RESEARCH ARTICLE

Received on: 01/12/2015
Published on:08/03/2016

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Conflict of Interest: None Declared !

Designing and Defining a Culture Medium for Growing Shell-less Chick Embryo *in- vitro*

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ABSTRACT

Background:
Chick embryos are mostly used as developmental model system to study the potential effects of various drugs and their mechanism of action. However inaccessibility of developing chick embryo in the egg has led to partial understanding of the effects and mechanisms of various drugs. Hence, an experiment to design and develop a culture vessel and culture medium to support growth of chick embryos was devised.

Materials & Methods:
A trial and error method using normal and daily laboratory glassware and naturally occurring nutrient rich food was performed. Chick embryos were grown in various containers through which developing embryo could be studied without having to break open through the container. The nutrients required for the development of the embryo was provided by daily and cheap food sources like milk, egg. The time period of the experiment allowed six trials to be performed which brought about partial success to the above objective of the experiment.

Results and Conclusion:
A protein rich combination of Milk whey, Albumen, Pannett-Compton saline where whey was higher in proportion was indicative of supporting the growing chick embryo and an improved culture vessel was designed which helped maintain the pH and also drain away the excess medium. It also helped restrict the position of the embryo in the vessel.

Keywords: *In -vitro* chick embryos, culture medium for chick embryo.

Introduction

All the potential drugs that come into the market are tested prior on various vertebrate model system. Out of the many vertebrate model system, the developmental model system used is of chick embryo. However the inaccessibility of the developing chick embryo in the egg has led only to the partial understanding of the various mechanisms and effects of the potential drugs. This has led to invention of many in vitro techniques for its cultivation and studies. The most simple and rudimentary technique was of pouring the entire of the contents of the egg into a bowl and taking precautions against evaporation and bacterial infection. Waddington (1932)[37] successfully transferred the developed blastoderm of the chick embryo to the surface of a clot of fowl plasma and embryo-extract maintained in a watch-glass in a moist chamber. Spratt (1947)[31] modified the technique by stiffening the clot using agar and containing diluted egg albumen or yolk. The embryos developed from these blastoderms are stated to be small and slow developing. Modified techniques like hanging drop technique was used to culture isolated chick blastoderm outside the egg. In this technique, the isolated blastoderm was floated over a cover glass and attached by using a drop of coagulated plasma and then this explant was inverted onto a cavity glass slide containing a drop of water and a cover glass trimmed with paraffin. Dennis New (1955)[9] introduced glass retaining rings to this technique and each were placed over the stretched blastoderm while attached to the vitelline membrane with only thin albumen as the culture medium. Many techniques were developed to grow chick embryos

outside their shells. Bruce Dunn (1974)[13] devised a method for growing chick embryos in plastic slings. Working with Boone, Ramsey and Dunn improved shell-less embryo culture methods (Dunn and Boone, 1976[14]; Ramsey and Boone, 1972[26]). Castellot et al. (1982)[6] simplified the method further by substituting disposable hot cups for plastic tripods. Denis New (1955)[9] published a method for the culture of avian embryos that aimed to emulate the relationship of the early blastoderm to its normal substrate (the vitelline membrane), on which it expands in the egg (New. 1959)[10] and to use pure egg albumen as the culture medium. The strong bacteriostatic properties of the albumen made it possible to dispense with strict sterile techniques. New (1955)[9] suggested the use of a slightly hypotonic saline, mildly buffered to neutrality with phosphates, Pannett-Compton solution (Pannett and Compton, 1924)[25], for explanting the embryos for culture. However, many others have tended to use Howard-Ringer's medium, which is an unbuffered salt solution. The pH of the explantation medium is probably unimportant because egg albumen is strongly alkaline in stored eggs (as high as 9.5) while the yolk is slightly acid (usually 6.5). Work is continuously being done to come with a foolproof culture vessel and culture medium to support the growth of the embryo to develop into chicken in *in vitro* conditions.

Recently Nusrat Zareen and Muhammad Yunus Khan (2008)[23] have used culture containers consisted of thin, clear, semipermeable polyethylene sheet secured with elastic rubber bands on the mouth of a cylindrical plastic or paper cup. Before transferring the egg's content into the culture container, the polyethylene sheet was sagged down a little by gloved fingers for comfortable accommodation of the contents. The brim of the cup was covered with a sterile Petri dish lid. Only cultures with the blastodisc positioned to uppermost side of the yolk were used in the experiments.

MATERIALS AND METHODS

Materials:

Embryonated eggs from Venkateshwar Hatcheries

Pannett-Compton Saline[22]

Materials used to setup the culture vessel- Glass bowls, Plastic cups, Petri-plates, Sandwich wrapping plastic foil.

Materials used for preparing the culture medium- Pannett-Compton saline, Milk, Milk whey, Egg Albumen[9], Egg yolk.

Trial and error method was used to design the culture vessel and culture medium.

Trial 1: Glass bowls were used to culture early chick embryo. All the glassware, chemicals were sterilized by autoclaving.

Medium used for explanation:

Sample 1	Pannett and Compton saline
Sample 2	Pannett and Compton saline+ Egg Albumen
Sample 3	Pannett and Compton saline+ Egg Albumen+ Egg Yolk
Sample 4	Pannett and Compton saline+ Egg Albumen+ Milk

Table 1: The table shows the composition of the medium of each sample in which the embryo was grown during trial 1.

Medium was filled in the bowls half its volume. The **24-48 hour embryo** was removed from the egg and suspended in the medium in the dialysis bag. The dialysis bag was suspended in the bowl by means of elastic bands. To ensure maximum sterility and closing the system glass bowl of a greater radius was placed inverted on top of it. The entire assembly of the culture vessel and medium along with the embryo was incubated in the incubator at 37.9° C and was observed daily for growth.

Trial 2: Modifications were made in the vessels and medium to eliminate most of the mistakes occurred in Round 1. Glass bowls were used to culture chick embryo. Medium used were modified:

Sample 1	Pannett and Compton saline+ Albumen
Sample 2	Pannett and Compton saline+ whey(substituted for milk)
Sample 3	Pannett and Compton saline+ yolk
Sample 4	Pannett and Compton saline+ whey+ yolk

Table 2: The table shows the composition of the medium of each sample in which the embryo was grown during trial 2.

The volume of the medium was reduced to increase air space. Instead of placing the explants inside the dialysis bag, the bag was cut open and the embryo was placed on it. The **24-48 hour embryo** was removed from the egg and suspended in the medium on the dialysis bag. It was suspended in the medium by means of a elastic band. Larger bowls which were to be used was replaced by petri plates to avoid inconvenience. This entire assembly was transferred into the incubator at 37.9° C and observed daily for keeping record of its growth. Apart from these, **0 hour embryos** were cultured in bowls in the medium composed only of saline. These samples were also transferred to the incubator.

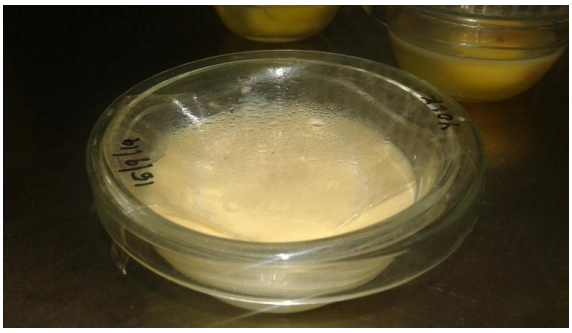

Figure 2: Embryo (24-48 hours) in the yolk sample.

Figure 3: Embryo(0 hour) in saline sample.

Trial 3: Modifications were made in the culture assembly. Glass bowls was made permanent. Medium volume was reduced drastically (10 ml medium was filled in the bowls)

MEDIUM	SALINE (ml)	ALBUMEN (ml)	
1	2.5	7.5	
2	5.0	5.0	
3	7.5	2.5	
4	1.0	9.0	
MEDIUM	ALBUMEN (ml)	SALINE (ml)	WHEY (ml)
5	1.0	2.5	7.5
6	1.0	7.5	2.5

Table 3: The table shows the composition of the medium of each sample in which the embryo was grown during trial 3.

72 hour embryo was excised from the eggs (0, 24, 48 hour embryo weren't feasible). Use of dialysis bag was avoided totally and the embryo was placed directly into the medium. Petri plates were used to close the culture bowls. This entire assembly was transferred into the incubator at 37.9° C and observed daily for keeping record of its daily growth.

Figure 4: Embryo (72 hours) embryo in sample 1.

Trial 4: Solidifying agent (Agar) was used to restrict the movements of the embryo in the liquid medium. The glass bowls were used to culture **72 hour chick embryo.** The combination of Whey+Albumen+Saline with whey-7.5 ml and saline-2.5 ml was used as the medium. This medium was poured upon the solidified agar (1%). The explant was placed on the Agar surface such that it was in continuous contact with the medium**.** Petri-plates were replaced by sandwich wrapping plastic foil (PVC). The glass bowls were covered using this plastic foil. This entire assembly was transferred into the incubator at 37.9° C and observed daily for keeping record of its growth.

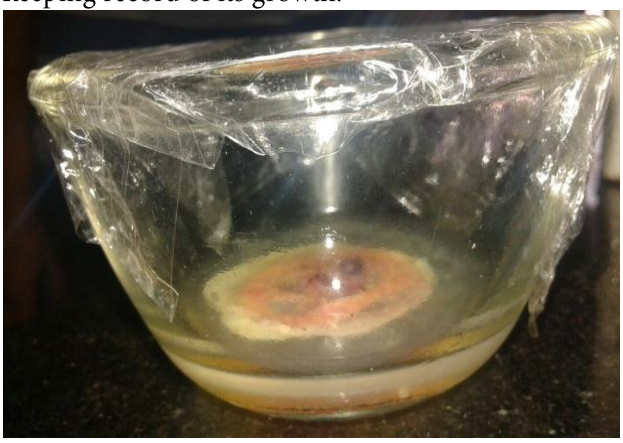

Figure 5: Embryo embedded on medium solidified using agar.

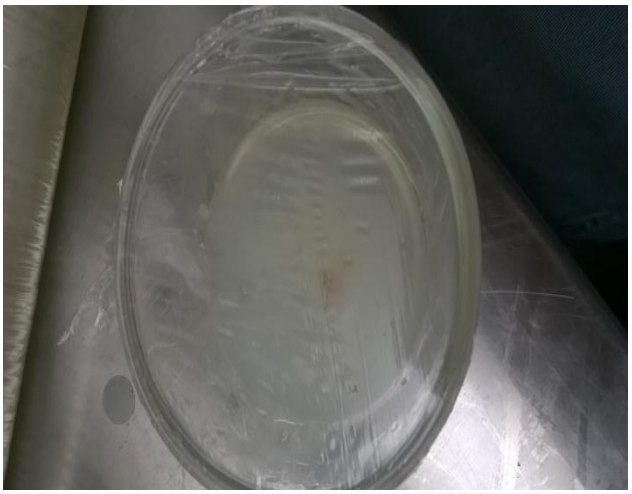

Figure 6: The sample in which petri-plates were substituted by wrapping plastic foil.

Trial 5: Round 3 was repeated to check the thin film formation on the chick embryo. The glass bowls were used to culture **72 hour chick embryo**. The combination of Whey + Albumen + Saline with whey-7.5 ml and saline-2.5 ml was used as the medium. Petri-plates were replaced by sandwich wrapping plastic foil (PVC). The glass bowls were covered using this plastic foil. This entire assembly was transferred into the incubator at 37.9° C and observed daily for keeping record of its growth.

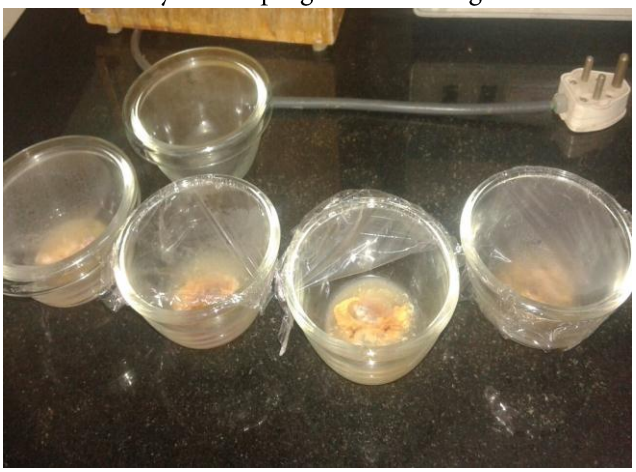

Figure 7: Samples for trial 5 being prepared.

Trial 6: Maintaining the pH of the medium at neutral (7) for optimum growth of the embryo was the objective of this round. The glass bowls was replaced by plastic cups to culture **72 hour chick embryo**. A thin layer of sterilized sponge was used to cover up the base of the cup. Upon the sponge, sterile Whatman filter paper was placed. A thin layer of solidified agar (1%) was placed on the filter paper which was made porous using a sterile pointer. The combination of Whey + Albumen + Saline with whey-7.5 ml and saline-2.5 ml was used as the medium. The medium was poured into the cup on the solidified agar such that the movement of the explant was restricted as well as it remained in constant contact with the medium. The plastic cups were covered using the plastic foil. This entire assembly was transferred into the incubator at 37.9° C and observed daily for keeping record of its growth.

Figure 8: The assembly shows three distinct layer- Lowermost sponge layer Whatmann Filter paper in the middle Soft Agar layer on the top

RESULTS

Trial 1: Out of the four cultured embryo, two samples showed live embryos on the second day

Sample	Observation
Saline	Heartbeat seen
Saline+Albumen	Heartbeat seen
Saline+Albumen+Yolk	No Heartbeat seen
Saline+Albumen+Milk	No Heartbeat seen

Table 4: The activity of the embryo in each sample of trial 1 after 24 hours.

However both the alive embryos didn't show any growth morphologically. The assumptive medium supported the embryo to stay alive but didn't support the growth of the embryos. Early chick embryo didn't survive in the medium composed of milk and yolk. The culture vessels used were very unstable. 100% sterility wasn't been ensured by inverted bowls. The use of dialysis membrane for suspending the embryo in the medium wasn't a good option because the transfer of the embryo into the bag wasn't a fool proof method without harming the embryo and it also was tedious.

Figure 9: Image of the embryo in the Pannett-Compton saline sample after 24 hours.

Figure 10: Image of the embryo in the Pannett-Compton saline and Egg albumen sample after 24 hours.

Trial 2:

7 embryos were explanted: 0-hour embryo and 24-48hour embryo.

0-hour embryo explants

Sample	Observation (after 24 hrs)	Observation (after 48 hrs)
1	Red spots observed	Blood vessels started forming
2	Yolk sac burst (discarded)	-
3	No change observed	Yolk sac burst (discarded)

Table 5: The activity of the 0-hour embryo in each sample of trial 2 after 24 hours and 48 hours respectively.

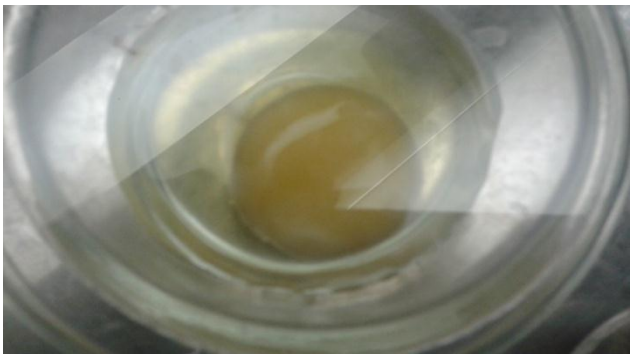

Figure 11: The 0-hour embryo in sample 1

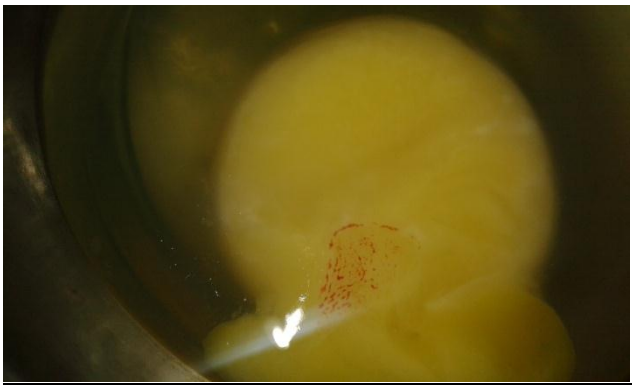

Figure 12: The 0-hour embryo in sample 1 after 24 hours.

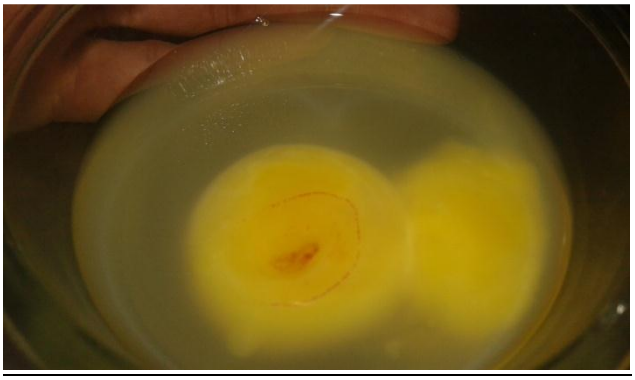

Figure 13: The 0-hour embryo in sample 1 after 48

24-48 hour embryo explants

Sample	Observation
Saline+ Albumen	Heartbeat seen+ very little increase in size
Saline+Albumen+Whey	Heartbeat seen+ very little increase in size
Saline+Albumen+Yolk	No Heartbeat seen
Saline+Albumen+Whey+Yolk	No Heartbeat seen

Table 6: The activity of the 24-48 hour embryo in each sample of trial 2 after 24 hours.

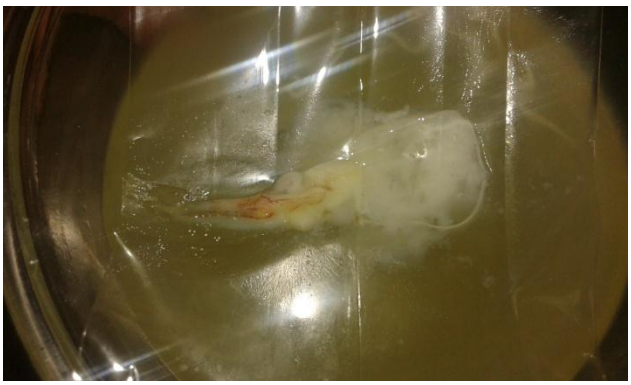

Figure 14: Embryo suspended in Pannett-Compton saline, Albumen and Whey during trial 2 after 24 hours.

The medium consisting of yolk was entirely avoided as it doesn't support the growth of the embryo. The embryos didn't survive for long after 24 hours of culturing. The medium composing of Pannett and Compton saline + Egg Albumen and Pannett and Compton saline + Milk Whey supported the growth and survival of the chick embryos only for 24 hours. However air space reduced by introduction of petri dishes to cover the vessel instead of inverted bowls. The dialysis bag was cut open mid way through and used for suspending the embryo on the medium.

Trial 3:

72-hour embryos were explanted

Sample	Observation
1	Heartbeat seen+ No increase in size
2	Heartbeat seen+ No increase in size
3	Heartbeat seen+ No increase in size
4	Heartbeat seen+ No increase in size
5	Heartbeat seen+ Limb development was seen
6	Heartbeat seen+ No increase in size

Table 7: The activity of the 72 hour embryo in each sample of trial 3 after 24 hours.

Figure 15: Embryo in sample 5 when transferred into the medium from the egg during trial 3

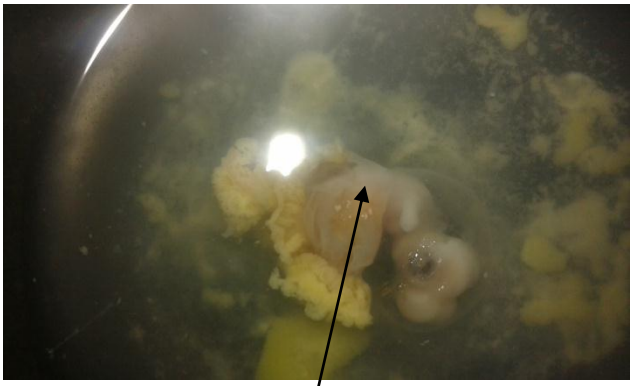

Figure 16: Embryo in sample 5 after 24 hours during trial 3 showing limb development

All the media supported the survival of the embryo. Minimal increase in size of the embryos was noticed however no appreciable increase in the size of the embryo was seen. However the medium composed of maximum volume of whey showed limb development after 24 hours of culturing. No embryo survived after 24 hours of incubation. Reason maybe lack of air supply. No dialysis bags were used in this round. This makes it cost efficient.

Trial 4:

Embryos were found dead after 24 hours of incubation. The layer was visibly thick enough to cover up the entire embryo. Gaseous exchange must have been hampered. Medium spoilage was detected.

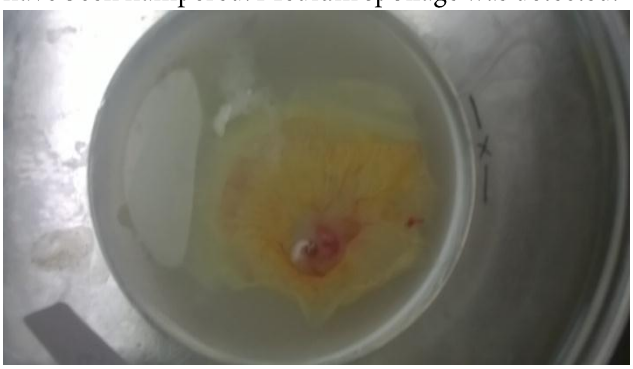

Figure 17:Sample when explanted

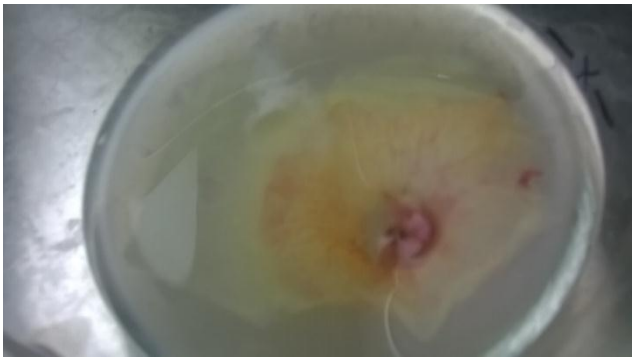

Figure 18: Sample showing thin layer formation after 24 hours. Embryo can't be observed properly due to the film formed on top of it.

Trial 5:

Embryo when grown on liquid medium (without solidifying agent) showed the thin layer formation. However as compared to Round 4 this film was very insignificant.

Embryos didn't survive for 24 hours. Embryo without the circulatory system and the supporting membranes showed perfect film floating on the medium while embryo with the circulatory system and membranes showed films which couldn't be distinguished from the membranes of the embryo. The pH of the medium was checked to know the effects of the film so formed. pH had dropped from 7 to 2. This film was mostly due to the spoiling of milk at high temperature. Air supply and film

formation remained a major problem in the culturing of the embryo.

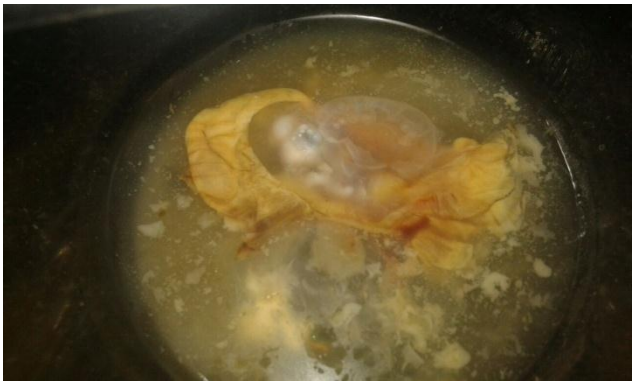

Figure 19: Sample showing the film formation after 24 hours.

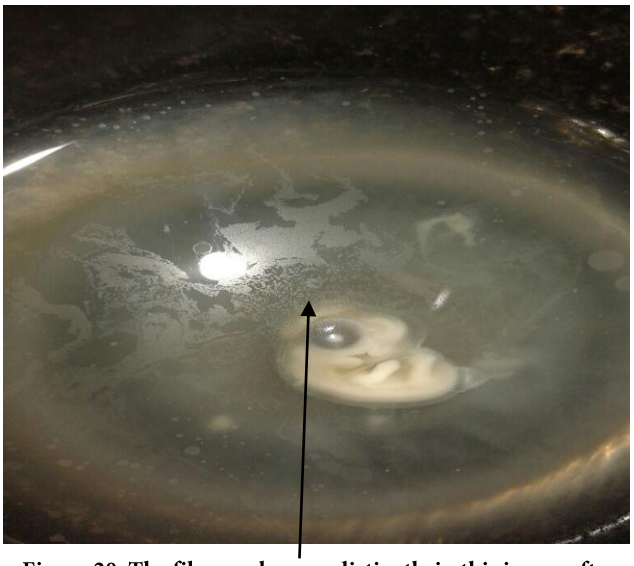

Figure 20: The film can be seen distinctly in this image after the extra-embryonic membranes are removed from the sample.

Trial 6:

Film formation was eliminated as the medium drained into the lower surface. pH remained constant at neutral (7). However the embryo didn't survive. Air supply still remains a problem.

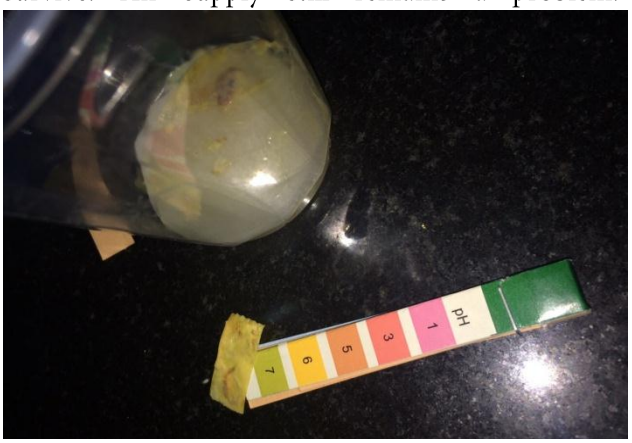

Figure 21: Figure showing the medium maintained at pH-7 after 24 hours.

DISCUSSIONS

Trial 1:

Age of embryo: 24 hour old embryos were used. However those embryos proved very delicate for handling. The transfer of such embryos in the dialysis bag proved a great challenge.

Medium: Albumen and Saline provided a support medium for the survival of the embryo but didn't provide enough nutrients for its growth. Milk didn't work as a medium because of its high fat content and its property of getting spoiled after keeping at such high temperature for very long period of time. It was considered that milk be substituted by whey (contains only milk proteins). Yolk started getting thicker on being removed from the yolk sac and mixed with the saline. However because it is the natural component of the egg, yolk was again considered as an option as a protein source in the medium for the next round.

Culture vessel: Inverted bowls proved to be very tedious in transporting the system from place to place and it failed to maintain 100% sterility. Even placing the embryo inside the dialysis bag was very tedious and often ended in dead embryos getting inside the bag. Also the bag proved to be a very small place for the developing embryo. Volume of the medium was decided to be reduced to increase air space for the growing embryo.

Changes to be brought about for Round 2:

Substitution of milk by whey as protein source in the medium.

Substitution of inverted bowls by petri dishes to enclose the system.

Cutting open the membrane mid way through and placing the embryo on it suspended in the medium. Volume of the medium was decided to be reduced to increase air space for the growing embryo.

Trial 2:

Age of embryo: 0 hour embryos were used. they showed very slow development. Primitive streak wasn't formed even after 48 hours of culturing. 24-48 hour old embryos were also sampled. However these too were very delicate to handle.

Medium: Albumen and Whey proved to be excellent protein sources for the developing chick embryo. However development was seen in 0- hour chick embryo without any supporting medium in the bowl. The development was however very slow and the primitive streak couldn't be observed even after 48

days. Only initiation of blood vessels was seen. Yolk proved to be a wrong choice of protein source in the medium. Hence yolk was entirely removed from being the protein source in the medium.

Culture Vessel: Modifications made to the culture vessels in Round 1 led to the elimination of many disadvantages. However air space still proved to be an issue. Use of dialysis bag seemed to be a tedious and expensive affair.

Changes to be brought about for Round 3:

Albumen and Whey volume requirements have to be investigated upon.

Use of dialysis bag was entirely avoided.

Using only 10 ml medium to increase the air space.

Trial 3:

Age of embryo: 72-hour old embryos were used. Advantages of these over 0, 24, 48 hour old embryos are that they can be suspended in the medium (10 ml) owing to its large size. No dialysis bag was required to provide support or suspend it on the medium.

Medium: Volume of the medium was 10 ml. This proved useful as it increased the air space and even the embryos could remain suspended in the medium. The combination of Whey+Albumen+Saline where whey was higher in proportion proved very conducive to the fast development of the embryo (limb development was seen).

Culture vessel: Petri dish should be substituted with something which allows continuous air supply so that the development of embryo cannot be hindered.

Changes to be brought about for Round 4:

Finding a suitable substitute for petri dish.

Checking the shelf life of Albumen and Whey in combination.

Culturing more embryos in the above medium to prove its validity.

Trial 4:

Age of embryo: 72-hour old embryos were used.

Medium: The combination of Whey+Albumen+Saline where whey was higher in volume was used for development of the embryo.

Culture Vessel: Petri dishes was substituted by Sandwich wrapping plastic foil (PVC) in consideration to bring about changes which favour air exchange.

Solidifying Agent: 1% Agar (soft agar) was used as solidifying agent. This proved useful to restrict unwanted movements in the position of the embryo. Also it brought into light a serious issue of medium spoilage by film formation.

Round 3 was decided to be done again with the same vessel and medium to study media spoilage

Trial 5:

Age of embryo: 72-hour old embryos were used.

Medium: The combination of Whey+Albumen+Saline where whey was higher in volume was used for development of the embryo.

Medium spoilage was observed. Due to human error it was unnoticed in the preceding trials. The effect of the spoilage was thin cloudy layer formation over the cultured embryo. This caused restricted gaseous exchange. Also a drastic pH drop was observed which could affect the growing embryo.

Culture Vessel: The use of glass bowls continued as culture vessels

Trial 6:

Age of embryo: 72-hour old embryos were used.

Medium: The combination of Whey+Albumen+Saline where whey was higher in volume was used for development of the embryo.

Culture Vessel:Glass bowls were replaced by Plastic cups in which arrangement of the sponge, filter paper and agar will be easier than glass.

In the plastic cup the medium was poured over the embryo which was embedded on the soft agar slab. The medium washed over the embryo supplying it with nutrients, then drained into the sponge through the filter paper. This avoids the problem of film forming as well as pH drop in the layer of medium in contact with the embryo.

Modifications to be included: Continuous air supply (Proper gaseous exchange) and continuous nutrient supply.

The experiment was designed to explant the embryo and allow maximum embryonic development. New explanted blastoderms (definitive primitive streak or head-process stages) and incubated them until embryonic development ceased. Maximum development shown by them was reaching the 10-somite stage and the ceasing development and growth[9]. Stern experimented on similar lines and recorded every 12 hours the stage of development attained. Using three different techniques to culture the embryo, the results observed were also different. But the maximum time the embryo showed development outside the egg was for 48 hours and showing maximum development of stage 16[8]. Nusrat

Zareen and Muhammad Yunus Khan repeated successful culturing, tracing the developmental process of the embryo upto the 15th day of embryonic life at least after which the survivability period varied in different embryo cultures. The most advanced age reached in this project was day 19 of the embryonic life, which in researchers' understanding is the latest developmental stage in shell less environment described as yet in which the entire egg contents was poured into the vessel without explanting the blastoderm or the embryo[23]. The experiment performed by the trial and error method was successful in maintaining the embryo for 48 hours allowing embryonic development by explanting the embryo in a medium designed for its growth as compared to just pouring the contents of the egg into saline.

CONCLUSION

Embryo survived in *in vitro* conditions but rarely showed any development.

A nutrient medium (protein rich feed) was designed and optimized which helped the embryo survive. The combination of Whey+Albumen+Saline where whey was higher in proportion constituted the medium (Whey-7.5 ml, Saline-2.5 ml, Albumen-1.0 ml).

An improved culture vessel was designed which helped maintain the pH and also drain away the excess medium. It also helped restrict the position of the embryo in the vessel.

ACKNOWLEDGMENT

We are thankful to Dr. R.G. Pardeshi, Principal, Fergusson College for providing us infrastructural facilities. We express our sincere gratitude to Dr. Mrs. Sonali Joshi, Head, Department of Biotechnology for giving us access to the laboratory and providing us with the necessary amenities and encouragement about the experiment. We also thank Venkateshwar Hatcheries, Pune for providing us with the embryonated eggs. We would specially thank our lab assistant Mr. Kailas Athawale for providing us the various lab equipments on time and all my friends for all their support and suggestions for helping us complete our journey.

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Cite this article as:
Abraham B. Mathew and Shampa Chakraborti. Designing And Defining a Culture Medium for Growing Shell-Less Chick Embryo *in vitro.* Asian Journal of Pharmacology and Toxicology, 04(13), 2016, 12-21.