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Fertilization and Subsequent Development of Bovine Embryos Following In-Vitro Fertilization in Commercially-Prepared Medium

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This study was conducted to compare the capability and efficiency of two different media of IVF in prompting subsequent development and quality embryo. Matured oocytes in-vitro were fertilized using frozen semen and co-incubated in two different media (BODM and FHK medium) and followed the subsequent development stage up to *in-vitro* culture with simple modifications. Results showed that oocytes fertilized in BODM medium appeared to have 60 % induced pronuclear formation during 12 h and 100 % during 18 h, whereas oocytes achieved 58.54 % in cleavage formation at 2-cell during 72 to 96 h post culture and a blastocyst formation of 6.66%. Moreover, for the embryo quality assessment through the total cell count, analysis showed a mean value of 189.00 per embryo. Consequently, upon the usage of FHK medium for fertilization, oocytes prompted 80 % during 12 h and 60% during 18h of pronuclear formation. In addition, 60.46 % of the total samples had exhibited cleavage formation of 2-cell stage during 72-96 h post culture and 28.37 % for the blastocyst formation rate. Moreover, embryo quality assessment showed that total cell count had a mean value of 182.22 per embryo. The study concluded that both media are efficient in embryo production *in-vitro*, however FHK medium is considered to be more efficient in the production of blastocyst in bovine species.

Keywords: *in-vitro* embryo production, medium, pronuclear formation, blastocyst, embryo

Introduction

In modern biotechnology, *in-vitro* embryo production (IVEP) is one of the most important tools in achieving the success rate of applied biotechnology techniques. IVEP has been modified and optimized to attain its efficiency to

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produce acceptable output in its indirect contribution to the increase of supplies in the industries of milk and meat. IVEP is always done in the laboratory and follows three subsequent stages which includes *in-vitro* maturation (IVM), *in-vitro* fertilization (IVF) and *in-vitro* culture (IVC). As for mammalian cells, it can only exist outside their natural *in-vivo* surroundings, only if the *in-vitro* environment mimics that of the living body. This emphasizes the importance of providing a chemical and physiological media in which cells will survive. Various researches have tried a number of different media and culture conditions ranging from simple buffered balanced solutions to a more complex media supplemented with amino acids and various cofactors, whereas some has been paying attention to sequential culture systems. Thus far a perfect media for the production of embryo is very vital.

Although using IVEP in bovine oocytes-sperm as a subject research have highly optimized and tested, it customarily familiarized and repeatedly done. However, it is continually work-on to further the technique using other ruminant donor. Yet, this study was conducted to compare two different media in the fertilization *in-vitro*. It is to justify the capability of commercialize medium (FHK) as a mating rendezvous compared to the conventionally (BODM) used fertilization medium. Comparison was acquired via formation of pronucleus, occurrence of cell division at 2 cells (cleavage), further development includes blastocyst formation and embryo quality assessment.

Objectives: To compare and determine the efficiency of two different *in-vitro* fertilization media in terms of the subsequent development from sperm penetration to embryo production.

Materials and methods

Ovary Collection and In-Vitro Maturation

Ovaries are collected from the slaughter house and brought in the laboratory submerged in 0.9% saline solution with gentamycin (9g NaCl: 1L of distilled H_2O) at ambient temperature. The follicles present on the ovaries were aspirated using a 21-gauge needle in a 10 ml syringe. Bovine cumulus-oocyte complexes were collected and washed with Tyrode's Albumin Lactate Pyruvate (TALP) working solution. Oocytes were grouped according to the morphological appearance and incubated in the *in-vitro* maturation medium (IVM) for 22-24 hours at 38 ℃ and 5% CO_2 in the air.

In-Vitro Fertilization

For *in-vitro* fertilization (IVF) two media were used. The conventional

Bracket Oliphant Defined Medium (BODM) was compared to the commercialized (FHK) medium. Frozen semen straws were thawed in 37℃ water for 15 seconds and washed using BODM and centrifuged at 15,000 rpm for five minutes to remove the cryoprotectants. Supernatants were discarded and the sperm pellets were used. The sperm concentration was adjusted to $2x10^6$ sperm/ml by adding the subsequent IVF medium. A group of 20 or more matured oocytes were co-incubated with spermatozoa in a dish containing 50 ul of the fertilization medium at 38.5℃ under 5% CO_2 in air. The experiment was replicated four times with not less than 40 oocytes used for every replication.

Penetration assay

Sperm penetration ability was checked at different time points (12h and 18 to 20h), using 10 oocytes each following the protocol of Matas et al., 2012, after the onset of IVF. Given the specified time points of gamete incubation, the cumulus cells and other adhering spermatozoa in the cattle oocytes were removed by repetitive pipetting and agitating in a tube containing TALP-HEPES (5 mg/ml in PBS). Oocytes and presumptive embryos were mounted on glass slides and fixed with acetic alcohol (acetic acid 1: ethanol 3) for atleast 3 days. It was then stained with 1% orcein in acetic acid, destained in mounting solution (glycerol: acetic acid: water; 1:1:3) and examined with x40 and x100 objectives. The penetrated oocytes were determined as those having sperm heads in the previtelline space, in the oocyte cytoplasm and with visible pronuclei. Pronuclear formations (at 12 and 18 to 20h) were also observed in the process.

In-Vitro Culture

After fertilization, the remaining presumptive zygotes were transferred to a modified synthetic medium following the protocol of Atabay et al., (2007). The adhering spermatozoa were removed by repetitive pipetting and washing of the zygotes. It was then cultured for a period of 6-7 days in 38.5℃, 5% CO_2, 5% O_2 95 %N_2. The culture medium was replaced on the 4^{th} day of culture. The cleavage from two cell and further development of embryos and blastocyst stage is recorded during *in-vitro* culture. The data is presented as the percentage of pronuclear formation, cleavage formation at 2-cell and blastocyst formation over the total cell cultured.

Embryo Quality Assessment

Samples of the embryos produced from each of the donor cattle were subjected to quality assessment using the differential staining method of Sripunya *et al.* (2009) with minor revisions. The blastocysts were washed using Phosphate Buffered Saline (containing 0.2% polyvinyl alcohol (PBS-PVA) and were incubated for 30-60 seconds. Embryos were transferred to 25 ug/ml Hoechst 33342 dissolved in absolute ethanol and incubated for at least 30 minutes at 37℃. The embryos were removed from the stain and washed three times on PBS-PVA and mounted on a glass slide. Mounting was done by adding glycerol droplets on the side of the embryo and flattened using coverslips. The embryo is viewed on a fluorescence microscope (Nikon Inverted Microscope Ti) and focused using UV light. The total cell number (TCN) of the embryo were counted and recorded. Images were taken using Nikon Imaging Software-Basic Research. The data is presented as the total cell number of the embryos produced in each IVF media.

Statistical Analysis

The obtained data were statistically analyzed using t- test to compare different values observed between the two media with the level of significance $P<0.05$.

Results and Discussion

Sperm Penetration and Pronuclear Formation

Sperm penetration is the ability of the sperm to penetrate into the Zona Pellucida of the oocytes. Thus, for the pronuclear formation, it is the formation of the pronuclei of sperm and oocytes after post fertilization process. In study 1, sperm penetration and pronuclear formation were determined. Table 1 shows the percentages of sperm penetration and pronuclear formation at two different time points (12h and 18h post IVF) of the two media. Results revealed that after 12h of oocytes fertilized in BODM medium, 60% of the samples displayed PN formation (Table 1, Figure 1) while FHK conveyed 80% (Table 1, Figure 3). It was also presented in the table that after 18h of culture, PN formation in the samples fertilized in BODM medium increased up to 100% (Table 1, Figure 2) while FHK medium reduced the number of formation at 62.5% (Table 1, Figure 4). The results demonstrated the ability of oocytes fertilized in FHK medium to induce PN formation at earlier time points over BODM medium. Whereas the

declined number of samples performed PN formation at 18h can be attributed to the possibility that some of the fertilized oocytes could have advanced to next stage of development, the syngamy or the fusion of male and female pronuclei.

Table1: Rates of sperm penetration and pronuclear (PN) formation during IVF using two different IVF media: BODM (locally) and FHK (commercially) prepared.

Media	Time(Hrs)	Pronuclear Rate	Percentage (%)
BODM medium	12	6/10	60
	18	14/14	100
FHK medium	12	8/10	80
	18	10/16	62.5

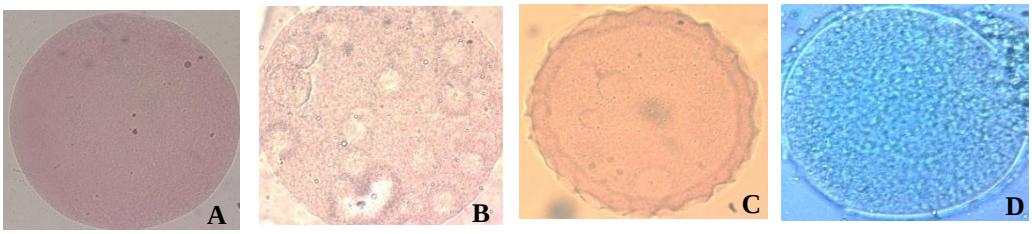

Figure 1. Pronuclear formation after 12 h and 18 h of co-culture in BODM medium (A, B) and FHK medium (C, D) respectively under bright field microscope with 40x-100x magnification.

Accordingly, the fertilization event in mammalian oocytes, the first evidence of sperm penetration is observed at 1 hour after fertilization *in-vitro*. Attachment of the spermatozoa in the ooplasm resulted in the initiation of 2nd meiosis and was reported to occur from 5-8 hours post insemination and further decondensation of penetrating sperm head in association with female chromosome decondensation were observed. Opposing and apposing pronuclei were frequently seen between 11-23 hour after insemination (Ocampo, 2001). Thus, a low incidence of male pronuclear formation was observed during 12 h (Funahashi, 1995). Syngamy, prophase stage of 1st mitotic division including first cleavage, was observed as early as 20 h after insemination (Ocampo, 2001).

Following fertilization, the sperm triggers a series of intracellular changes which initiate oocyte activation and pronuclear formation. Oocyte activation can also be induced artificially by several chemicals. The sperm nucleus is transformed into the male pronucleus through the interaction of

oocyte cytoplasmic factors (Chian *et al.*, 1996). Hence, the two-time point observation of PN formation (12 h and 18-20h) in the present study corresponds to the above previous findings.

Cleavage and Blastocyst Development

The early formation of pronucleus of oocytes and sperm after insemination has great impact on the succeeding embryonic development. In study 2, cleavage and blastocyst formation were determined. Table 2 shown the rate of cleavage and blastocyst development during subsequent culture of oocytes following IVF, wherein there were 202 and 211 presumptive zygote cultured in BODM medium and FHK medium correspondingly. As shown on the table 2, 58.54% of samples derived from BODM medium exhibited cleavage rate at 2-cell while samples derived from FHK medium attained 60.46%. Consequently, higher percentile rate of blastocyst formation was observed on samples derived from FHK medium, which have 28.37% while lesser on samples derived from BODM medium with a value of 6.66%.

T-test revealed no significant difference on the cleavage rate using the two media. Hence in the blastocyst rate, FHK medium was appeared significantly different over the BODM medium, since it performs early development. According to Lundin *et al.* (2001), early embryo cleavage is a strong indicator of embryo quality.

There are several differences between the cleavage in placental mammals and the cleavage in other animals. Mammals have a slow rate of division that is between 12 and 24 hours. These cellular divisions are asynchronous. Zygotic transcription starts at the two-, four-, or eight-cell stage. Cleavage differs from other forms of cell division in that it increases the number of cells without increasing the mass (Forgacs, 2005).

Table 2: Subsequent in vitro development of bovine oocytes following IVF subjected in two different medium

Media	Number of oocytes cultured	Cleavage Rate at 2 cell	Blastocyst rate
BODM medium	202	58.54%	6.66%
FHK medium	211	60.46%	28.37%

Multiple media provide satisfactory maturation, fertilization and cleavage rates for buffalo sperm and oocytes (Hammam *et al.*, 2010). Early-cleaving zygotes are more likely to develop to the blastocyst stage than their

late-cleaving counterparts (Leckniak, 2008) after the occurrence of syngamy subsequent development occurred producing a diploid hollow ball of cell known as the blastocyst.

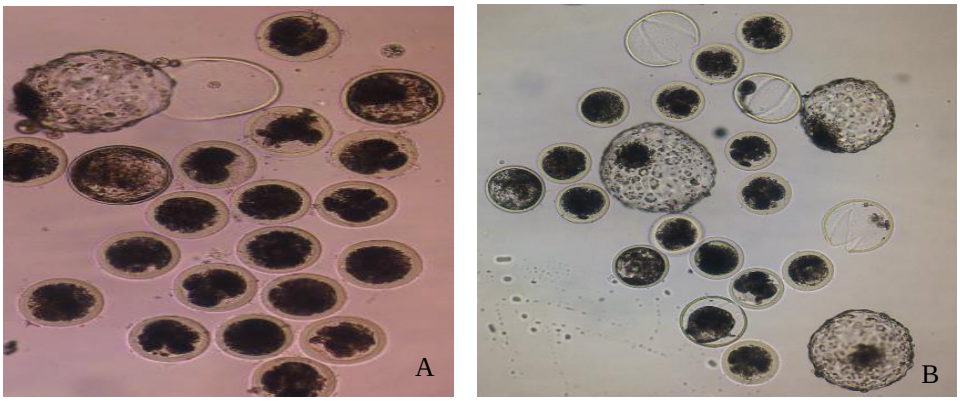

Figure 2. Cleavage and hatched blastocyst from oocyte fertililized in BODM (A) and FHK (B) mdium respectively.

Thus, on the collection of blastocyst during six days, it is possible to find embryos in advanced stages of development, including hatched blastocysts but most embryos reach the blastocyst stage on Day 7. A small proportion of embryos are delayed, reaching the blastocyst stage on Day 8 but their quality and feasibility is poor, as demonstrated by their lower resistance to cryopreservation (Gasparrini *et al.*, 2001). This may be considered as the cause of voiding the viability of produced blastocyst at late hour in BODM medium due to its late cleaving formation.

Embryo Quality Assessment through Total Cell Count

In Study 3, embryos were assessed through total cell count. Table 3 shows the number of embryo produced and the total cell count of blastocyst produced from two different IVF media. As shown in the table, there were two embryos with 189.00 total cell count using BODM medium, while there were nine embryos produced with a total cell count of 182.22 using the FHK medium.

T-test revealed no significant difference observed in terms of cell count between BODM (189.00) and FHK (182.22) medium. On the other hand, Figure 3 present the embryo stained in Hoechst derived from respective fertilization medium. The cell count values obtained after culture *in-vitro* are within the expected range suggesting the suitability of environment provided to the two treatment groups during further culture *in-vitro* (Figure 3).

Table3: Comparison of Total Cell Count of Blastocyst following IVF in FHK or BODM medium

	Number of Embryo	Total Cell Count
BODM Medium	2	189.00
FHK Medium	9	182.22

Note: Total values has no significant difference at P<0.05.

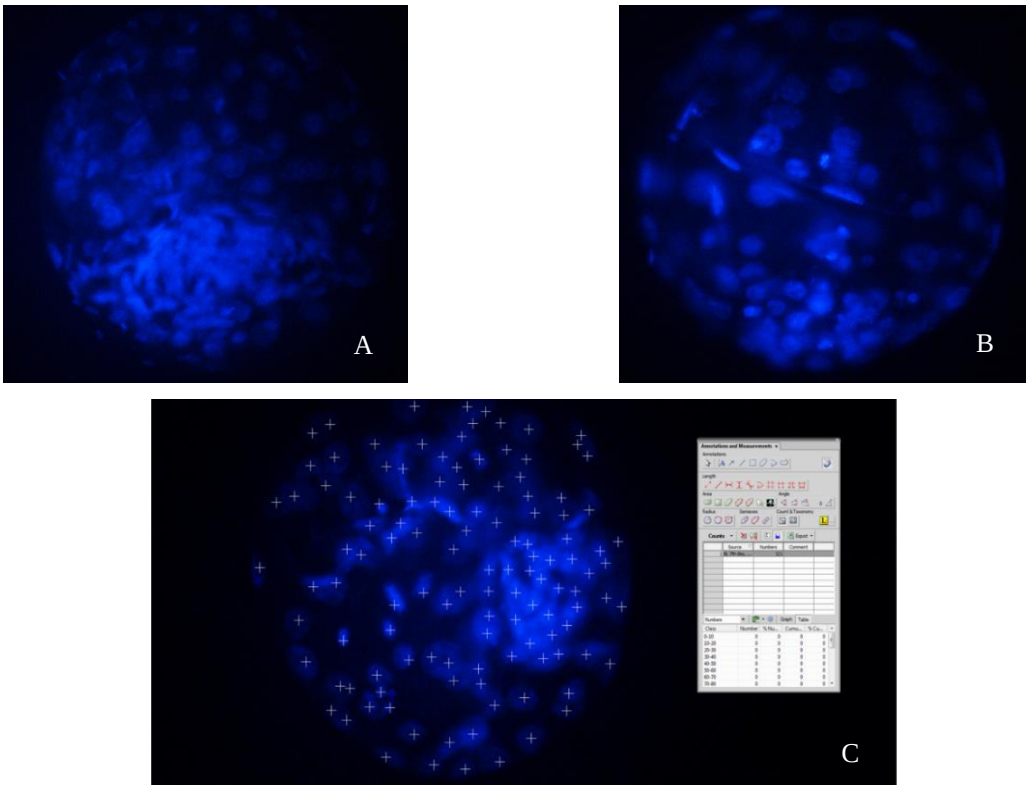

Figure 3. Embryo derived from the oocytes fertilized from BODM (A) and FHK (B)medium using Hoechst Staining and the cell counting of embryo using the NiS software for inverted microscope Ti (C) **Note:** Image was stained by Hoechst and viewed at x40-x100 under fluorescence microscope

For the assessment of quality embryo, total cell of the embryo is counted just to justify their viability for transfer. The total cell count can be determined by the addition of the inner cell mass (interior part) and trophoblast cells (exterior part) of the cell. For the standard range, embryo is said to be qualified when it has at least hundred total cell counts, for the assurance that it can survive after the transfer.

There are many studies which support the efficiency of adding

supplementation to the media. The two fertilization media have the same composition, hence for the FHK medium, is supplemented by HEPES, caffeine, heparin and L-cysteine, which has incomparable impact in the production of embryo. HEPES is widely used in cell culture for the reason that it is better at maintaining physiological pH (Baicu *et al.*, 2002) while, according to Ibrahim *et al.* (2015) caffeine has a hyperactivation effect on sperm cells at specific mM concentration. Moreover, Heparin is a natural component of ooplasm and it plays an important role in the rearrangement of the oocyte chromatin and as an oocyte maturation factor (Flores, 2008). It also acts anticoagulation and defense at such sites against invading bacteria and other foreign materials (Nader *et al*., 1999). Lastly, L-cysteine is an alpha amino acid that can be used as tools to enhance the efficiency of bovine oocytes, in penetrating the sperm, formation of pronuclei and further development (Rahim *et al*., 2011).

Summary

This study is conducted to determine the efficiency of two IVF media and analysed the fertilizing ability of the frozen-thawed semen from Gylorando cattle. This study aimed to determine the sperm penetration and pronuclear formation, as well as evaluation of subsequent development, and lastly, assessment of the embryo produced derived from oocytes fertilized in two different media. The research study used *in-vitro* embryo production technique which includes *in-vitro* maturation, *in-vitro* fertilization and *in-vitro* culture. The results at 12 hr point of observation, FHK medium has a higher induction of pronuclear formation with a percentage of 80% compared to BODM medium with 60%. Thus, for the 18 hr, BODM medium shown 100% of pronuclear formation while FHK has 62.5%, on the other hand, there is no significant differences between the two medium in terms of cleavage rate, thus FHK medium has a significantly higher blastocyst compared to BODM medium. Moreover, for the assessment of embryo using the Total Cell Count, both media produced significantly comparable quality of embryo.

Conclusion

Taken together for the conclusion of the present study, the rate of pronuclear formation was greater in oocytes cultured in FHK medium at 12 h, however at 18 h, a higher rate of PN formation is observed in oocytes cultured in BODM medium. The number of cells cultured in FHK that reached 2-cell stage of cleavage formation is comparable to that of cells cultured in BODM medium. However, a greater blastocyst rate was observed in cells cultured in

FHK medium. Finally, the assessment of embryo through total cell count revealed comparable results of both media. The superiority of FHK medium over BODM medium in terms of blastocyst production is therefore established. Given the fact that production of blastocyst is really important in the laboratory, consideration of using FHK for the IVEP has a great help in conduction of future studies, since it is a ready-to-use medium, much time is saved for the experiment.

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