Original article

Comparison of Embryo Culture in Sequential Versus Single Culture Media

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Abstract

Background: Embryo quality plays a significant role in determining the success in ART (Assisted Reproductive Technology). In-vitro embryo culture in varying culture media per se has its own impact on the embryo quality. Due to the varying compositions in Sequential and Single embryo culture media, this study was carried out to compare the outcomes of pre-implantation embryo culture using these media.

Aim & Objective: This prospective double blinded study was aimed at comparing the fertilization rate and cleavage rate of in-vitro culture of mice pre-implantation embryos in commercially available Sequential culture media versus Single culture media.

Methodology: Number of Groups: 2 (Group 1 – Sequential culture media from Vitrolife & Group 2 – Single culture media from Irvine Scientific) Sample size: 35 sibling oocytes in each group. IVF was done by inseminating a concentration of 50,000 spermatozoa per oocyte. Fertilization, cleavage, growth and development of the embryos in the two groups were noted. Data collected was subjected to statistical analysis and conclusions were drawn.

Result: Fertilization rate in Group 1 was 60% and in Group 2 was 45.7%. P value (0.5464) was not significant. Cleavage rate was 100% in both the groups. Cleavage rate at 18 – 24 hours (First Cleavage) was 76.19% in Group 1 and 87.5% in Group 2. Cleavage rate at 34 – 38 hours (Second Cleavage) was 76.19% in Group 1 and 87.5% in Group 2. P value (0.8093) for first and second cleavage was also not significant.

Conclusion: The fertilization and cleavage rates were not altered using Sequential culture media or Single culture media for in-vitro mice embryo culture. Thus, this study concludes that both the fertilization and cleavage rates are not affected by the type of culture media used.

Key Words: Sequential culture media, Single culture media, Embryo culture, Cleavage stage embryo, Blastocyst.

Introduction

Birth of Louise Brown (the first IVF baby), the fruitful outcome of the efforts of Patrick Steptoe and Robert Edwards in 1978 has laid a rapid and impressive progression in the field of ART.[1] Since then, the number of babies born out of ART accounts for about 5 million as reckoned by International Committee for monitoring Assisted Reproduction Technologies (ICMART).[2] In-Vitro Fertilization (IVF), a technique currently being practiced in ART involves culturing of pre-implantation embryos in-vitro in embryo culture media. To improve pregnancy and live birth rates, the embryos are cultured in appropriate culture media. All currently available commercial media formulations have their foundation from 'Whitten's medium'.[3]

Researchers concluded that the requirement of nutrition and ions vary at different stages of embryo development in - vivo.[4] Formulation of the culture media was initiated based on the protein and carbohydrate requirements of the embryo, as per the in-vivo observations.[4]

Formulation of these media isbased on two philosophies: (i) back-to-nature (ii) let-the-embryo-choose.[5] Sequential culture media from VitroLife has fertilization media(IVF media), cleavage media (G1 media) and blastocyst media (G2 media); whereas single media (aka monoculture media), Irvine Scientific media consists of one medium - used from fertilization till blastocyst stage.

It is now established that the formulation of sequential media is based on the amount of nutrients present in the fallopian tube and uterus at different stages of embryo development.[6] This allows stage specific changes of media mimicking the natural environment. In single media formulation, all the components remain at set concentrations and follows the belief that the embryos will utilize the required nutrients as they develop through different stages.

On reviewing the literature, the outcome of culturing embryos in sequential or single culture media does not vary significantly and no conclusive reports have been published contributing to the increase in ART outcomes.[5] This study is thus aimed in identifying the fertilization rate, cleavage rate, growth and development of mice pre-implantation embryos in sequential versus single culture media.

Aim

The aim of this study is to compare the fertilization rate, cleavage rate, growth and developmental potential of in-vitro culture of mice pre-implantation embryos in commercially available **Sequential culture media** versus **Single culture media.**

Objective

Primary objective

Assess and compare the fertilization rate and cleavage rate obtained from culturing mice embryos in commercially available sequential and single culture media.

Secondary objective

Assess the growth, development up to blastocyst stage and the quality of the embryos in both the groups.

Materials and Methods

This study is a prospective double blinded study carried out in the Department of Andrology and Reproductive Medicine, Chettinad Hospital and Research Institution. Blinding of the dishes containing embryos was done and two other embryologists were made to observe and the findings were recorded. The study procedures were carried out in Animal House located within the institution for a duration of 6 months. Swiss Albino mice, whose gametes are the prototype of humans[7] were used for this study. Ethical clearance was obtained from Institutional Animal Ethics Committee (IAEC4/Desp.No.28/Dt.10.10.16). Swiss Albino mice between 6-8 weeks of age were used for the study.

Methodology

Incubation of culture media

Culture media were pre-incubated before the procedure. Commercially available culture media were used. Few culture media that were used in this study involved overnight incubation and few media were incubated on the day of oocyte retrieval depending on the buffers used in the culture media. Sequential media which has different media for the use from fertilization till blastocyst cuture (G-IVF, G1, G2) and Ovoil from Vitrolife; Single culture media from Irvine Scientific were incubated overnight. Phosphate Buffered Saline (PBS) from Sage and QUINN'S sperm wash media - Conventional sperm preparation media were incubated on the day of oocyte collection from mice.

Dish preparation

Culture dishes from Falcon and Nunc were used in this study. 60 mm dish with PBS media was taken for collecting ovaries and testes from mice. 4 - well dish (2 nos) were taken and loaded with Single culture media and Sequential culture media respectively.

Retrieval of Cumulus Oocyte Complex from female mice

Ovarian hyperstimulation for female mice was done to obtain adequate number of oocytes. Drugs are administered intra-peritoneally (Figure 1a). 5 IU of lyophilized PMSG (Pregnant Mare Serum Gonadotropin) was administrated for ovarian stimulation. 48 hours after stimulation, 2.5 IU of lyophilized hCG (human Chorionic Gonodotrophin) as used to trigger ovulation. Reconstitution of these medicines was done using Sodium Chloride and a volume of 0.5 ml was administrated intra-peritoneally. 12 hours from the trigger, oocytes were collected after euthanizing the mice by exposing to higher doses of halothane.

Euthanized mice were dissected under sterile environment and the ovaries were collected in dish containing PBS (Figure 1b). Under stereo microscope, the oocyte cumulus complexes were retrieved from the ampulla and were placed in the incubator (Figure 1c).

Figure 1(a): Intra-peritoneal drug administration in Swiss Albino mice

Figure 1(b): Ovary along with oviduct placed in PBS at 37° C.

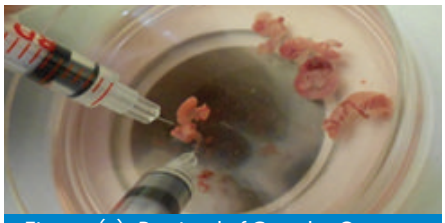


Figure 1(c): Retrieval of Cumulus Oocyte Complex from ampullausing two hypodermic needles (18G) attached to a one mL syringes.

Retrieval of spermatozoa from male mice

Eight weeks old male mice were euthanized by exposing to higher doses of halothane. Cauda epididymis along with the vas deferens of the male mice was dissected. Using forceps, excess adipose tissue was removed. The epididymis was nicked using needle and it was milked to obtain spermatozoa (Figure 2). The dish was then placed at 37°C to allow the spermatozoa to disperse. The spermatozoa concentration was assessed and prepared for IVF (Invitro Fertilization).

Figure 2: Retrieval of spermatozoa from epididymis.

IVF and Embryo culture using Sequential culture and Single culture media

Group – 1 (Sequential culture media)

For Sequential culture, a four-well dish was taken and was filled with Sequential media and overlaid with oil (Table 1).

Well - 1	0.5 ml G – IVF + 0.5 ml oil
Well - 2	0.5 ml G – IVF + 0.5 ml oil
Well - 3	0.5 ml G – 1 + 0.5 ml oil
Well - 4	0.5 ml G – 2 + 0.5 ml oil

Table 1: Four-well dish containing Sequential culture media

35 oocyte cumulus complexes (sibling oocytes) were transferred to well-1 containing Fertilization media (G – IVF media). Insemination of spermatozoa was done at zero hours. A standard concentration of 50,000 spermatozoa per oocyte was inseminated. The dish was then incubated at 37°C in 6% CO_2 incubator. 3 – 4 hours later, the oocytes from well-1 were transferred to well-2 containing the same media to prevent polyspermy. The dish then was placed back in the incubator for further incubation at 37°C in 6% CO_2 incubator. 6 – 8 hours after insemination, fertilization check was done by assessing the presence of two pro-nuclei, two polar body and cytoplasmic halo. After checking for the signs of fertilization, the fertilized oocytes were then transferred to well-3 containing Cleavage media (G-1 media). 18 - 24 hours after insemination, first cleavage check **(Cleavage 1)** was done. 34 - 38 hours after insemination, second cleavage check **(Cleavage 2)** was done. Embryos were then transferred to well-4 containing Blastocyst media (G-2 media). Further development culturing up to 96 hours post insemination was done and documented.

Group – 2 (Single culture media)

A four well dish was taken and was filled with Single media and overlaid with oil (Table 2).

Well - 1	0.5 ml single media + 0.5 ml oil
Well - 2	0.5 ml single media + 0.5 ml oil
Well - 3	0.5 ml single media + 0.5 ml oil
Well - 4	0.5 ml single media + 0.5 ml oil

Table 2: Four-well dish containing single culture media

35 oocyte cumulus complexes (sibling oocytes) were transferred to well-1. Insemination of spermatozoa was done at zero hours. A standard concentration of 50,000 spermatozoa per oocyte was inseminated. The dish was then incubated at 37°C in CO_2 incubator. 3 - 4 hours later, the oocytes from well-1 were rinsed in well-2 to prevent polyspermy and were transferred to well-3. The dish was then incubated at 37°C in CO_2 incubator. 6 – 8 hours after insemination, fertilization check was done. 18 - 24 hours after insemination first cleavage check **(Cleavage 1)** was done. 34 - 38 hours after insemination, second cleavage check **(Cleavage 2)** was done. Observations made were documented. Further development culturing up to 96 hours post insemination was done and documented.

Statistical Interpretation of Data

The fertilization and cleavage rates were assessed and statistically interpreted. P-value ≤0.05 was considered statistically significant. Fisher's exact test was employed to analyze the statistical significance in the data collected by assessing both the groups.

Results

Sample size was 35 numbers in each group. Number of oocytes fertilized were 21 in Sequential culture group and 16 in Single culture group. Fertilization rate in sequential and single media was calculated and is shown in Table 3.

Group	Sample size (No of Oocytes)	Number of Fertilized Oocytes	Fertilization rate
Group - 1: Embryo culture in sequential culture media	35	21	60 %
Group - 2: Embryo culture in single culture media	35	16	45.71%

Table 3: Fertilization rate in Group 1 & 2

Number of cleaved embryos was noted, and cleavage rate was calculated. Out of 21 oocytes that were fertilized, all the 21 got cleaved in group 1. In group 2, all 16 fertilized oocytes were cleaved. All the oocytes that were fertilized underwent cleavage and hence, 100% cleavage rate was obtained in both the groups (Table 4).

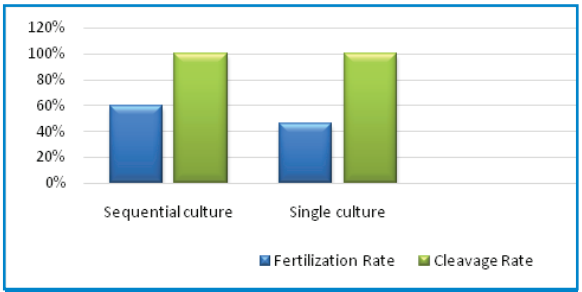
Group	Number of Fertilized oocytes	Number of embryos Cleaved	Cleavage rate
Group – 1: Embryo culture in sequential culture media	21	21	100 %
Group – 2: Embryo culture in single culture media	16	16	100 %

Table 4: Cleavage rate in Group 1 & 2

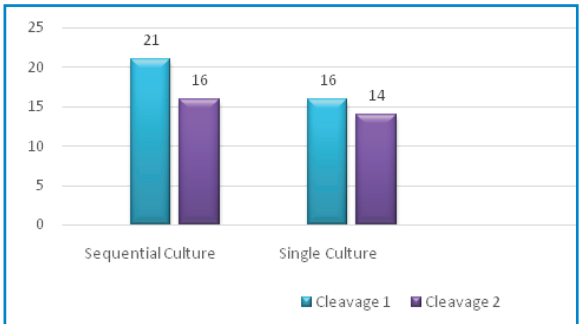
First and second cleavage (Cleavage 1 and Cleavage 2 respectively) in both the groups were assessed and cleavage rate was calculated (Table 5).

Group	Cleavage 1 (No of embryos)	Cleavage 2 (No of embryos)	Cleavage rate
Group – 1: Embryo culture in sequential culture media	21	16	76.19%
Group – 2: Embryo culture in single culture media	16	14	87.5%

Table 5: First and Second Cleavage rate for Group 1 & 2.



Bar chart 1: Comparing the Fertilization rate and Cleavage rate in both the groups.



Bar chart 2: Comparison of First and Second cleavage rate for Group 1 & 2.

Using Fisher's exact test, P value was calculated. The output revealed that there was no statistical significance in fertilization rate comparing group 1 & 2 (Table 6).

Group	Sample size (No of Oocytes)	Number of Fertilized Oocytes	P value
Group – 1: Embryo culture in sequential culture media	35	21	0.5464
Group – 2: Embryo culture in single culture media	35	16	

Table 6: P value for fertilization rate of Group 1 & 2

First and Second cleavage embryos was subjected to Fisher's exact test and P value was obtained. P value obtained was statistically not significant (Table 7).

Group	Cleavage 1 (Number of embryos)	Cleavage 2 (Number of embryos)	P value
Group – 1: Embryo culture in sequential culture media	21	16	0.8093
Group – 2: Embryo culture in single culture media	16	14	

Table 7: P value for First and Second Cleavage rate in Group 1 & 2.

This study involved a sample size of 35 oocytes in each group. Incubator maintaining 37°C with 6% CO_2 was used to incubate the culture media and the dishes containing oocytes and embryos.

Group 1 contained Sequential culture media and Group 2 contained Single culture media. At zero hours, IVF was performed by inseminating 50,000 spermatozoa per oocyte.6 – 8 hours later, fertilization was checked. Presence of two pro-nuclei and two polar body was considered fertilized. In Group 1, 21 oocytes were fertilized and in Group 2, 16 oocytes were fertilized. The fertilization rate was calculated which was 60% in Group 1 and 45.7% in Group 2. P value comparing the fertilized oocytes obtained in Group 1 and 2 was not significant (P value 0.5464).

18 - 24 hours after insemination, First cleavage check was performed. The presence of 2 – 4 blastomere (Figure 3, 4) on with or without fragmentation was considered as Cleavage 1. Fertilized oocytes in both the groups underwent cleavage and thus 100% cleavage rate was obtained. 34 – 38 hours later, Second cleavage (Cleavage 2) was assessed in which, 16 embryos reached 4 – 8 cell stage in Group 1 and 14 embryos reached the same in Group 2. Second cleavage rate in Group 1 was 76.5% and in Group 2 was 87.1%. P value obtained was not statistically significant (P value 0.8093).

The growth and development of the embryos were observed further in both the groups till 96 hours. The quality of the embryos until the development of blastocyst was the same. The number of embryos reaching up to blastocyst stage was also comparable in both the groups.

Discussion

Culture media has a greater contribution in determining the quality of the embryo which affects the success rate. This led to the formulation of Sequential culture media and Single culture media. Availability of two media do not provide a clear-cut choice for the Embryologists to choose culture media in routine practice. Thus, researches compared the outcome of culturing embryos in the available two media. Upon reviewing the literature three types of outcome were noted in studies done comparing single and sequential media, (i) both sequential and single culture media are equal, (ii) Sequential media is superior and (iii) Single media is superior.[9,10]

Literature shows no conclusive results in the outcome of culturing embryos in single or sequential culture media. Hence, to overcome the complications involved in choosing the right culture media, this study was performed in mice model. Commercially available Single culture media (Irvine Scientific) and Sequential media (Vitrolife) were used in this study.

Conclusion

The use of Single and Sequential culture media for in-vitro mice embryo culture did not alter the fertilization rate, cleavage rate and in-vitro growth and development until the development of blastocyst. The outcome of this study thus clearly demonstrates that there is no significant alteration in the use of both sequential and single culture media for in-vitro embryo culture. Extended studies in human with large sample size involving the assessment of clinical pregnancy, live birth, neonatal outcome is essential to draw a more definite conclusion.

Figure 3 (a): Cleavage stage embryo showing 2 blastomeres (Cleavage 1)

Figure 4: Cleavage stage embryo showing 4 blastomeres (Cleavage 2)

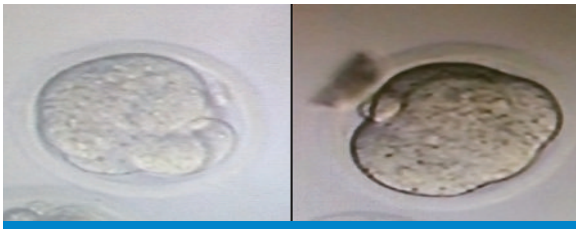


Figure 5: Morula

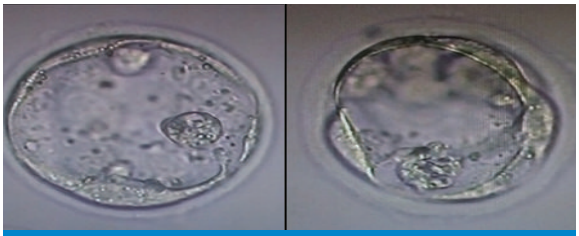


Figure 6: Blastocyst

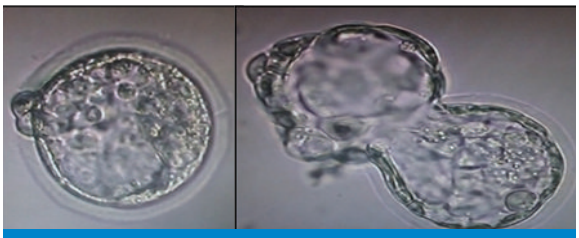


Figure 7: Hatching blastocyst

Figure 8: Hatched-out blastocyst

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Conflicts of Interest

No conflicts of interest were observed.