Original Article

Influence of Different Buffers on Embryo Growth in In-vitro fertilization

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Abstract

Introduction: The human reproduction is achieved as the domino effect of successful synchronization and communication between the embryo and the uterine endometrium. Here the embryo is thought to be the most important factor for achieving a pregnancy in assisted reproductive technology (ART). These embryos were cultured in culture media containing various vital components, of which the buffers for maintaining the pH play an imperative role. The bicarbonate buffer being the oldest & most commonly used buffer in Assisted Reproductive Technology (ART) for handling gametes and culturing them as embryos is now replaced by 4-2-hydroxyethyl-1-piperazine ethane sulfonic acid (HEPES) & 3-N-morpholino propane sulfonic acid (MOPS) organic based buffers for collection and manipulation of gametes.

Aim: This study was aimed to observe the growth potential of embryos in culture media containing organic buffers such as HEPES & MOPS.

Materials and Methods: The male and female Swiss albino mice which imprints similar genetic pattern as humans were used for the study. The female mice were stimulated for super-ovulation, sacrificed, Cumulus Oocyte Complex (COC) were retrieved from ampulla. The male mice of appropriate age were selected sacrificed and spermatozoa were retrieved from the testis. These retrieved cumulus complex were inseminated with spermatozoa at standard concentration (In-Vitro Fertilization) and culture of embryos were done as groups I, II, III in culture media containing bicarbonate (CO_2), HEPES & MOPS respectively.

Study design: Prospective study

Study duration: October 2016 to May 2017; for a period of 8 months

Study setting: Dept. of Reproductive Medicine & Andrology, Chettinad Super Speciality Hospital, Chennai.

Result: The fertilization rate in bicarbonate, HEPES, MOPS buffer based culture media were 60%, 51.42% & 0% respectively. The oocytes inseminated in MOPS based culture media failed to survive.

Conclusion: This study shows that HEPES can be used as substitute to bicarbonate but cannot be replaced.

Key words: ART- Assisted Reproductive Technology, HEPES- 4-2-hydroxyethyl-1-piperazine ethane sulfonic acid, MOPS- 3-N-morpholino propane sulfonic acid, CO_2 – Carbon dioxide, COC – cumulus oocyte complex.

Introduction

Since the birth of first IVF baby Louise Brown from the work of Dr. Robert Edwards and his team, the field of IVF has taken tremendous growth.[1] Earlier, the pioneers in IVF used a simple salt solution along with glucose and phosphate supplementation for embryo culture.[2] Subsequently, the following researchers added numerous components to aid in embryo development such as amino acids, nutrients, minerals, and growth factors.[3]

At present, culture media used in IVF are available commercially, manufactured by different companies who aim to increase the success rates.

The most commonly used IVF media are based on three main types of buffers namely bicarbonate, HEPES, MOPS. These buffers are essential to maintain the pH of the media which in turn is maintained by physiologic temperature. The maintenance of pH and temperature are essential to provide a physiologic environment for the gametes being cultured.[4]

Improper maintenance of pH levels are detrimental to the gametes and embryos which in turn results in improper or delayed development.[5] The maintenance of carbon dioxide [CO_2] levels and temperature is very important to maintain the pH levels with culture media based on bicarbonate buffer with a special CO_2 incubator, whereas in case of organic buffers like HEPES and MOPS just the temperature maintenance is good enough.

The usage of different buffer based media for different category of works in IVF has impact on the gametes both on intercellular and laboratory processes.[6] Very few comparative studies are available to demonstrate

the effects of different buffers used in culture of gametes right from collection of oocytes, insemination with spermatozoa, its fertilization potential and growth.[7-9]

Hence this study was aimed to compare the effect of different buffers such as bicarbonate, HEPES, MOPS on the fertilization and growth potential of embryos using commercially available medias G-IVF (Vitrolife), Conventional sperm preparation media (Quinn's sperm wash media) and G-MOPS (Vitrolife) respectively.

Materials & Methods

This prospective experimental study was conducted from October 2016 to May 2017 for a period of 8 months at Chettinad Hospital & Research Institute, Kelambakkam with support from the Animal house, Chettinad Health City, Kelambakkam. The ethical committee approval was obtained from IAFC. The Swiss Albino mice breed was used for study purpose.

Figure 1(a): Swiss Albino mice

Figure 1(b): Intra-peritoneal injection in mice

The female mice were subjected to ovarian stimulation with 5 IU of lyophilized PMSG, reconstituted with bacterio static sodium chloride (0.5 ml) and injected intra-peritoneally. The injected mice were maintained in the specific pathogen free (SPF) condition of 12:12 hours light: dark(photo effect), in the temperature of 21°C to 24°C. These injected mice were isolated to prevent it from mating. After 48 hours, intra-peritoneally, hCG trigger was given with 2.5 IU of lyophilized hCG, reconstituted with bacteriostatic sodium chloride (0.5 ml). The timing of oocyte retrieval was planned 12 hours after hCG trigger. The commercially available culture media used for the study were maintained in a stable environment according to manufactures instructions and incubated appropriately. Their pH was tested using pH paper before culture of embryos.

On the day of oocyte retrieval, mice were euthanized using halothane and the ovaries were dissected. The oocyte cumulus complexes were retrieved from ampulla. The retrieved COC were incubated in G-IVF media until the spermatozoa were retrieved from the testis of the male mice.

Figure 2(a): Retrieval of oocytes from ampulla

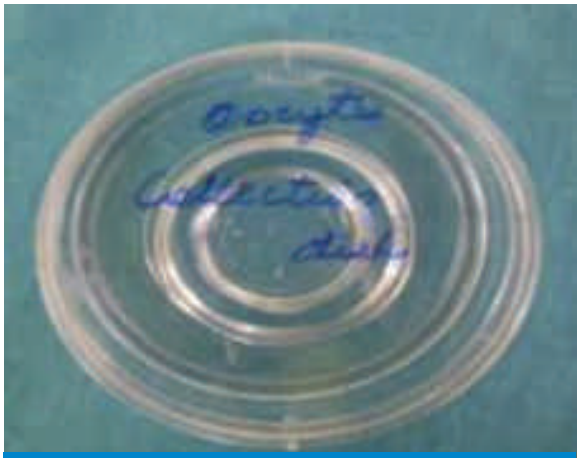

Figure 2(b): Collection of COC in a dish

Figure 2(c): Retrieval of sperms from testis

The male mice of appropriate age were sacrificed and testis dissected for retrieval of spermatozoa from epididymis. The animal carcasses were disposed properly as per instruction from IAEC.

The sample size of 35 oocytes was added in each group. In-Vitro fertilization was done by insemination of spermatozoa at standard concentration (50000:1) with the oocytes in commercially available culture media G-IVF, Quinn's sperm wash media, G-MOPS as Group I, II, III respectively (Table 1).

GROUP	CULTURE MEDIA
I	G-IVF (bicarbonate @ 6% CO_2)
II	Quinn's Spermwash media (HEPES)
III	G-MOPS (MOPS buffer)

Table 1: Group I, II, III for the study

After 4 hours, these oocytes were transferred into fresh culture medium overlaid by oil. This is done to prevent polyspermy and reduce the production, exposure to ROS. These ROS (reactive oxygen species) were proven to be detrimental to oocytes and affect fertilization rate.

After 6 hours of insemination, oocytes were assessed for fertilization. The presence of two pronuclei and two polar bodies were considered as criteria for fertilization. After fertilization check, these oocytes are transferred to cleavage dish for culture with appropriate culture media as grouped above. The assessment of cleavage was also done after 16 hours and recorded. At the stage of assessment of fertilization and cleavage check, the oocytes and embryos were blinded and the assessment was done by the same observer for all the stimulation cycles. Therefore, there was no inter-observer variation.

The statistical analysis was done using SPSS software. The fertilization and cleavage rate in all the three groups were compared. The fertilization rate was calculated as the percentage of no of oocytes fertilized with total no of oocytes inseminated. The cleavage rate was calculated as no of oocytes cleaved with no of oocytes fertilized. In addition to that, no of embryos reaching 2 to 4 cell stage and 4 to 8 cell stage were compared in each group using Fisher's exact test. A p-value of lesser than or equal to 0.05 was considered to be statistically significant.

Results

GROUP	Sample size (No of oocytes)	Number of Fertilized embryos	Fertilization rate
1 (Bicarbonate)	35	21	60 %
2 (HEPES)	35	18	51.42 %
3 (MOPS)	35	0	0 %

Table 2: Compares fertilization rate among the three groups

GROUP	Sample size (No of oocytes)	Number of Cleaved embryos	Cleavage rate
1 (Bicarbonate)	35	21	100 %
2 (HEPES)	35	18	100 %
3 (MOPS)	35	0	0 %

Table 3: Compares cleavage rate among the three groups

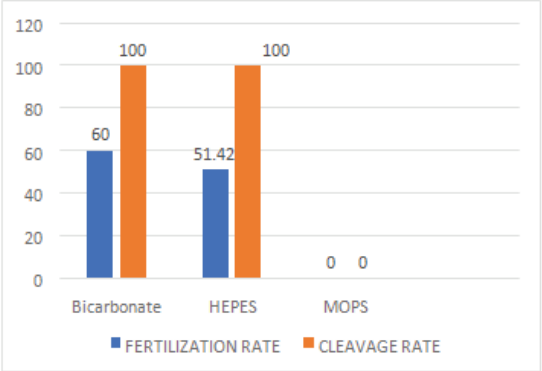

Figure 3: Comparison of fertilization and cleavage rate among the three groups

On analyzing Fisher's test between bicarbonate and HEPES (table 4) buffer based media, a p-value of 0.6307 was obtained. This shows that these two groups are not statistically significant. Hence, HEPES based buffer is as good as bicarbonate buffer for culturing embryos.

Group	Number of fertilized & cleaved embryos	Number of unfertilized oocytes	P value
Bicarbonate	21	14	0.6307
HEPES	18	17	

Table 4: Fisher's test for bicarbonate and HEPES buffer

P-value is 0.6307 which is not lesser than 0.05. Hence, statistically not significant.

On analyzing Fisher's test among bicarbonate, MOPS (table 5) and HEPES, MOPS buffer (table 6) based culture media; a p - value of 0.0001 was obtained which is statistically significant. This explains that bicarbonate and HEPES based buffer is better compared to MOPS based culture media for culturing embryos.

Group	Number of Fertilized & Cleaved embryos	Number of unfertilized oocytes	P value
Bicarbonate	21	14	0.0001
MOPS	0	35	

Table 5: Fisher's test for bicarbonate and MOPS buffer

Group	Number of Fertilized & cleaved embryos	Number of unfertilized embryos	p value
HEPES	18	17	0.0001
MOPS	0	35	

Table 6: Fisher's test for HEPES & MOPS buffer

P-value is 0.0001 which is less than 0.05. Hence, statistically significant.

Group	Number of cleaved embryos	Day 1	Day 2
Bicarbonate	21	21	16
HEPES	18	18	15

Table 7: Embryos cleaved on day 1 and day 2

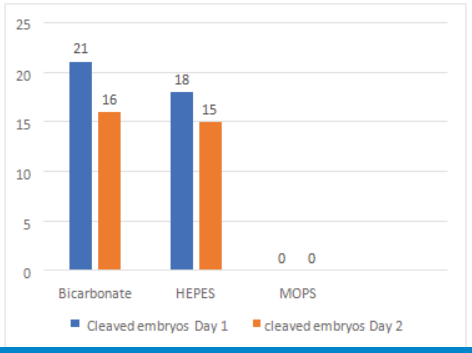

Figure 4: No of cleavage embryos on day 1 and day 2

	Bicarbonate	HEPES	MOPS
DAY 1	100 %	100%	0
DAY 2	76.19%	83.33%	0

Table 8: Compares cleavage rate on day 1 & 2 among the three groups

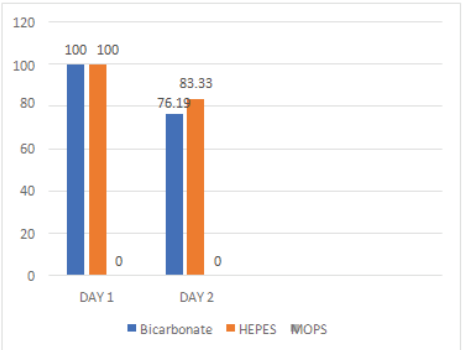

Figure 5: Comparison of cleavage rate on day 1 and day 2

On day 1, the fertilization and cleavage rate is compara-tively higher in group 1 (bicarbonate) than group 2 (HEPES) & group 3 (MOPS).

On day 2, the cleavage rate was significantly higher in group 2 (HEPES) than group 1 (bicarbonate) & group 3 (MOPS).

Discussion

The optimal maintenance of pH and temperature is crucial in IVF as gametes are very sensitive to fluctuations. In bicarbonate buffer, the maintenance of CO_2 levels helps to maintain the stability in pH. In organic buffers containing zwitterions such as HEPES and MOPS, the maintenance of temperature is essential to maintain a stable pH but the effect of these zwitterions on gametes is not fully studied.

Previous studies involving the effect of buffers used in culture did not emphasize on the fertilization, cleavage rate and the growth of embryos. In this study, the fertilization and cleavage rate is compared between media containing bicarbonate, HEPES, MOPS buffers. These are crucial and may pave way for another alternative.

The bicarbonate buffer being the most commonly used was made as the control arm. The effect of HEPES and MOPS were compared with bicarbonate with regard to fertilization, cleavage rate. Although many studies were involved in using HEPES and MOPS as media for spermatozoa preparation, sperm cryopreservation, and handling of gametes during ICSI, very few studies were involved with culturing of gametes in HEPES and MOPS.[10-14] Only few studies were available to substantiate the effect of HEPES and MOPS compared to bicarbonate.

With regard to HEPES buffer and embryos growth, previous studies showed that HEPES was able to support fertilization,[15] embryo growth[16] and development at room atmosphere (37°C). Few studies also indicated low fertilization rate, delayed development while using HEPES buffered media.[17-19]

In a previous study, it was found that when the concentrations of MOPS were around 20 to 25 Mm, it enabled the growth of mouse embryos till blastocyst stage at room atmosphere (37°C). Few studies also showed the detrimental effects of MOPS on gametes.[20,14,21] In this study, we used the commercially available MOPS media (Vitrolife).

In this study, fertilization was assessed after 6 to 8 hours of insemination with spermatozoa. The appearance of 2 pronuclei and 2 polar body was taken as the criteria for fertilization. In group 1, out of 35 oocytes inseminated with spermatozoa, 21 were fertilized at the time of fertilization check. In group 2 out of 35 oocytes, 18 were fertilized. In group 3, out of 35 oocytes numbers of oocytes fertilized were zero. These oocytes were dark and shrunken at the time of fertilization check. The fertilization rate was 60 % in group 1 (bicarbonate), 51.42% in group 2 (HEPES), 0% in group 3 (MOPS) (Table 8). On comparing the fertilization rates, bicarbonate group had a statistically higher fertilization rate than HEPES group.

The assessment of cleavage was done after 24 hours of fertilization check. Comparing the cleavage of embryos out of 35 oocytes inseminated, 21 (100 %) fertilized oocytes cleaved in group 1(bicarbonate). In group 2 (HEPES), 18 (100 %) were cleaved whereas in group 3 (MOPS) it was 0 (Table 9).

On comparing the growth of embryos, 2 to 4 cell stage were seen in 21 (100 %) fertilized oocytes in group 1. In group 2, 18 (100 %) oocytes reached 2 to 4 cell stage. On day 2, 18 (76.19%) embryos reached 4 to 8 cell stage in group 1 and 15 (83.33 %) embryos in group 2 (Table 14). Although the cleavage rate in Day 2 embryos was found to be higher in group 2 (HEPES), there was delayed cleavage observed in group 2 (HEPES) compared to group 1 (bicarbonate).

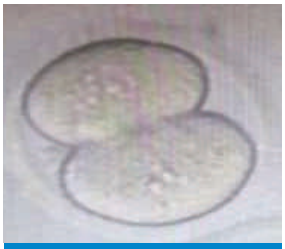

Figure 6(a): 2cell embryo

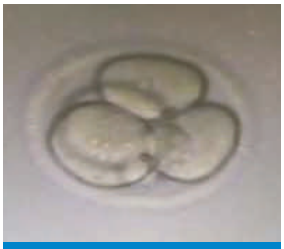

Figure 6(b):4 cell embryo

Figure 6(c): Compacting embryo

Figure 6(d): Morula

Figure 6(e): Blastocyst

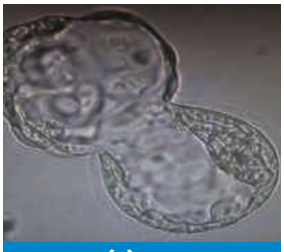

Figure 6(f): Hatching blastocyst

On further observations, four embryos reached the stage of compaction and formation of morula in group 1 whereas in group 2 two embryos developed till compaction and morula. On extended culturing, four high quality blastocysts were formed in bicarbonate based buffer and two embryos in group 2 (HEPES) were arrested at the stage of morula.

Conclusion

Though many studies substantiate the culturing of embryos in MOPS right from oocyte collection to handling of gametes during ICSI, upon culturing of embryos MOPS doesn't serve as a good medium for their growth and culture. The addition of few more components to overcome the problems for culturing in MOPS buffered media should be studied.

Thus, HEPES can be used in addition to bicarbonate buffers for culturing of embryos. But arrest at the stage of morula seems to be a disadvantage. The sample size is 35 in each group. Involving more sample size, analyzing the reason for arrest at the stage of morula and methods of overcoming has to be involved in future studies. The embryos cultured with HEPES should be transferred and implantation, obstetric, perinatal outcome should be analyzed in future.

The bicarbonate being the most commonly used buffer is the gold standard. HEPES buffered media being used widely for handling of gametes can also be used for culturing of embryos as the fertilization and cleavage rate remains close to that of bicarbonate buffer. Hence, HEPES can used only as an alternative to bicarbonate buffered media but cannot be replaced for culturing of embryos. Still studies have to be performed in future to estimate the implantation rate, obstetric and perinatal outcome for its use in humans.

Authors declare no conclict of interest.

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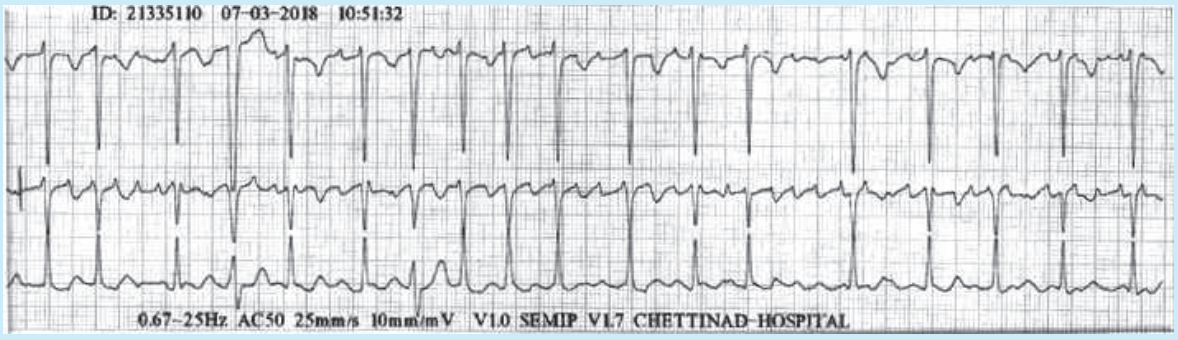
Interesting ECG



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