

## CULTURE SYSTEM FOR CHICKEN EMBRYOS USING A PLASTIC FILM AS CULTURE VESSELS

Liana Mihaela FERICEAN, I. BANATEAN-DUNEA, Mihaela OSTAN,  
Silvia PRUNAR, F. PRUNAR, Olga RADA

*Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania", Faculty of Agricultural Sciences, Aradului St. 119, Timisoara-300645, Romania*

*Corresponding author: liana.fericean@gmail.com*

**Abstract.** *The aim of this work was to develop a shell-less culture system for chicken embryos. There were examined the conditions required for embryonic hatching by comparing factors such as the addition of calcium lactate and optimal moisture monitoring, and the plastic wrap along with a glass were used as culture medium. Observations on the development of the chickens (*Gallus gallus domesticus*) embryo were made from day 1 until the hatching age of the chick, day 21. In our study, the embryos were transferred to the artificial environment after different numbers of days, starting on day 0 (without being preincubated), 24, 48, 55 and 72 hours after preincubation. A number of 10 eggs were used in each sample. As study material we used eggs from mixed breed, kept free chickens from private households. The data obtained showed that the pre-incubation period until the transfer of embryos to the culture vessel influenced the viability and success of the embryo. Transfer of embryos to the culture vessel without preincubation is not recommended because the embryos did not survive more than three days. The transfer of the embryos to the culture vessel at 24 hours led to the survival of the embryos until the fifth day, reaching the maximum mortality on the fourth day. The highest viability was obtained at 72 hours of preincubation, and when the embryo was transferred to the culture vessel after 80 hours, it was difficult to transfer without destroying the yolk membrane.*

**Keywords:** *shell free culture, chicken embryos*

### INTRODUCTION

The development of the embryo remains a miracle even when we manage to decipher some of its secrets. For our understanding, the evolution of the embryo in the bird's egg could be the most suggestive way to have an image of what happens when a new life appears.

After Kamihira et al., 2005 and Kyogoku et al., 2008 a shell-less culture, where a chick embryo is taken from an eggshell and cultured in an artificial environment, could be helpful for the preservation of rare birds, if it can be applied to saving damaged eggs. Andacht et al.(2004) consider that the windowing of an eggshell to facilitate gene introduction and other manipulations significantly reduces the hatchability. This technique also potentially plays a role in the education of school children in life sciences, through the direct observation of embryonic development. (Yutaka et al., 2014).

Numerous papers have been reported for quails on using preincubated embryos in surrogate eggshells as culture vessels it was obtained successfully live birds that reached sexual maturity. Most researchers have studied the effect of embryonic manipulation on chick hatchability (Perry 1988, Ono et al., 1994; Kamihira et al., 1998; , Kamihira et al. (1998) Kamihira et al.,2004 Borwornpinyo et al., 2005, Ono et al., 2005;

Dragan et al, 2011; Kato et al., 2013 consider that the development of avian embryo depends is influenced by the maternal nutrition packaged into the eggshell.

## MATERIAL AND METHODS

**Biological material.** As biological material we used eggs from mixed breed, kept free chickens from private households (Fig. 1).

**Method of culture.** To reconstitute the culture medium by using an artificial vessel was used a transparent glass, transparent plastic foil, Petri dish lid, calcium supplements and water.

The plastic foil was molded on an oval surface in the shape of an egg and removed the wrinkles when made the culture vessel, because the presence of wrinkles leads to lower survival rates. After molding the plastic wrap in the bowl, was added lactic calcium, which we hydrated with distilled water.

At the bottom of the glass was made a hole with a diameter of 1 cm, and the hole was covered with a sterile cotton bandage. A solution with disinfectant (50 ml) was introduced into the bottom of the beaker to maintain a clean, bacteria-free environment (Figure 2).

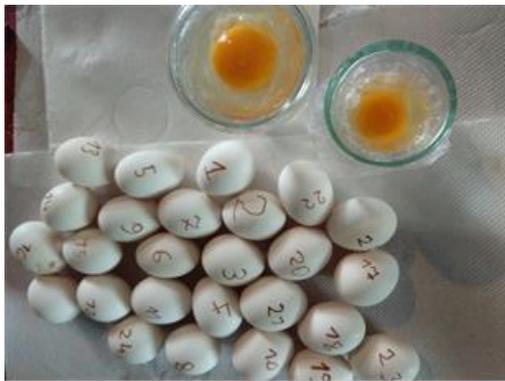


Figure 1. Eggs from mixed-breed chickens



Figure 2. Preparation of the artificial vessel

Chicken eggs were preincubated for 0 - 72 h at 38°C and 60 % humidity and then transferred to the artificial vessel (Table 1). A number of 10 eggs was used in each sample.

Table 1.

Number of samples and preincubation period

Nr samples	Sample	Preincubation hours
1.	A	0 hours
2.	B	24 hours
3.	C	48 hours
4.	D	55 hours
5.	E	72 hours

**Statistics.** All statistical analyses were conducted with STATISTICA 10.0. software. The variables were analyzed statistically by a repeated- measures ANOVA, with preincubation time as grouping factor. Duncan's multiple range test with  $p < 0.05$  was used to evaluate the significance of differences between the experimental variants.

## RESULTS AND DISCUSSIONS

The study aims to develop a shell-less culture system for chicken embryos. There were examined conditions necessary for embryonic hatching by comparing factors such as the addition of calcium lactate and optimal moisture monitoring, and the plastic film that was used as a culture.

The embryos were transferred to the artificial environment on different days, the hen's eggs were pre-incubated for 0-72 hours and then transferred to the artificial vessel.

In our study, the embryos were transferred to the artificial environment on different days, starting with day 0 (without being preincubated), 24, 48, 55 and 72 after pre-incubation.

The analysis of variance showed that the pre-incubation period until the transfer of the embryos to the culture vessel influenced very significantly the viability and success of the embryo (Table 2).

Table 2

Analysis of Variance								
Analysis of Variance								
Marked effects are significant at $p < .05000$								
	SS	df	MS	SS	df	MS	F	p
Embryos viability (days)	2182,920	4	545,7300	43,80000	45	0,973333	560,6815	0,00

In sample A the embryos were transferred without pre-incubating the eggs, directly from the shell into the culture vessel. Transfer of embryos to the culture vessel without pre-incubation is not recommended because the embryos survive only up to the third day. (Table 3 and Figure 3a). The transfer of embryos in the culture vessel at 24 hours (sample B) led to the survival of the embryos until the fifth day, reaching the maximum mortality on the fourth day (figure 3b).

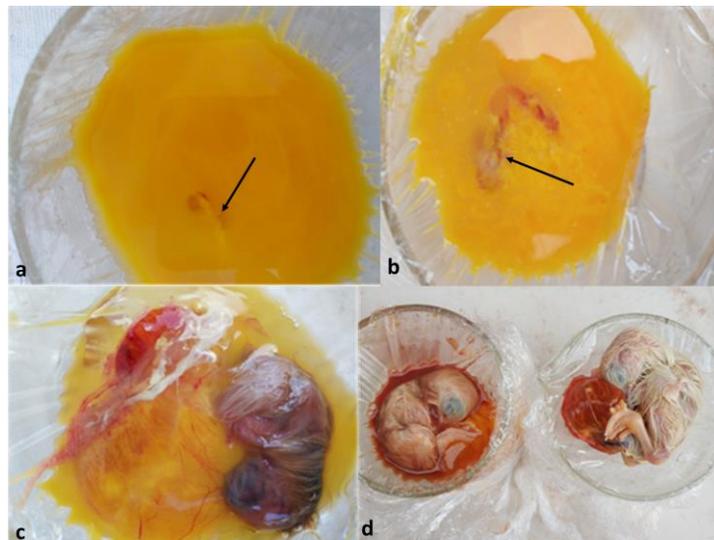


Figure 3 The viability of the embryos depending on preincubation period: a. without preincubation, embryo died after 3 days; b. 24 h preincubation, embryo died after 4 days ; c. 55h preincubation, embryo died after 17 days d. 72 h preincubation, embryo died after 19 days

The transfer of embryos in the culture vessel after 48 hours incubation (sample C) increased embryos viability to 9 days, the maximum mortality was reached on the eighth day (table 4, Figure 4).

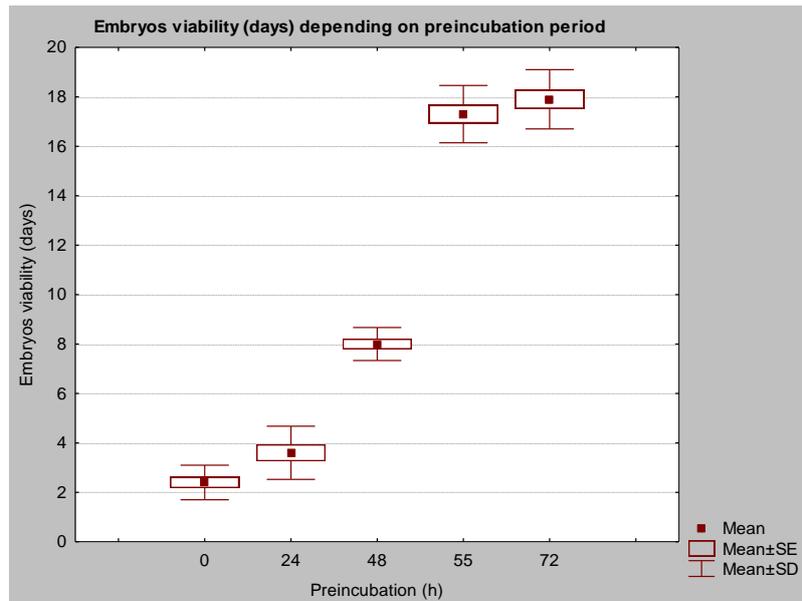


Figure 4. The viability of the embryos (days) depending on preincubation period (h)

Table 4

The viability of the embryos (days) depending on preincubation period (h)

Preincubation(h)	Embryos viability (days)				
	Average	SD	SE	Minimum	Maximum
0	2,40 d	0,699206	0,221108	1,0	3,0
24	3,60 c	1,074968	0,339935	2,0	5,0
48	8,00 b	0,666667	0,210819	7,0	9,0
55	17,30 a	1,159502	0,366667	15,0	19,0
72	17,90 a	1,197219	0,378594	16,0	20,0

Legend: SD = standard deviation; SE standard error; Significance of differences Duncan Test: a – maximum value; b – significant difference in comparison with a, s.o

The transfer of embryos in the culture vessels after 55h incubation enhanced the viability of embryos of up to 19 days, maximum mortality was reached on days 17 (figure 3c; 4) and 18, no chick hatched. The increasing of the incubation period with 7h conducted to a double time viability of the embryos, with a significant difference in comparison with the previous experimental variant (table 4). A longer period of preincubation didn't assured a significant longer viability. The transfer of embryos in the culture vessel after 72 hours had the highest viability of embryos up to 20 days, no chickens hatched in artificial conditions, maximum mortality was reached on days 17 and 18. There was no significant difference between the results obtained in the last two experimental variants.

According to Rowlett, Simkiss, (1987) and Borwornpinyo et al., (2005) embryo transfer had the highest viability at 55-56 hours after incubation. In our study, the highest viability was obtained at 72 hours of preincubation, and when the embryo was transferred to the culture vessel after 80 hours, it was difficult to transfer without destroying the yolk membrane.

In the experiment above was observed that is a positive significant correlation between the preincubation time and embryos viability ( $r = + 0.89588$ ;  $p = 0.0000$ ).

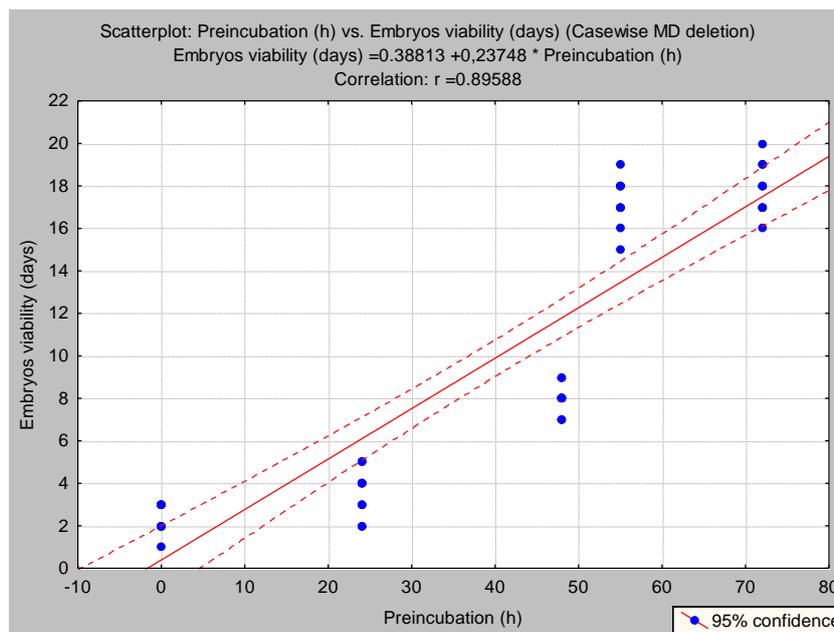


Figure 5. The correlation between the preincubation time (h) and the viability of embryos (days)

A previous study by Kamihira et al. (1998) using quail eggs as study material mentions the importance of highly effective lactic calcium supplementation for embryonic development, and the viability of embryos became highest when 250-300 mg of lactic calcium was added.

Thus, calcium supplementation in embryos is essential for shell less cultures, as normal chicken values within eggshells provide most of the calcium in the eggshell during embryonic development to hatching (Rowlett and Simkiss, 1987; Kamihira et al., 1998). By supplementing with lactic calcium, the necessary for the minimal development of the embryo was ensured. To avoid moisture loss and dehydration of embryos by perspiration, 2.5-3 ml of distilled water was first added.

### CONCLUSIONS

We can conclude that the pre-incubation period until the transfer of the embryos in the culture vessel influenced the viability on the day of culture, as well as the success of the embryo.

Transfer of embryos to the culture vessel without preincubation is not recommended because the embryos did not survive past the third day.

The transfer of the embryos to the culture vessel at 24 hours led to the survival of the embryos until the fifth day, reaching the maximum mortality on the fourth day. In conclusion, we do not recommend the transfer of embryos to the culture vessel after 24 hours.

In our study, the highest viability was obtained at 72 hours of preincubation, and when the embryo was transferred to the culture vessel after 80 hours, it was difficult to transfer without destroying the yolk membrane.

Calcium supplementation in embryos is essential for shellless cultures, as normal values of chickens inside the eggshells are provided by most of the calcium in the eggshell during embryonic development to hatching.

#### **Acknowledgments**

We thank Prof dr. Corneanu Mihaela for their assistance in statistical analysis of manuscript

#### **BIBLIOGRAPHY**

- ANDACHT T., HU W. and IVARIE R. 2004 - Rapid and improved method for windowing eggs accessing the stage X chicken embryo. *Molecular Reproduction and Development*, 69: 31-34.
- BORWORNPINYO S., BRAKE J., MOZDZIAK P.E. and PETITTE J.N. 2005. Culture of chicken embryos in surrogate eggshells. *Poultry Science*, 84:1477-1482.
- DIANA DRAGAN, ALEX BIRAU, OLGA RADA, LIANA MIHAELA FERICEAN. 2019. Observation regarding the pellets and food behaviour in captivity of *Bubo bubo* *Research Journal of Agricultural Science*, 51(4)
- KAMIHIRA M., OGUCHI S., TACHIBANA A., KITAGAWA Y. and IJIMA S. 1998. Improved hatching for in vitro quail embryo culture using surrogate eggshell and artificial vessel. *Development, Growth & Differentiation*, 40: 449-455.
- KAMIHIRA M., NISHIJIMA K. and IJIMA S. 2004. Transgenic birds for the production of recombinant proteins. *Advances in Biochemical Engineering/Biotechnology*, 91: 171-189.
- KAMIHIRA M., ONO K., ESAKA K., NISHIJIMA K., KIGAKU R., KOMATSU H., YAMASHITA T., KYOGOKU K. and IJIMA S. 2005. High-level expression of scFv-Fc fusion protein in serum of genetically manipulated chickens by using a retroviral vector. *Journal of Virology*, 79: 10864-10874.
- KATO A., MIYAHARA D., KAGAMI H., ATSUMI Y., MIZUSHIMA S., SHIMADA K. and ONO T. 2013. Culture system for bob white quail embryos from the blastoderm stage to hatching. *Journal of Poultry Science*, 50: 155-158.
- KYOGOKU K., YOSHIDA K., WATANABE H., YAMASHITA T., KAWABE Y., MOTONO M., NISHIJIMA K., KAMIHIRA M. and IJIMA S. Production of recombinant tumor necrosis factor receptor/Fc fusion protein by genetically manipulated chickens. *Journal of Bioscience and Bioengineering*, 105: 454-459. 2008.
- ONO T., MURAKAMI T., MOCHII M., AGATA K., KINO K., OTSUKA K., OHTA M., MIZUTANI M., YOSHIDA M. and EGUCHI G. A complete culture system for avian transgenesis, supporting quail embryos from the single-cell stage to hatching. *Developmental Biology*, 161:126-130. 1994.
- ONO T., NAKANE Y., WADAYAMA T., TSUDZUKI M., ARISAWA K., NINOMIYA S., SUZUKI T., MIZUTANI M. and KAGAMI H. Culture system for embryos of blue-breasted quail from the blastoderm stage to hatching. *Experimental Animals*, 54: 7-11. 2005.
- YUTAKA TAHARA, KATSUYA OBARA 2014. A Novel Shell-less Culture System for Chick Embryos Using a Plastic Film Culture Vessels *The Journal of Poultry Science*
- PERRY M.M. A complete culture system for the chicken embryo. *Nature*, 331: 70-72. 1988.
- ROWLETT K. and SIMKISS K. Explanted embryo culture: In vitro and in vivo techniques for domestic fowl. *British Poultry Science*, 28:91-101. 1987.
- ROWLETT K. and SIMKISS K. Respiratory gases and acid-base balance in shell-less avian embryos. *Journal of Experimental Biology*, 143: 529-536. 1