



## IMPLEMENTATION OF EX- OVO CHICK EMBRYO DEVELOPMENT MODEL FOR EVALUATION OF ANGIOGENESIS AND VASCULOGENESIS STUDIES

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Article Received on 16/08/2015

Article Revised on 06/09/2015

Article Accepted on 28/09/2015

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### ABSTRACT

The need of continuous monitoring throughout the experimental period initiated authors to test the *ex-ovo* embryo development model for CAM angiogenesis to test the drugs. The experimental design involved comparison of traditional *in-ovo* model (using window technique) with experimental *ex-ovo* shell less culture. HBSS which is conventionally

used as medium for test drugs/chemicals was introduced at 48, 72, 96 and 120 hrs of development and embryos were studied at 144hrs when CAM development is complete. In data analysis primary, secondary and tertiary (capillary networking) vitelline veins were numerically compared. The data shows *ex-ovo* shell less culture is of equal competence of traditional *in-ovo* window method with an advantage of intermediate monitoring when drugs are used.

**KEYWORDS:** Angiogenesis, vasculogenesis, CAM, *ex-ovo* model, *in-ovo* model, window method

### INTRODUCTION

*In vivo* models of angiogenesis include the chick chorioallantoic membrane (CAM), rabbit cornea assay, sponge implant models, matrigel plugs and conventional tumor models.<sup>[1, 2]</sup> The chick embryo provides well characterized *in vivo* biological system. But this system cannot be monitored throughout the development and hence to utilize continued monitoring privilege

with the same development status the model of an *ex-ovo* culture of the chick embryo is developed where, intact *in-ovo* relationship between the embryo and yolk sac/albumin is preserved outside the egg shell and shell membranes i.e. in an artificial container. To allow various experimental manipulations during growth period and to enable to develop 'testing model system' where many compounds can be tested for their properties and effects *ex-ovo* model of CAM angiogenesis is studied in present work.

CAM assay with *in-ovo* development has so far been used for the evaluation of pro-angiogenic or anti-angiogenic effect of numerous plants and animal extracts,<sup>[3, 4, 5, 6]</sup> and the shell less culture model is used to study glucose induced malformations in the developing embryos during early periods of life.<sup>[7]</sup> to study various stages of neurodevelopment of chick embryos,<sup>[8]</sup> to evaluate state specific effects of ethanol on neurodevelopment of chick embryos.<sup>[9]</sup> to observe changes in behavior caused by nicotine and substance from cigarette smoke,<sup>[10]</sup> An *ex-ovo* chick embryo culture system suitable for imaging and microsurgery applications has been developed.<sup>[11]</sup> A computer based video imaging system is recently used for analysis of critical phase of cardiac looping in live chick embryos in shell less cultures.<sup>[12]</sup> Human stem cell xenograft is also studied using *ex-ovo* culture of chick embryo.<sup>[13]</sup>

In the present study, *ex-ovo* chick embryo development is designed to analyze vasculogenic and angiogenic development so that it can be adapted to test any anti- or pro- angiogenic and /or vasculogenic drugs.

## MATERIAL AND METHODS

### Materials

Fertilized eggs (0 hrs) of *Gallus gallus* chick were obtained from local poultry (Quality poultry products, Malgao, Tal. Miraj, Dist. Sangli, Maharashtra). They were surface sterilized and incubated in pre-sterilized incubator at 37.5°C in 70-75% relative humidity. The sterile conditions were maintained throughout the experiment.

### Preparation of Culture Assembly

For the holding the cultures of shell less embryos, autoclavable glass bowls of dimensions: 9.0 cms of surface diameter, 4.0 cms of base diameter and depth of 4.5 cms were used with their lids. (Fig 1)

### Preparation of shell less culture

A modified method of Hamamichi and Nigoshiri.<sup>[10]</sup> was used for the preparation of shell less culture. Briefly, fertilized eggs were pre-incubated up to 48 hours (HH stage 12).<sup>[14]</sup> They were kept in horizontal position for half hour in the incubator itself to ensure the proper position of the embryo. Then the eggs were carefully transferred into the Laminar Air Flow Unit (Micro-Filt India) for culture preparation. Before the preparation of the cultures, albumen from an unfertilized egg was poured in the bowl. This albumen acts as a shock absorber to provide the cushion to the growing embryo and also to prevent desiccation of the egg.<sup>[6]</sup>

Each egg was opened into the bowl by cracking against edge of bowl. The bowl was then covered with lid (Fig.1). Aseptic conditions were maintained throughout the experiment along with minimal and careful handling. Cultures with proper position as well as growth of the embryo were selected for further incubation. Normally incubated eggs and shell less cultures of chick embryos were sacrificed at 144 hrs of development (6 days) for the analysis of growth parameters and morphometric parameters. The shell less cultures were photographed at intermediate stages. During the experiments, HBSS was introduced through a syringe using minute window onto the growing embryonic disc.

Mortality and morphological abnormalities with hemorrhage, vascular damage etc. if any can be monitored during incubation period. Embryo weight, CAM weights were determined after desired intervals. CAM growth was evaluated by measuring CAM area.



**Figure 1. The experimental setup for the shell less culture.**

A: The eggs procured from local poultry, and the culture assembly; B: the growing shell less culture; C: Incubator used for maintaining the cultures.

**Table 1. Experimental design for the *ex-ovo* culture method.**

Group	Experimental condition	Hrs of initiation of HBSS / HBSS + drug treatment	Final hrs of sacrifice
Group I	Normal (in ovo)		144
Group II	A. Normal (in ovo)+HBSS	48	
	B. Normal (in-ovo)+HBSS	72	
	C. Normal (in ovo)+HBSS	96	
	D. Normal (in ovo)+HBSS	120	
Group III	shell less (ex-ovo)		
Group IV	A. shell less (ex-ovo)+HBSS	48	
	B. shell less (ex-ovo)+HBSS	72	
	C. shell less (ex-ovo)+HBSS	96	
	D. shell less (ex-ovo)+HBSS	120	

### Evaluation of vasculogenesis and angiogenesis

The assessment of vasculogenesis and angiogenesis on CAM was carried out by focusing on morphometric and microscopic parameters including quantitation of primary vitelline veins (largest), main trunks; secondary (branches of primary veins) and tertiary (all other vessels) blood. Primary vitelline veins were analyzed for alterations in vasculogenesis; while secondary and tertiary blood vessels were evaluated for angiogenic response.

### Experimental groups

In the design, HBSS treated embryos are treated as controls. If any drug is to be used, it can be dissolved in HBSS (vehicle). For *ex-ovo* embryos, *in-ovo* embryos are treated as controls. Following groups of animals are assessed for the vasculogenic/ angiogenic response. Group I contains normal group of developing *in-ovo* embryos sacrificed at 144 hrs of development for further analysis. Group II contains four subgroups as IIA, IIB, IIC, and IID, each of the embryos from these groups were administered with HBSS (vehicle for any drug and hence vehicle control). When drug /any extract is to be used add additional group IIA+ containing HBSS + drug. In these two groups IIA and IIA+, HBSS and HBSS + drug treatment is initiated at 48 hrs of development. This was also true for IIB, IIC and IID conditions where HBSS was administered at 72, 96 and 120 hrs respectively and were sacrificed at 144hrs of development. Same experimental schedule was conducted for *ex-ovo* status i.e. group III and group IV (Table 3).

For each group 10 eggs were analyzed. For *in-ovo* development, only 0.5 ml HBSS could be accommodated and hence same amount was maintained in *ex-ovo* development. For HBSS administration, the time intervals were decided according to the developmental thresholds related to vasculogenesis or angiogenesis or significantly related to it. Such changes are illustrated in the Table 4.

## RESULT AND DISCUSSION

The results (Table 1) indicate that the pattern of *in-ovo* and *ex-ovo* models' response to HBSS resembled each other. Administration of HBSS at 120 hrs of chick development hardly influenced the embryonic or CAM growth judged by weight. Administration of HBSS at 48 hrs showed (9.89%) and (11.36%) decrease in the embryo weight in both *in-ovo* and *ex-ovo* status but 1.14 and 1.09 fold increase in CAM weight respectively in *in-ovo* and *ex-ovo* status. At 72hrs, HBSS administration resulted in 8.79% and 10.22% decrease in embryo weight in *in-ovo* and *ex-ovo* status respectively which indicates marginal decrease in embryo weight without any alterations in embryo. CAM weights increased 1.07 fold (*in-ovo*) and 1.05 fold (*ex-ovo*) indicating normalization of CAM weight. At 96 hrs, HBSS administration showed 7.69% (*in-ovo*) and 9.09 % (*ex-ovo*) decrease in embryo weight while CAM weight showed 1.14 fold (*in-ovo*) and 1.12 fold (*ex-ovo*) increment.

The results indicate that HBSS given at 48 hrs decreases embryo growth marginally without any abnormalities as noted at 144hrs (this decrease in weight is regained after 144hrs as the chicken hatched from *in-ovo* eggs were normal, data not presented). Administration at 72hrs, though embryo weight was gained it remained marginally low as compared to normal in *in-ovo* and *ex-ovo* status. The corresponding CAM weights showed marginal increase in both *in-ovo* and *ex-ovo* status. Administration of HBSS at 96 hrs marginally increased the CAM weight with coupled marginal decrease in embryonic weight without any abnormalities (in all the above cases on hatching the *in-ovo* chicken were normal). These observations indicate similarity in the developmental pattern observed at studied early developmental stages after HBSS administration to *in-ovo* and *ex-ovo* embryos. Thus in both the status the embryonic growth as well as CAM growth was normal.

**Table 2. Developmental thresholds at different incubation hours of embryonic growth**

Sr. No.	Hrs of development	Developments
1	48 hrs (HH stage 12)	Well defined area vasculosa (AV). Few vitelline vessels extended vessels extended in area vasculosa(AV)
2	72 hrs (HH stage 20)	Vitelline vessels are branched and capillary network begins to appear at the periphery of CAM. Progress of CAM area initiated.
3	96 hrs (HH stage 24)	Anterior, posterior and lateral vitelline vessels are well developed. Further progress of CAM area extension. Network of capillaries extended and can be observed microscopically.
4	120 hrs (HH stage 26)	All the details observed at 96 hrs are further progressed
5	144 hrs (HH stage 29)	CAM development at its maximum. Angiogenic network completed.

**Table 3. Morphometric analysis of *in-ovo* and *ex-ovo* status of embryos**

Groups	Experimental condition	Hrs of initiation	Emb. Wt (gms)	CAM wt (gms)	CAM/mb. Wt Ratio	CAM area (cm <sup>2</sup> )
Group I	Normal ( <i>in ovo</i> )	-	0.91±0.08	0.70±0.12	0.77	31.92
	B. Normal ( <i>in ovo</i> )+HBSS	72	0.83±0.03	0.75±0.02	0.99	33.63
	C. Normal ( <i>in ovo</i> )+HBSS	96	0.84±0.08	0.80±0.09	1.16	30.78
	D. Normal ( <i>in ovo</i> )+HBSS	120	0.90±0.02	0.71±0.05	0.79	32.48
Group III	Shell less ( <i>ex-ovo</i> )	-	0.88±0.02	0.72±0.09	0.82	30.78
Group IV	A. Shell less ( <i>ex-ovo</i> )+HBSS	48	0.78±0.08	0.79±0.04	1.82	33.04
	B. Shell less ( <i>ex-ovo</i> )+HBSS	72	0.79±0.04	0.76±0.07	0.99	32.45
	C. Shell less ( <i>ex-ovo</i> )+HBSS	96	0.80±0.08	0.81±0.06	1.03	31.11
	D. Shell less ( <i>ex-ovo</i> )+HBSS	120	0.89±0.07	0.74±0.06	0.83	30.78

Values indicate wt in gms  $\pm$  S.D. The standard deviations are within 10% limit and the weight differences between *in-ovo* and *ex-ovo* status are statistically not significant.

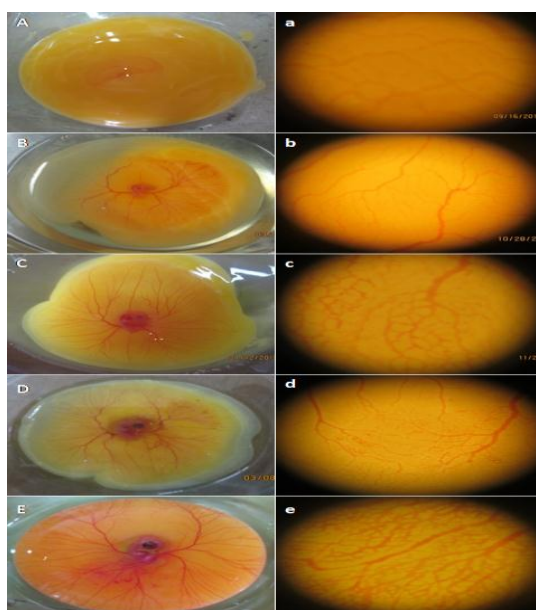
The total CAM area on which network of blood vessels formed by vasculogenesis and angiogenesis processes were calculated at 144 hrs and were compared in both the experimental conditions; since it influences growth and development of embryos at early stages. It is vital for survival, growth and homeostasis in the vertebrate embryo. Evaluation of primary vitelline veins and secondary blood vessels (sprouts of primary veins) on CAM and their distribution in both the experimental conditions indicated no change in number of primary and secondary vessels under the influence of *in-ovo/ex-ovo* status or HBSS administration. While tertiary vessels or capillary network showed significant increase (1.38 fold) with HBSS administration at 72 hrs and 96hrs, the administration at 48 hrs and 120 hrs hardly influenced their number or networking *in ovo* experimental condition. Similar alterations in blood vessels were also noted in *ex ovo* experiments. The differences between results of shell less culture and normal *in ovo* experimental condition with respect to the

vasculature parameters are statistically non-significant (Table 2). CAM vasculature was clearly recognized with well established dendritic pattern of vessel formation in both the experimental conditions (Fig. 2 and Fig. 3). The results indicated that embryonic growth and development in both the experimental conditions were parallel, without affecting any vasculogenesis and angiogenesis parameters.

**Table 4. HBSS influenced alterations in the tertiary vessels of CAM in *in-ovo* and *ex-ovo* status.**

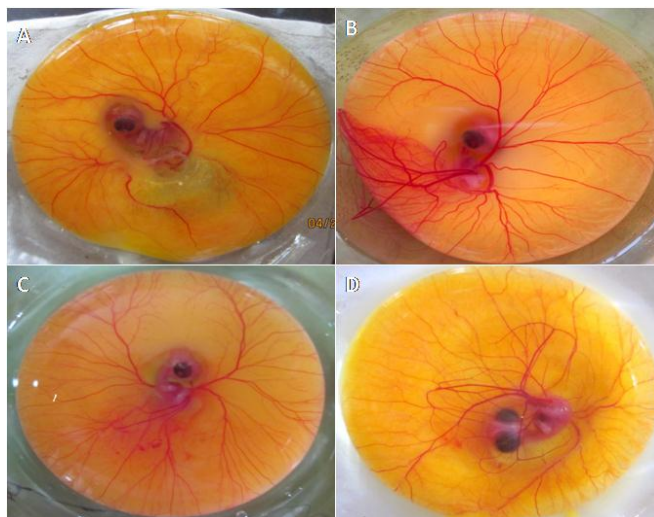
Group	Experimental condition	Hrs of initiation	Vasculogenesis	Angiogenesis	
			Primary vessels	Secondary vessels	Tertiary vessels
Group I	Normal ( <i>in ovo</i> )	-	6 $\pm$ 0.14	21 $\pm$ 1.60	160 $\pm$ 13.02
Group II	A. Normal ( <i>in ovo</i> )+HBSS	48	6 $\pm$ 1.02	21 $\pm$ 0.69	169 $\pm$ 5.58
	B. Normal ( <i>in ovo</i> )+HBSS	72	6 $\pm$ 0.12	22 $\pm$ 0.79	222 $\pm$ 10.28
	C. Normal ( <i>in ovo</i> )+HBSS	96	7 $\pm$ 0.02	22 $\pm$ 0.76	221 $\pm$ 12.57
	D. Normal ( <i>in ovo</i> )+HBSS	120	6 $\pm$ 0.13	20 $\pm$ 0.9	189 $\pm$ 6.23
Group III	Shell less ( <i>ex-ovo</i> )	-	6 $\pm$ 0.21	18 $\pm$ 1.11	155 $\pm$ 8.46
Group IV	A. Shell less ( <i>ex-ovo</i> )+HBSS	48	6 $\pm$ 0.09	21 $\pm$ 1.09	167 $\pm$ 7.22
	B. Shell less ( <i>ex-ovo</i> )+HBSS	72	6 $\pm$ 0.73	22 $\pm$ 0.12	211 $\pm$ 9.76
	C. Shell less ( <i>ex-ovo</i> )+HBSS	96	7 $\pm$ 1.02	22 $\pm$ 0.82	219 $\pm$ 7.65
	D. Shell less ( <i>ex-ovo</i> )+HBSS	120	6 $\pm$ 0.53	19 $\pm$ 1.24	179 $\pm$ 6.2

Values in the bracket indicate fold change over normal embryos of that status. The values of all parameters indicate values  $\pm$  S.D. The standard deviations are within 10%. The differences between *in-ovo* and *ex-ovo* status are statistically not significant.



**Fig. 2. Images showing stages of development of chick embryo at various hours of incubation**

(A: 48 hrs, B: 72 hrs, C: 96hrs, D: 120hrs, E: 144hrs). The next row of figures indicate the development of blood vasculature on CAM at the respective hours of incubation (a: 48 hrs, b: 72 hrs, c: 96hrs, d: 120hrs, e: 144hrs).



**Fig. 3. Images showing comparison of vascular development in different experimental groups of the eggs.** The groups are normal (A), normal with HBSS (B), shell less (C) and shell less with HBSS (D). The eggs of the treatment groups were opened at 144 hrs for comparison.

## CONCLUSION

Thus results and analysis of data with experimental design involving different hours of development in which angiogenic thresholds are related and indicate that the model of *ex-ovo* development is equally competent to test drugs which are possibly pro- / anti- angiogenic. In present experimental data where drug vehicle (HBSS) is used for study, same can be extended for any drug or extract with graded doses so that lowest effective dose can be deduced. Thus authors recommend this experimental model for pro-/ anti-angiogenic efficacy testing of various compounds.

## ACKNOWLEDGMENTS

Authors are thankful to University Grants Commission, WRO, Pune for partly providing financial assistance for the experimental work. Authors are also thankful to the Head of the Zoology Department and Principal of Smt. Kasturbai Walchand College, Sangli, Maharashtra, India for providing laboratory facilities.



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