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RESEARCH PAPER

Comparative Evaluation of Developmental Competence of Immature Cattle Oocytes in Three Different Culture Media

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ABSTRACT

The present study evaluates the effect of different embryo culture media on early embryonic developmental competence in cattle. *In vitro* techniques for the development of cattle embryo involves *in vitro* maturation of oocytes, *in vitro* fertilization, and culture of presumptive zygotes for early embryonic development. The cattle ovaries were collected from Kolkata slaughterhouse and immature oocytes were aspirated by follicular aspiration technique. After collection, these COCs were thoroughly washed in washing media before transferring them into IVM media droplets where they were cultured for 24 hours in CO_2 incubator at 5% level of CO_2 and at 38.5 °C with the maximum humidity. The *in vitro* matured oocytes were co-incubated with processed and capacitated sperm for 14-18 hr. After fertilization, presumptive zygotes were washed to remove the cumulus layer surrounding the zygotes, and cultured in IVC media. Three different culture media *i.e.* (TCM-199, BO-IVF and mCR2aa) were used for early embryonic development. The cleavage rate was higher in TCM-199 (74.47±9.63^a) and mCR2aa (71.64±10.03^a) medium as compared to BO-IVF medium (53.56±3.64^b). Morula development rate was significantly higher in TCM-199 (35.39±1.467^c) and BO-IVF (26.83±2.18^d) medium as compared to mCR2aa (22.83±2.9^d) medium. Blastocyst formation rate was observed significantly higher (P<0.05) in BO-IVF medium (14.17±2.85^f) compared to TCM-199 (4.63±0.6301^e) but not with mCR2aa (10.72±5.42^f) culture medium. From the present study it could be concluded that all three culture media are able to produce blastocyst, but BO-IVF and mCR2aa media showed higher potential to produce blastocyst in contrast to TCM-199 media.

HIGHLIGHTS

- Blastocyst formation rate was observed significantly higher (P<0.05) in BO-IVF medium (14.17±2.85^f) compared to TCM-199 (4.63±0.6301^e) but not with mCR2aa (10.72±5.42^f) culture medium.

Keywords: TCM-199, BO-IVF, mCR2aa, IVM, IVC, cleavage, blastocyst

In Vitro Embryo Production (IVEP) has become the most emerging part in the field of reproductive biotechnology. IVEP is a part of assisted reproductive biotechnology which has a great potential for speeding up the genetic improvement. Numerous *in vitro* studies developed good quality embryos from the cattle oocytes using different culture media Rosenkrans *et al.* (1994). IVEP has emerged as an alternative assisted reproductive biotechnological tool over the *in vivo* production of cattle embryos due to maximized production of offspring from superior and disseminate germplasm (Berglund 2008). *In vitro* development of the feasible embryos is the key application of IVEP. In recent years, *in vitro* production (IVP) of bovine embryos has become a generalized technology implemented in the increased production of livestock Stroebech

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et al. (2018). It has advanced greatly during the last two decades from the first calf born from IVF embryo Brackett *et al.* (1982). IVEP allows cheaper production of predictable embryo supply. Some studies reported the repeated recovery of primary oocytes using ovum collection procedures (OPU) Mutembei *et al.* (2008). IVEP technology does not only offer optimization of high-quality dams (mother), but also allows the preservation and doubling of the genetically similar cattle by making embryos available for cloning and nuclear transfer Muasa *et al.* (2010). The progress in the IVEP technology can be used to change the economic benefits of farmers. Recently use of sexed semen (SS) from superior bulls showed the increased IVEP in cattle Gonzalez-Marin *et al.* (2017).

IVEP involves three main steps: maturation of primary oocytes obtained from large antral follicles, fertilization of ripened secondary oocytes with frozen thawing semen, and embryo culture up to one week to the formation of blastocyst that can be transferred to the synchronized recipients or can be cryopreserved for future use. In contrast to the conventional embryo transfer (ET), the production of embryos with the aid of IVEP has several advantages; first, IVEP can be used effectively in animals not responding to the super ovulation treatment. Second, IVEP can be used to explore the genetic potential of the females to resist against terminal diseases that would not be achieved by conventional ET. Beyond the potential use of the IVEP in livestock regimes, embryos are also necessary for the establishment of other aspects of biotechnology, such as cloning, stem cell production and transgenesis, etc. After artificial insemination (AI) and several ovulation and embryo transfer schemes like multiple ovulation transfer technology (MOET), IVEP represents the third generation technique targeting better animal reproduction control. Many studies have been made to determine the most appropriate conditions for the development of IVM, IVF and IVC to maximize the level of production and quality of embryo in bovine. Therefore, there is a need to develop an efficient embryo culture system that can support the embryonic development of the oocytes that will result in the production of viable embryos that benefit from both investigations and commercial efforts. The culture media and its composition play an important role in the development competition of embryos produced *in vitro.* To optimize the IVEP, it is important to understand the physiology of the embryo *in vivo.* Supplementation of several growth factors in the culture media improvised the maturation of oocytes, fertilization with thawed semen and culture of embryos with variable results Gasparrini *et al.* (2006). The main important developmental events are carried out during the post embryonic development Kharche *et al.* (2011). Embryos from the pre-implementation stage can be developed in different ways consisting of simple balance solutions and carbohydrates to complex constituents, such as tissue culture media (TCM-199) with additional serum supplements. Comparative study on the effectiveness of three different culture media (TCM-199, BO-IVF and mCR2aa) in the development of cattle embryo to the blastocyst stage is not well defined. Our objective was to determine the best embryo culture medium for *in vitro* cattle embryonic development.

MATERIALS AND METHODS

Chemicals, Cell Culture Media and Supplements

Molecular biology grade chemicals and reagents were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and HIMEDIA Biosciences Pvt. Ltd., Mumbai, India unless otherwise stated. BO-SEMENPREP and BO-IVF media were purchased from IVF BIOSCIENCE. All the plastic wares, petri dishes, centrifuge tubes and experiment related tools were purchased from Tarsons Products Pvt. Ltd. (Kolkata, India). The 0.22 μm disposable syringe filters were purchased from Milipore Corp., Bedford, MA, USA.

Collection of Ovaries

Fresh cattle oviducts and ovaries were collected from Kolkata slaughter house. These were washed 3-4 times with antibiotic fortified (400 $IU.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin) isotonic warm saline (32-35°C). The washed oviducts and ovaries were then put in a thermo flask containing warm saline. The collected ovaries were transported to the laboratory within 3 h of slaughter. In the laboratory, the ovaries were rinsed twice with antibiotic fortified warm saline and were then

trimmed to remove the extra tissue and were washed several times with antibiotic fortified saline solution (30-35°C).

Collection of oocytes

Immature oocytes were collected from visible surface follicles (>3 mm diameter) of ovaries by using aspiration method with a 19 gauge hypodermic needle attached to a 5 ml disposable plastic syringe containing aspiration medium (TCM-199 + DPBS + 0.3% BSA + 50 $\mu g.ml^{-1}$ Gentamicin sulfate). The contents of the syringe, which included the aspirated oocytes, follicular fluid, granulosa cells and other debris, were poured in 65 mm glass petri dish. Searching of oocytes was carried out under a zoom-stereo microscope at 40X magnification and were shifted to 35 mm petri dish containing washing medium (TCM-199 + 10% FBS + 0.8 mM sodium pyruvate + 50 $\mu g.ml^{-1}$ gentamicin sulfate). The aspirated oocytes were graded according to the following criteria: grade A (>5 layers of cumulus cells), grade B (3-5 layers of cumulus cells), grade C (<3 layers of cumulus cells) and grade D (partial/no layer of cumulus cells). All A, B and C grade COC's (cumulus oocyte complexes) with homogenous and evenly granulated cytoplasm were used for maturation.

In-Vitro Maturation of oocytes

The usable quality oocytes were collected from the searching dish and washed 4-6 times with the washing medium (TCM-199 + 10% FBS + 0.8 mM sodium pyruvate + 50 $\mu g.ml^{-1}$ gentamicin sulfate), and then twice with the maturation medium (TCM-199 + 10% FBS + 5 $\mu g.ml^{-1}$ FSH-P + 0.8 mM sodium pyruvate + 5% Follicular fluid + 50 $\mu g.ml^{-1}$ gentamicin sulfate). For *in vitro* maturation, groups of 20-25 COC's were placed in 100 µl droplets of maturation medium overlaid with sterile mineral oil in 35 mm Petri dishes and cultured for 24 h in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C.

Sperm preparation and *in vitro* fertilization

The spermatozoa used for IVF throughout the study were from the same donor that had been tested for IVF earlier. The spermatozoas were prepared for fertilization using swim-up method. Briefly, three straws of frozen-thawed bull semen were used for IVF procedure. Two semen straws were suspended in 1.2 ml of Working Bracket Oliphant (WBO) media Brackett *et al.* (1993) containing 10 $\mu g.ml^{-1}$ heparin, 0.57mM caffeine sodium benzoate and 1.23mM sodium pyruvate and another semen straw was suspended in Bioscience-IVF media (1.2 ml) and incubated for swim-up at 38.5°C for 18 min. After incubation, progressively motile sperm cells were taken by collecting the supernatant (800 µl) from the 1.5 ml eppendorf tubes and centrifuged at 2000 rpm for 5 min. After centrifugation, the supernatant was decanted and the pellet was dissolved again in 1ml of WBO media and centrifuged at 2000 rpm for 5 min. Finally, the pellet was dissolved in 1 ml of Fertilization Bracket Oliphant (FBO) medium for second wash at 2000 rpm for 5min. After centrifugation, the supernatant was decanted and 50-100 µl of FBO and Bioscience-IVF media were added to the respective treated pellets and mixed properly. The matured COC's were inseminated with capacitated motile spermatozoa (1-2 million $spermatozoa.ml^{-1}$) in respective FBO drops (50 ml) and Bioscience-IVF drop (50 ml) and placed in 5% CO_2 incubator at 38.5°C for 15-18 h with maximum humidity.

In Vitro Culture of Presumptive Zygotes

After the 15-18 h of sperm-oocytes co-incubation, the cumulus cells were washed off from the oocytes by gentle pipetting in washing medium. The zygotes were washed 3-4 times in washing media and cultured in three IVC culture medium i.e. TCM-99, BO-IVF and mCR2aa medium for 48 h in 5% CO2 incubator with maximum humidity at 38.5°C. After 48h of incubation the cleaved embryos were shifted to 100 µl droplets of three different blastocyst medium i.e. TCM-199, Bioscience-IVF and mCR2aa and co-incubated with healthy vibrant oviductal cells in 5% CO_2 incubator with maximum humidity at 38.5°C for 8 days.

Experimental Design and Statistical Data Analysis

In the present study three different culture media *i.e.* (TCM-199, BO-IVF and mCR2aa) were used for early embryo development. Data analysis was done by IBM® Statistical Package for the Social Sciences (SPSS). Effect of different culture media on developmental competence of *in vitro* embryo production in cattle was determined by applying

multivariate test under general linear model. Descriptive statistics were performed to calculate mean and standard errors. Fisher's LSD (Least Significant difference) was done to identify the significant difference between the mean values. Graph preparation was done by Graph pad prism software.

RESULTS AND DISCUSSION

Comparative study of TCM-199, BO-IVF and mCR2aa as culture media for the early embryonic developmental stages

A total of 372 immature oocytes were taken in this experiment to analyse the effect of different culture media on cattle embryonic development. It has been found that there is significant differences among the three medias ($p<0.05$) particularly in cleavage rate, 4-cell stage, 8-cell stage, morula stage and blastocyst stage (Table 1). The cleavage rate (74.47±9.63) was highest in TCM-199 media but not significantly higher than mCR2aa media. The results indicate that four-cell stage, eight-cell stage and morula formation rate were significantly higher in TCM-199 media as well as BO-IVF media. The blastocyst formation rate (4.63 ± 0.6301) in TCM-199 was significantly lower than other two medias i.e. BO-IVF and mCR2aa. In BO-IVF media the cleavage rate (53.56±3.64) was significantly lower than other two medias i.e. TCM-199 and mCR2aa but on the other hand blastocyst formation rate (14.17±2.85) was significantly higher in BO-IVF media as compared to TCM-199 media but not to mCR2aa (Fig. 1). Similarly in mCR2aa media the cleavage rate (71.64±10.03) was significantly higher as compared to the BO-IVF media. Whereas, 4-Cell stage, 8-Cell stage and morula formation rate in mCR2aa media was significantly lower with TCM-199 media but not with BO-IVF media. On the other hand blastocyst formation rate (10.72 ± 5.42) in mCR2aa media was not significantly lower than BO-IVF media but significantly higher than TCM-199 media.

The similar study was also reported by Palasz *et al.* (2008) that, use of TCM-199 with or without bovine serum albumin (BSA) to culture the cattle oocytes did not show any significant level of difference in cleavage rates i.e. (74.8% and 79.8%) respectively. The blastocyst formation rate obtained in their experiment was about 14.9%. The use of hyaluronan (HA), a glycosaminoglycan and BSA

Table 1: Comparative study of TCM-199, BO-IVF and mCR2aa as culture media for early embryonic development

Culture media	Total no. of Cleaved oocytes (%)	Total no. of 4-Cell stage embryo (%)	Total no. of 8-Cell stage embryo (%)	Total no. of morula stage embryo (%)	Total no. of blastocyst stage embryo (%)
TCM-199	74.47±9.63^{a}	82.8±9.07^{a}	54.9±4.9b^{b}	35.39±1.467^{c}	4.63±0.6301^{e}
BO-IVF	53.56±3.64^{b}	56.37±7.07^{b}	31.83±1.3^{c}	26.83±2.18^{d}	14.17±2.85^{f}
mCR2aa	71.64±10.03^{a}	54.88±10.79^{b}	28.92±3.48^{c}	22.83±2.9^{d}	10.72±5.42f

Values (Mean±SEM) inTCM-199, BO-IVF and mCR2aa with different superscript differ significantly (p<0.05).

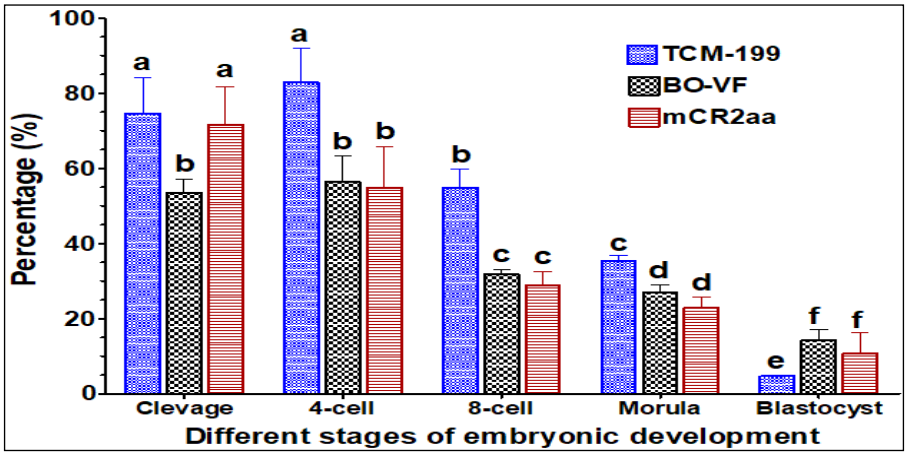

Fig. 1: The graph represents the comparative effect of TCM-199, BO-IVF and mCR2aa as culture media on different stages of embryonic development in cattle

in addition to TCM-199 showed an increase in blastocyst formation rate (32.1%). These results suggested that TCM-199 without any additional supplements showed no significant differences in cleavage rate but the blastocyst formation rate was less in comparison to the other groups. A.P Gandhi *et al.* (2000) cultured the immature cattle oocytes in TCM-199 and obtained the cleavage and blstocyst rate of about 64.2% and 30.5% respectively. In his experiment he determined that there were no significant differences with TCM-199 and mSOF medium in terms of cleavage and blastocyst rate. Puja *et al.* (2016) reported the cleavage rate (38.46%) and blastocyst rate (9.57%) of cattle embryos in mCR2aa culture medium. Y.H Choi *et al.* (2002) showed that the cleavage rate and blastocyst rate of cattle embryos was about 78% and 2% respectively in mCR2aa medium.

CONCLUSION

All the three different culture media (TCM-199, Bo-IVF and mCR2aa) were able to produce cattle embryos of different developmental stages. The blastocyst formation rate was found higher in BO-IVF medium and lower in TCM-199 medium. The mCR2aa medium did not show any significant difference to the BO-IVF medium. Both BO-IVF and mCR2aa medium could be used to improve the *in vitro* embryo production in cattle. TCM-199 can be enhanced with additional supplements to increase the cleavage and blastocyst development rate in bovine.

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