Novel Chick Embryo Window Culture Model and the Effects on Development

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INTRODUCTION: The chicken embryo is an established animal model that enables studies of functional musculoskeletal tissue development.[1,2,3] Advantages of the chick embryo model over mammalian embryo models include isolation from maternal influences, easier access to the embryo, and affordability. We have extensively used this model to study embryonic tendon development, mechanobiology, and wound healing from early to late stages of development.[1,2,3,4] In some studies, we injured the calcaneal tendon[2] or gripped the leg in a bioreactor,[3] both which required direct access to the embryo. The standard protocol to do so is to "side-window" the egg, in which a hole is cut in the side of the shell by day 3 (D3) of the 21-day developmental period, before the vascular chorioallantoic membrane (CAM) adheres to the shell.[5] However, this method leads to poor near-hatch viability of 33%[3]. Here, we introduce a novel "aircell-window" technique to create a window in the shell over the aircell, a natural air pocket between the CAM and shell. We demonstrate that this technique can be performed after D3 and results in improved viability and more normal development than side-windowing. This aircell-windowing technique is useful for studies requiring direct embryo access, especially for late-stage musculoskeletal development studies.

METHODS: Fertilized White Leghorn chicken eggs were used for all groups. *Side-windowing:* At D3, 3 mL of albumen is extracted from a needle-sized hole drilled near either end of the egg and then sealed. A 2-cm diameter hole is cut in the side of the shell, the hole covered with tape, and the egg then incubated side-window up in a non-rocking incubator (**Fig. 1a**). *Aircell-windowing:* At D6 or D9, the aircell is outlined, and a smaller hole is cut within the outline 2 mm from the edge of the aircell. To access the embryo, a hole in the CAM is created via cautery to minimize bleeding. The window is covered with tape, and the egg is incubated aircell-window up in a non-rocking incubator (**Fig. 1b**). *Control:* Non-windowed eggs incubated in a rocking incubator served as controls. *Measurements:* Embryos were sacrificed at D18 via decapitation. Viability was measured as the percentage of embryos viable at sacrifice. Both 3rd toe and beak length were measured with calipers to stage embryos.[6] The whole embryo and dissected body parts were weighed. *Statistical analysis:* Based on data normality and variance equality, either ANOVA (with Tukey's or Dunnet's post hoc) or non-parametric Kruskal Wallis test (with Dunn's post hoc) was performed. Because of the binary nature, no statistical analysis was performed for D18 viability.

Results: D6 aircell-windowed eggs (D6-Aircell) had similar viability as D3 Side-windowed eggs (D3-Side). D9 aircell-windowed eggs (D9-Aircell) had higher viability than both D3-Side and D6-Aircell (**Fig. 2a**). 3rd toe and beak length measurements reflected that D3-Side significantly impacted gross development of chick embryos, whereas Aircell group embryos developed more normally (**Fig. 2b, c**). Similarly, whole body and average leg mass measurements suggested that D3-side was underdeveloped whereas the Aircell groups developed normally (**Fig. 2d, e**). The liver masses of the D3-Side and D6-Aircell groups were smaller than that of controls. In contrast, the liver mass of the D9 aircell-windowed group was no different from the controls and larger than D3-Side and D6-Aircell (**Fig. 2f**). The lung mass of D3-Side and D6-Aircell were lower than the control, D9-Aircell was no different than the control, and windowed groups were no different from one another. (**Fig. 2g**). The heart masses of all windowed groups were significantly lower than those of controls but not different from one another (**Fig. 2h**).

Discussion: We selected days of windowing based on the standard side-windowing protocol (at D3)[5] and the timing of limb movements,[7] as movement is needed for tendon development.[4] Albumen removal, time of non-rocking, and separation of the CAM and inner shell membrane likely contributed to differences in viability and development between the windowing techniques. Albumen provides water and nutrients to the embryo, and partial removal of albumen decreases embryo weight near hatch.[8] Thus, albumen removal may have contributed to the low embryo mass in D3-Side eggs. Aircell eggs may have had normal embryo masses because no albumen was removed. Periodic egg rocking, naturally performed by hens, promotes proper adhesion of the CAM to the shell and nutrient distribution.[9] Absence of rocking from D3-D8 increases mortality in broiler chickens.[10] D3-Side and D6-Aircell were no longer rocking starting at D3 and D6, respectively, which may have contributed to their poor viability. In contrast, D9-Aircell was rocking until D9, likely reducing negative impacts of non-rocking. The aircell-windowing technique is attractive for late developmental stage chick embryo studies in which limb tendons (and other musculoskeletal tissues) need to be accessed, because it results in more normal development than side-windowing and with higher viability.

SIGNIFICANCE: Here we establish a novel aircell-windowing technique that improves late-stage viability and decreases negative impacts on development as compared to the standard windowing technique, thereby enabling studies of late-stage embryos with reduced loss of life.

REFERENCES: [1] Marturano et al. 2013 *Proceedings of the National Academy of Sciences, 110(*16*)*; [2] Nguyen et al. 2023, *Scientific Reports, 13(1);* [3] Stein et al. 2019, *Tissue Engineering Part C: Methods, 25(*11*);* [4] Pan et al. 2018, *Philosophical Transactions Royal Society B, 373*; [5] Korn et al. 2007 *Journal of Visualized Experiments, (8)*; [6] Hamburger et al. 1951 *Journal of Morphology, 88(1)*; [7] Wu et al. 2001, *Journal of Experimental Zoology, 291(2);* [8] Willems et al. 2014, *World's Poultry Science Journal, 70(*3*)*; [9] Cutchin et al. 2009, *Journal of Applied Poultry Research, 18(*3*)* [10] Elibol et al. 2004, *British poultry science, 45(5)*

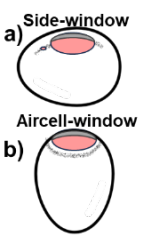

Fig. 1: Side- (a) and aircell-window (b) models.

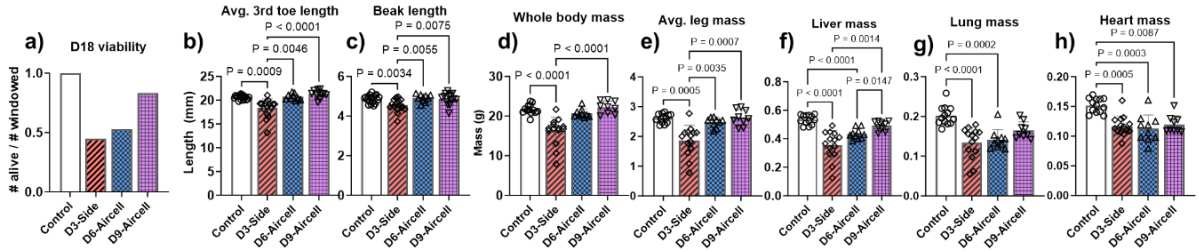

Fig. 2: D18 embryo viability (a), average 3rd toe length (b), beak length (c), whole body mass (d), average leg mass (e), liver mass (f), lung mass (g), and heart mass (h) for control, D3-Side, D6-Aircell, and D9-Aircell embryos. N ≥ 18 embryos per group were used for viability, and N ≥ 9 embryos were used per group for other measurements. P<0.05 was considered significant.