胚を同じ培養日数または同じ発生ステージで自然発生胚と比較した結果，人工容器で培養した胚には，発生の遅れや体重，第三趾長が小さい事が分かった。また，胚盤葉期から人工容器で培養し，死亡した胚を同じ培養日数または同じ発生ステージで孵卵 60 時間後から培養した胚を比較した結果，発生の遅れや体重，第三趾長が小さい事が分かった。また，胚盤葉期から人工容器で培養した胚にのみ眼に関する奇形が見られた。そして，このことから培養初期の外胚葉へのダメージが奇形発生と培養初期の死亡率に関係があると推察された。

キーワード：人工容器，鳥類胚培養，奇形，ニホンウズラ

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