BIOLOGY, MEDICINE, & NATURAL PRODUCT CHEMISTRY ISSN 2089-6514 (paper)
Volume 15, Number 1, April 2026 | Pages: 293-297 | DOI: 10.14421/biomedich.2026.151.293-297 ISSN 2540-9328 (online)

Optimizing Injection Techniques for Avian Embryos: A Practical Model for Experimental Embryology

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Manuscript received: 07 December 2025. Revision accepted: 13 April 2026, Published: 21 April 2026.

Abstract

Background:Avian embryos such as chickens and ducks offer an ethical and practical model to study early development and test experimental procedures. However, differences in *in-ovo* injection techniques can affect embryo survival and the consistency of results. This study aimed to find the most effective injection method to improve the success and reproducibility of avian embryo research. Methods: A total of 60 fertilized eggs (30 chicken and 30 duck eggs) were used. They were randomly assigned into six groups: control, horizontal injection, and vertical injection (10 eggs per group for each species). Each treated egg received 0.25 mL of sterile 0.9% saline through a fine 27G syringe and was incubated at 37±0.5°C with 55–60% humidity for five days. Embryo growth was evaluated daily based on the formation of blood vessels, which can be seen by examining the eggs, and differences in egg weight. After the fifth day, the eggs are broken open and the embryos are observed by body length, morphology formation. Results: Vertical injection through the pointed end of the egg resulted in the highest normal development rates (70% in chickens and 80% in ducks). In contrast, horizontal injection caused almost complete growth failure, while control embryos developed normally. Statistical analysis showed a significant effect of injection orientation on embryo survival ($p < 0.01$). Conclusion: Injecting vertically through the pointed end of the egg is a reliable and low risk method for manipulating avian embryos. This optimized approach ensures better embryo survival and can be applied in future studies involving developmental biology, toxicology testing, stem cell research, and gene editing experiments.

Keywords: Avian embryo; in ovo injection; embryology model.

INTRODUCTION

Optimizing experimental techniques in avian embryos, particularly in ovo injection, is essential to ensure consistent survival and normal development throughout incubation. Improper injection angle, entry point, or penetration depth can cause damage to extra embryonic membranes, contamination, or hemorrhage, thereby reducing embryo viability and reproducibility of experimental outcomes (Cloney, K., et al., 2020; Chen, L., et al. 2021). Establishing a standardized, low mortality method will enhance the reliability of avian embryos as models for drug testing, molecular delivery, and developmental manipulation.

Recent advances have refined avian embryo handling through ex ovo culture systems, chorioallantoic membrane (CAM) assays, and precision in ovo microinjection techniques that enable localized delivery of genetic or pharmacological agents (Moreno, J.I., et al., 2017; Henley, T., et al. 2019; Samela, A. et al, 2024). These models bridge the gap between ethical restrictions in mammalian embryology and the need for dynamic, accessible systems to study organogenesis and experimental teratology (Smith, S.M., et al. 2012; Szabó, R., et al. 2024; Wachholz, G. E., et al. 2021). This allows for experiments or interventions to understand how these processes occur. This also allows for experiments or interventions to understand how these processes occur.

The use of chicken embryos enables the development of innovative techniques for developmental analysis and therapeutic testing. Researchers can apply multiple methods to monitor and manipulate embryonic growth. (Kaplan Arabaci., et al, 2025; Samela, A. et al, 2024; Smith, S.M., et al. 2012; Szabó, R., et al. 2024; Wachholz, G. E., et al. 2021). This approach allows for the identification of specific factors involved in tissue formation and development, closely mirroring processes in human embryos. Embryonic research offers significant potential for innovation in the field of medical health with technological advances and an increasing understanding of cell biology (Soczyńska, J., et al, 2025). Integration of basic research will provide solutions for understanding and treating various diseases. This is

related to embryonic development and improves the effectiveness of future therapies.

MATERIALS AND METHODS

Study Design and Ethical Approval

This experimental study was conducted using fertilized eggs of chicken (*Gallus gallus domesticus*) and duck (*Anas platyrhynchos domesticus*) to evaluate optimal in ovo injection techniques for embryological intervention. The study protocol received ethical clearance from the Research Ethics Committee, Faculty of Medicine, Universitas Islam Negeri Syarif Hidayatullah Jakarta (Approval No. B-088/F12/KEPK/TL.00/12/2023).

Sample Allocation and Experimental Groups

A total of 60 fertilized eggs were used in this study. Those eggs consisted of 30 chicken and 30 duck eggs, which were obtained within 24 hours post laying from certified local breeders at Reni Jaya Pamulang, Tangerang Selatan, Indonesia. All eggs were visually inspected to ensure intact shells and uniform size. Eggs were randomly allocated into six groups. Each group containing 10 eggs:

- Control A: Chicken (no injection)
- Control B: Duck (no injection)
- Treatment 1A: Chicken with horizontal injection
- Treatment 1B: Duck with horizontal injection
- Treatment 2A: Chicken with vertical injection
- Treatment 2B: Duck with vertical injection

All eggs were incubated at 37±0.5°C and 55–60% relative humidity in a forced air incubator equipped with automatic rotation every 2 hours. Temperature and humidity were monitored using a calibrated digital hygrometer.

Injection Procedure

The control groups received no injection, to see the normal development of the embryo. We can see the structure of the egg in Figure 1.

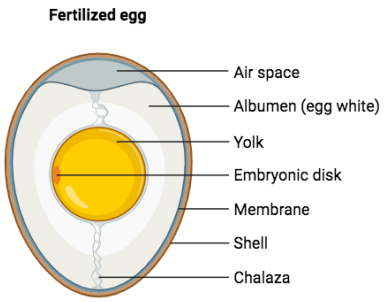

Figure 1. The Structure of Egg (created in BioRender.com).

In treatment group 1, both chicken or duck egg injection was performed horizontally through the lateral surface of the eggshell, penetrating the inner membrane along the equatorial axis. In treatment group 2, injection was carried out vertically through the pointed pole, penetrating the inner membrane toward the albumin. Each egg received 0.25 mL of sterile 0.9% NaCl solution, administered with a 0.5 mL insulin syringe fitted with a 27-gauge needle. This volume was selected based on prior in ovo optimization studies reporting minimal mechanical disruption and high embryo viability (Cloney, K. et al., 2020; Chen, L., et al. 2021). All treatment shown at Figure 2.

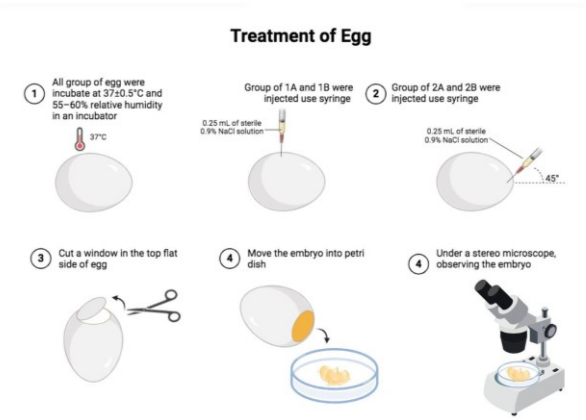

Figure 2. Treatment of Egg (created in BioRender.com).

The saline solution served to evaluate the mechanical effect of injection, such as needle penetration and fluid displacement, without introducing pharmacological variables. Before injection, the shell surface was disinfected with 70% ethanol. After injection, the puncture site was sealed with sterile band aid to prevent contamination and dehydration. Then, all of the eggs are placed again at the incubator, with the same temperature and humidity.

Incubation and Observation

Post treatment, all eggs were incubated for 5 days (120 hours). Embryonic development was documented every 24 hours, by weight change, and also saw the dark spot shadow when light by flash light. On the fifth day, the shell of the egg was broken to observe the embryo. Observation was using a stereomicroscope equipped with an HD digital camera. Embryos were extracted for morphometric and viability assessment. Morphological features including body curvature, limb bud appearance, vascularization, and heartbeat, were recorded.

Developmental Assessment and Quantitative Criteria

Embryos were classified into three developmental categories (good, moderate, poor) based on both morphological and quantitative parameters (Table 1). Embryo measurements were performed using ImageJ (NIH, USA).

Table 1. Developmental Categories of Embryo.

Category	Morphological description	Quantitative criteria
Good	Normal morphology	Crown rump length ≥ 2.3 cm, ≥ 20 somite pairs
Moderate	Partial development with abnormal size or reduced movement	Crown-rump length 1.5–2.2 cm, 10–19 somite pairs
Poor	Arrested or failed development, no heartbeat, or hemorrhage	Crown-rump length < 1.5 cm, 0 somites

Data Analysis

Descriptive statistics were used to summarize embryo viability and development outcomes. Group comparisons were analyzed using the Chi-square test with significance set at $p < 0.001$.

RESULTS AND DISCUSSION

Embryo Viability and Morphological Development

A total of 60 embryos were analyzed after 5 days of incubation. All control eggs remained viable with normal morphological progression. Table 2 summarizes the developmental outcomes for each experimental group.

Table 2. Distribution of embryonic development outcomes across treatment group.

Group	Poor n (%)	Moderate n (%)	Good n (%)	Total (n)
Control A (Chicken)	1(10)	3(30)	6(60)	10
Control B (Duck)	0(0)	3(30)	7(70)	10
Treatment 1A (chicken, horizontal)	10(100)	0(0)	0(0)	10
Treatment 1B (duck, horizontal)	9(90)	1(10)	0(0)	10
Treatment 2A (chicken, vertical)	0(0)	3(30)	7(70)	10
Treatment 2B (duck, vertical)	0(0)	2(20)	8(80)	10

Embryo viability was significantly affected by the injection orientation. Vertical injection (Treatment 2) yielded the highest proportion of normally developed embryos (≥70%) in both species, whereas horizontal injection (Treatment 1) resulted in almost complete developmental failure. Chi-square analysis confirmed a significant association between injection orientation and developmental outcome $p < 0.001$). Chi-square analysis confirmed a strong association between injection group and developmental outcomes ($\chi^2 = 53.03$, df = 10, $p < 0.001$), with a large effect size (Cramer's V = 0.665) (Table 3).

Table 3. Summary of statistical analysis for the association between treatment group and developmental outcome.

Test	X^2	df	p-value	Effect Size (Cramer's V)	Interpretation
Chi-square	53.03	10	<0.001	0.665	Strong association

Morphological Observations

At day 5, embryos in the control and Treatment 2 groups showed active organogenesis characterized by a distinct head region, developing eye vesicles, limb buds, and well-defined vitelline vascularization (Figure 3). In contrast, Treatment 1 embryos exhibited growth arrest, hemorrhage, and loss of structural integrity (Figure 3). Average crown rump length in normally developed embryos was 2.4 ± 0.2 cm, consistent with the expected value for 5 days avian embryos.

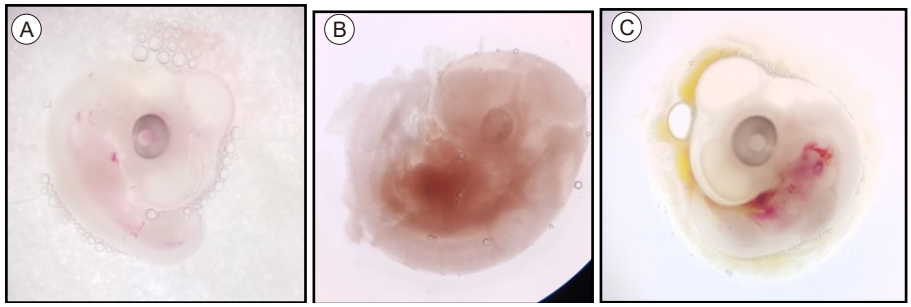

Figure 3. Morphological observation. (A. Control Group; B. Treatment 1; C. Treatment 2)

DISSCUSSION

Optimization of Injection Orientation

The present study demonstrates that vertical injection through the pointed pole of the egg produces markedly higher embryonic survival and more consistent development compared with horizontal injection. The improved viability is likely due to reduced damage to the blastoderm and extra-embryonic membranes, minimizing shear forces across the germinal disc. Similar observations were reported by Cloney, K., et al. (2018), who emphasized that injection depth and orientation are critical determinants of embryo survival in ovo models.

The results of this study indicate that vertical injection through the pointed pole of the egg results in significantly better embryo survival and development rates compared to horizontal injection. This difference is primarily due to the structure of the egg (Figure 1). The blastoderm of avian eggs is located directly beneath the blunt pole. The pointed pole has a thicker membrane and is relatively distant from the area of early embryonic development (Morishita, TY., et al, 2021). As we know that blastoderm are the embryonic precursor. (Lee Hyo-geon, 2016) Therefore, injection through the pointed pole minimizes the risk of blastoderm damage, thereby reducing the likelihood of early developmental disorders (Yasuda, T., et al, 2020). In contrast, horizontal injection has the potential to penetrate extra embryonic membranes, including the chorioallantois, yolk sac, and blood vessels, which are highly vulnerable during early embryogenesis. Damage to these structures can cause microhemorrhages, impaired nutrient distribution, and mechanical stress on the embryo, affecting viability and growth (Wick, S., et al. 2018).

Species Specific Response

Both chicken and duck embryos followed a similar pattern of response, although duck embryos exhibited slightly higher tolerance to vertical injection (80 % “good” outcomes) than chicken embryos (70 %). This may reflect minor anatomical differences in eggshell curvature and yolk to albumen ratio between species, which influence fluid distribution after injection (Biesek, J. 2023) The findings highlight that although general injection principles are transferable, species specific optimization remains essential for reproducible embryological experimentation.

Implications for Embryological and Biomedical Research

Optimized in ovo injection methods are fundamental for diverse applications, including:

- Delivery of gene editing reagents (e.g., CRISPR/Cas9 vectors),
- Introduction of stem or progenitor cells,
- Teratogenicity and drug toxicity screening, and
- Targeted tracing of developmental signaling pathways.

By minimizing procedural mortality, the vertical technique enhances model reliability while maintaining ethical and cost advantages compared to mammalian embryos. This supports the broader adoption of avian embryos as ethical, scalable, and transparent models for developmental and translational research. (Moreno, J.I., et al., 2017; Henley, T., et al. 2019)

Limitations and Future Directions

The study relied primarily on morphological endpoints; histological and molecular confirmation (e.g., Hamburger–Hamilton staging, gene expression analysis) were not performed. Future studies should integrate quantitative histomorphometry and molecular markers to confirm tissue differentiation stages and investigate the influence of injection depth and timing. Moreover, testing the optimized method across different incubation durations (e.g., 3–7 days) would provide a comprehensive viability curve.

CONCLUSIONS

This study successfully identified an optimal in ovo injection technique for avian embryo research. Vertical injection through the pointed pole of the egg provided the highest embryo viability and normal morphological progression in both chicken and duck models. The procedure minimizes mechanical trauma, prevents contamination, and maintains embryonic integrity throughout early organogenesis.

These findings establish a standardized and reproducible technique that can serve as a foundation for diverse experimental applications including molecular delivery, gene editing, and developmental toxicology, while adhering to ethical alternatives to mammalian embryo use. Future research integrating histological and molecular analyses will further validate this model as a reliable system for developmental and translational biomedical studies.

Acknowledgements: We would like to thank all of the parties which support our research, the members of the Department of Medical Biology, the members of Faculty of Medicine UIN Syarif Hidayatullah Jakarta, and also Institute of Research of Community Services (LP2M) UIN Syarif Hidayatullah Jakarta.

Authors’ Contributions: Nurin Nadzifatil Fitriyah designed the study, led the research until data analysis and wrote this manuscript. Suryani and Din Fitri Rochmawati carried out the laboratory work. Ahmad Nafi Ulumi, Abd El Rahman Irwan Maulana, Putri Shavina Shaqynia Nasution, Fa’iqotur Rokhmah, were held as research assistants. All authors read and approved the final version of the manuscript.

Competing Interests: The authors declare that there are no competing interests.

Funding: The authors declare that there is no funding support for this study.

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