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The Developmental Competence of the Bovine Embryos Processed in IVC-Stroebech Medium *vs*. Mcr2aa Medium

Research Article

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ABSTRACT

Fetal bovine serum (FBS) is preferred for its ability to provide a variety of molecules that can support the growth of embryos. However, the use of serum can lead to the development of large offspring syndrome, which is a significant problem. This study aimed to examine bovine embryo development derived from *in vitro* fertilized oocytes in two culture media: IVC-Stroebech medium, a chemically defined medium; and the modified medium of Charles Rosenkrans with amino acids (mCR2aa), an undefined medium containing serum. The process of *in vitro* maturation was carried out at 38.5 ˚C in IVC-Stroebech medium. The process of *in vitro* fertilization was carried out for 18 hours using Bracket and Oliphant's (BO) medium. As to the experiment, cumulus-free presumptive zygotes were randomly assigned to two different culture medium treatments. The first treatment was a two-step culture system using mCR2aa medium. The first step involved using mCR2aa medium without FBS for the first 48 hours of culture. After that, for the remaining days of culture, the second-step mCR2aa, which is composed of 10% FBS, replaced the medium. The embryos were incubated at 38.5 ˚C under 5% CO2 5% O2 and 90% N2 for 10 days. Statistical analysis revealed a significantly higher hatched blastocyst rate in the IVC-Stroebech medium in comparison with the mCR2aa medium. There was not any significant difference (P>0.05) in the formation rate of morulas and blastocysts between the IVC-Stroebech medium and the mCR2aa medium. Findings recommended the use of IVC-Stroebech medium for bovine *in vitro* embryo culture.

KEY WORDS blastocysts, define media, *in vitro* culture, *in vitro* fertilization, undefined media.

INTRODUCTION

In recent years, significant progress has been observed on the *in vitro* embryo production (IVEP) in bovine (Ferré *et al*. 2020). However, the developmental percentage of oocytes grown into the blastocyst stage is only around 30-40% (Shahzad *et al*. 2020). It is worth mentioning that the quality and quantity of embryos obtained through IVEP are significantly influenced by the conditions of *in vitro* culture (Poniedziałek-Kempny *et al*. 2019). The serum presence or absence can play a significant role in culture conditions that impact quality differences in a particular context. The buildup of lipid droplets in the cytoplasm of *in vitro*-produced embryos is associated with the presence of serum (Paschoal *et al*. 2014). The use of serum in IVC media has both positive and negative effects due to its constituents (Mesalam *et al*. 2019). Serum, is beneficial in embryo and oocyte culture as it contains albumin that helps to osmolality balance and acts as a free radical scavenger (Romão *et al*. 2016). The serum provides important nutrients like vitamins, growth factors, amino acids, macromolecules, and hormones that are necessary for the growth and develop-

ment of the embryos and can prevent oocyte adhesion and hardening *in vitro* (Romão *et al*. 2016; Kim and Yoon, 2019).

Long run exposure to the serum has significant effects on the morphology and biochemistry of embryos (Rizos *et al*. 2003). Serum is a supplement that is not well-defined chemically and can be contaminated by various microorganisms such as fungi, bacteria, viruses, and mycoplasma (Rauch *et al*. 2011). It can be stated that an example of aberrant effects on fetal growth is seen in cattle and sheep, and it is called "large offspring syndrome". This syndrome was observed because of the serum used in the preimplantation culture medium (Zhu *et al*. 2018).

Genetic engineering techniques have been used to identify, clone, and produce the crucial growth elements and nutrients needed by various types of cells (Gstraunthaler, 2003) and resulted in the creation of many chemically defined, serum-free media that are suitable for constant cell lines as well as certain types of cells in the initial culture (Taub, 1990). These media have been designed and are now available for use. Serum-free media are more specific to certain types of cells (Murillo *et al*. 2017). Researchers have developed defined IVC media to ensure that culture conditions are consistent and free from contamination. This type of media also makes it easier to compare results between different laboratories and improves the reliability of analysis. It is revealed that the quality of blastocysts, i.e early-stage embryos, is influenced by the conditions in which they are cultured (Mesalam *et al*. 2019).

The search for a replacement for fetal bovine serum (FBS) is mainly focused on the field of IVC and demands culture conditions to be defined, safe, and free of animal components (Rauch *et al*. 2011). Pryor *et al*. (2016) utilized BO-IVM, BO-IVF, and BO-IVC media (IVF Bioscience Co.) to mature, fertilize, and develop bovine embryos in a laboratory setting. They found that these modified, premade media resulted in the production of higher-quality embryos in different culture conditions. Hajian *et al*. (2020) conducted a study and observed that the BO medium resulted in a higher rate of blastocyst formation through somatic cell nuclear transfer (SCNT) compared to the synthetic oviductal fluid (SOF) medium. However, both media showed similar clinical pregnancy rates when it came to the post-implantation developmental competence of the SCNT-derived embryos. Nevertheless, compared to the SOF medium, a lower rate of pregnancy loss was observed for the BO medium.

In the current research, the developmental competence of the embryos and the hatchability of resulted blastocysts produced from defined (IVC-Stroebech medium) and undefined (mCR2aa) culture media were comparatively investigated.

MATERIALS AND METHODS

Location

This study was conducted in the embryo biotechnology laboratory of the Iranian Research Organization for Science and Technology (IROST). The laboratory was kept at a temperature between 28 ˚C and 30 ˚C. Before the study, all surfaces were exposed to ultraviolet radiation overnight.

Chemicals

Sigma Chemical Co. and Gibco in the USA have been the origin of all the chemicals and media used in the experiment, while the plasticware used was purchased from Falcon (Paignton, UK). The researchers utilized a serum-free *in vitro* culture system that was commercially available and designed specifically for bovine, known as IVM-Stroebech medium and IVC-Stroebech medium, which was purchased from Stroebech Media.

Collecting oocyte and *in vitro* maturation (IVM)

Oocyte collection and IVM of oocytes were done as described by Jafarnejad *et al*. (2018). Briefly, bovine ovaries were obtained from a slaughterhouse located in the vicinity and moved to the laboratory within two hours. When they arrived, they were washed three times with phosphate-buffered saline (PBS) containing 100 IU/mL penicillin and 50 mg/mL streptomycin. The washing process took place at a temperature of 30-34 ˚C. It is also worth accounting for the process of obtaining cumulus-oocyte complexes (COCs). The complexes were retrieved by aspirating them from follicles of size 2-8 mm using a syringe connected to an 18-gauge needle. After the follicular materials were drawn out, they were carefully transferred into a 90 mm petri dish containing washing media (TCM-199, Sodium pyruvate, gentamicin, l-glutamine, and FBS). The petri dish containing oocytes was then observed using a stereo-zoom microscope. Oocytes with an extra 3 layers of cumulus cells that were compact unexpanded and had a uniform and evenly-granulated cytoplasm were taken to maturation droplets. COCs in IVM-Stroebech medium droplets (100-µl droplets that 15 COCs per droplet) were covered with sterile mineral oil and their maturation was carried out at 38.5 ˚C under 5% CO2 air atmosphere for 24 h with maximum humidity.

Sperm processing and *in vitro* fertilization (IVF)

Finding the first polar body indicated that the oocytes had progressed to the metaphase II (MII) stage. Spermatozoa samples were prepared as described by Muzaffar *et al*. (2012). The matured oocytes were washed twice using a washing Bracket and Oliphan medium (BO) medium, which contained 137.0

µg/mL of sodium pyruvate, 1.942 mg/mL of caffeine sodium benzoate, and 10 µg/mL of heparin. Each droplet contained 15 oocytes and had a volume of 100 µL. The frozen spermatozoa straws from Holstein bulls were purchased from Nahadehaye Dami Jahed Co. (Karaj, Iran). Semen straws were thawed by immersion in warm water (37 ˚C) for 30 s pooled, and processed by washing twice with the BO medium. The pellet was resuspended in 0.5 mL of the capacitation and fertilization BO medium (containing washing BO medium and 10 mg mL^{-1} fatty-acid-free bovine serum albumin). Finally, the concentration of sperm suspension was adjusted to 1×10^6 cells per milliliter.

In vitro culture (IVC)

IVC of presumptive zygotes was done as described by Karimian *et al.* (2018). Following an 18-hour incubation period, the cumulus cells and corona radiata cells were eliminated by pipetting. The resulting zygotes were then washed to separate any sperm cells that were still attached. Presumptive zygotes without cumulus were placed in two different culture mediums. The first culture medium is called IVC-Stroebech medium (Key Ingredients: Insulin, Hyaluronic Acid, EDTA, Calcium L-Lactate, Sodium Selenite, L-Carnitine, Ethanolamine, Sodium Pyruvate, Albumin, Transferrin, Myo-Inositol, Zink Sulphate, and other Inorganic Salts, Essential and Non-Essential Amino Acids, Vitamins, Sodium Bicarbonate, Ala-Glutamine, Glucose, Phenol Red, and Gentamycin) and the second one is a modified version of the medium of Charles Rosenkrans with amino acids, abbreviated as mCR2aa. The allocation of the zygotes to these two culture mediums was done randomly. The mCR2aa culture medium is a two-stage process that involves two different types of media. The first stage uses a medium containing 0.6% Bovine serum albumin (BSA) and is used for the first 48 hours of cell culture. Following this, the medium is replaced with the second stage mCR2aa medium, which contains 0.6% BSA and 10% FBS. This second medium is used for the remaining 6 days of cell culture. The embryos were placed in a container and covered with clean mineral oil. The container was kept in a warm environment at a temperature of 38.5 ˚C, with a specific mixture of gases (5% CO2, 5% O2, and 90% N2) for 10 days. The environment was kept humid to support the growth of the eggs. After 5, 8, and 10 days, the rates of development for different stages, including morula, blastocyst, and hatched blastocyst, were recorded.

Statistical analysis

Quantitative data analysis through a T-test was run to address the research objectives (SPSS, 2011). In doing so, initially, descriptive statistics including mean and SEM were measured, followed by inferential measures through a T-test to investigate the significance level at $P < 0.05$.

RESULTS AND DISCUSSION

The present assessed whether defined (IVC-Stroebech medium) and undefined (mCR2aa) culture media had a significant impact on the development potential of embryo *in vitro*. The developmental rates of presumptive zygotes under different media are shown in Figure 1.

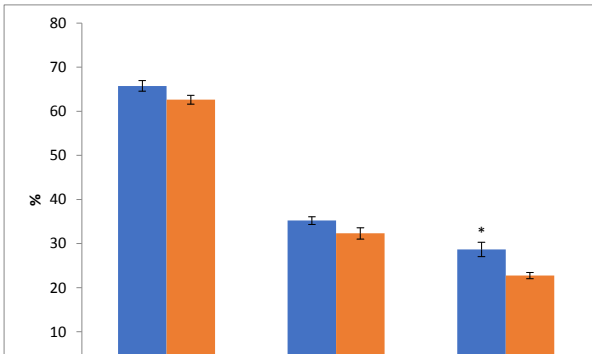

Figure 1 Effect of IVC-Stroebech medium and mCR2aa on *in vitro* development of bovine and sheep
Values are as mean ± SEM
* Indicate statistical difference (P<0.05)

Numerically, more zygotes and morula were formed in IVC-Stroebech medium (88.82±2.58 and 65.75±1.19, respectively) as compared to mCR2aa medium (82.76±1.76 and 62.63±1.0, respectively). Similarly, the blastocyst formation rate did not show any significant difference (P>0.05) between IVC-Stroebech medium and mCR2aa media (32.21±0.85 and 32.31±1.28, respectively). However, the IVC-Stroebech medium had significantly higher hatched blastocysts as compared to the mCR2aa medium (28.67±1.62 and 22.73±0.71, respectively) (P<0.05) (Table 1, Figure 2).

The culture media used for IVC of embryos are designed to meet the metabolic needs of the embryo (Naitana and Ledda, 2020).

Protein sources, antioxidant molecules, and growth factors were employed for enriching the culture media achieved by adding bovine serum albumin (BSA) or serum (either 5% or 10%) to the media. Our results showed that comparing the mCR2aa medium with the IVC-Stroebech medium did not significantly (P>0.05) affect the mean cleavage rate of oocytes and also morula and blastocyst formation. The percentage of hatched blastocysts, however, was greater for zygotes cultured in the IVC-Stroebech medium than for those cultured in the mCR2aa medium (P<0.05).

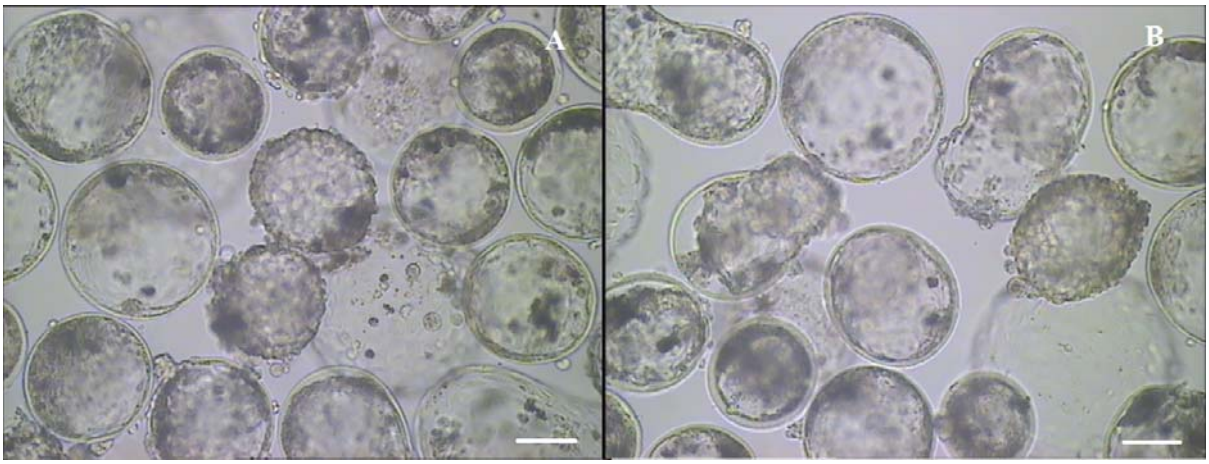

Figure 2 Hatched and hatching blastocysts 8 days after culture. A) IVC-Stroebech medium and B) mCR2aa Scale bar = 100 µm

The blastocyst rate of two different co-culture systems i.e., serum-free culture medium and serum and granulosa cell monolayer-free culture medium revealed that the blastocysts grown in culture medium without serum and granulosa cell monolayer showed a higher hatching rate, indicating a higher relative blastocyst rate compared to the serum-free system (Shahzad *et al*. 2020). Four different types of media were compared to see which ones were best at supporting pre-implantation embryos to arrive at the blastocyst stage (Goel *et al*. 2016). The comparison showed that mKSOMaa, TCM-199 with oviductal epithelial cells (OEC) co-culture, and mCR2aa had similar development competence and were equally effective in supporting the embryos' growth to the blastocyst stage. Adding serum to the mKSOMaa medium starting from day 4 improved the production of blastocysts (Goel *et al*. 2016). The use of serum not only enhanced the development of blastocysts but also increased the proportion of hatched blastocysts. Adding 10% FBS to SOF or CR1 starting from day 4 after insemination can improve the rates of blastocyst formation and increase the success of embryo hatching (Wan *et al*. 2009). FCS (fetal calf serum) resulted in the earlier development of blastocysts and led to an increase in embryos reaching the blastocyst stage by day 7 (Gómez and Díez, 2000).

It can be argued that the embryo's quality and development can be influenced by the serum type used during the culturing process. Embryos cultured using FCS resulted in lower quality and blastocyst rates compared to those cultured with BSA (Garcia *et al*. 2015). Additionally, the embryos produced with BSA showed higher hatching rates, suggesting that serum plays a crucial role in determining the quality and development capacity of embryos. Electron microscopy showed that blastocysts developed in the presence of FCS had a considerable amount of LDs in the cytoplasm of Trophectoderm and the Inner Cell Mass, as opposed to those grown in the same medium without serum (Gómez *et al*. 2008). The blastomeres of ovine embryos cultured in SOF medium supplemented with human serum showed a significant level of mitochondrial degeneration (Abe *et al*. 2002). This degeneration was more noticeable than the embryos cultured in a serum-free SOF medium containing bovine serum albumin and amino acids (SOFaa-BSA) instead of serum. The existence of serum might have an effect on mitochondrial metabolism, leading to an imbalance in the oxidation-reduction (redox) state (Sudano *et al*. 2011). This can hinder lipid metabolism and result in a surplus buildup of intracellular lipids.

The poor-quality IVEP embryos showed mitochondrial changes, which included a decrease in the total number of mitochondria and a proportion increase in immature mitochondria (Romão *et al*. 2016). These changes were linked to the use of a serum-containing medium during culture. The study also found that these changes affected lipid metabolism, leading to reduced ATP production as a result of fewer lysosome-like vesicles, lipid droplets, and cytoplasmic vesicles. Lipids play a crucial role in generating ATP through fatty acid oxidation, which is considered to be a vital energy source for embryos (Dunning *et al*. 2010). The process of lipid beta-oxidation was critical for the early development of embryos. The fatty acid composition of early-stage embryos (2-cell) and blastocysts was found to differ when embryos were cultured in either serum-supplemented or serum-free medium. The inclusion of serum in the medium led to the retention of elevated levels of the saturated fatty acids palmitic and stearic (Abe *et al*. 2002). Additionally, there was a noteworthy increase in the long-chain unsaturated fatty acids oleic and palmitoleic. Nonetheless, the amount of myristic acid detected was sig-

nificantly lower. This implies that the accumulation of long-chain saturated and unsaturated fatty acids in embryos exposed to serum may be attributable to the serum.

CONCLUSION

In this study, the use of IVC-Stroebech medium-defined culture medium compared to mCR2aa containing FBS was studied in the culture of bovine embryos. In the formation of morulas and blastocysts, the IVC-Stroebech medium was not significantly different from the mCR2aa culture (P>0.05). While the IVC-Stroebech medium resulted in significantly higher hatched blastocysts than the mCR2aa culture medium. Findings recommended to use of IVC-Stroebech medium for bovine *in vitro* embryo culture.

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