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Research article

Optimizing chemically-defined culture medium for the production of bovine (*Bos taurus*) embryos *in vitro*

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Abstract

The study optimized and established an efficient protocol for *in vitro* production of bovine pre-implantation embryos. Specifically, it developed a protocol that will yield higher blastocyst rate and better embryo quality using the modified Synthetic Oviductal Fluid and modified Potassium Simplex Optimization Medium culture media. Aspirated and selected oocytes from slaughterhouse-derived ovaries were placed into an *in vitro* maturation medium for 22 h. *In vitro* fertilization was allowed for 16 to 18 h and presumptive zygotes were washed and placed on the six different treatment culture media containing different combinations of modified Synthetic Oviductal Fluid and modified Potassium Simplex Optimization Medium culture media and supplemented with bovine serum albumin and fetal calf serum. In sequential culture media, embryos were transferred after 3-4 days to the second treatment culture media. Cleavage rate and development to blastocyst was determined at 2 and 7 days after *in vitro* culture, respectively. Blastocysts were stained with Hoechst 33342-Propium Iodide differential staining to assess embryo quality. The rate of cleavage was not significantly different among all treatments; however, sequential culture of embryos in modified Synthetic Oviductal Fluid + 3mg/ml BSA for 1-3 days and Potassium Simplex Optimization Medium + 5% FCS for 4-7 days yielded higher blastocyst development and blastocyst cell number. Based on the results, it can be concluded that the use of modified Potassium Simplex Optimization Medium supplemented with fetal calf serum in the later part of *in vitro* culture is beneficial in bovine embryo development.

Keywords: Bovine embryos, bovine serum albumin, culture media, fetal calf serum

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Introduction

Successful blastocyst development *in vitro* has been achieved in a variety of culture media such as Charles Rosenkrans Medium, Potassium Simplex Optimization Medium and Synthetic Oviductal Fluid Medium. However, the rate of development of bovine oocytes to the blastocyst stage following maturation, fertilization and culture *in vitro* is limited to about 30-40% (Marquant-Leguienne et al., 1998). Synthetic Oviductal Fluid is the most commonly used medium for embryo culture and usually used as a single medium through the entire culture period with bovine serum albumin as macromolecule component. However,

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addition of foetal calf serum in Synthetic Oviductal Fluid Medium during the latter part of culture period increased speed of embryonic development than when bovine serum albumin is added in the culture system. Recently, Synthetic Oviductal Fluid is being used in combination with Potassium Simplex Optimization Medium for a sequential culture system. Synthetic Oviductal Fluid then was subsequently modified with the addition of glucose, lactate, pyruvate, amino acids and vitamins (mSOF) and then with the addition of sodium citrate and myo-inositol.

Another medium called Potassium Simplex Optimization Medium has gained popularity worldwide. It contains glycyl-glutamine as a replacement for glutamine to prevent ammonia buildup and it is now called modified Potassium Simplex Optimization Medium (mKSOM).

Successful culture *in vitro* of sheep and cattle embryos (Tervit et al., 1972), and mouse embryos (Lawitts and Biggers, 1991) was reported utilizing Synthetic Oviductal Fluid and Potassium Simplex Optimization Medium. *In vitro* culture protocols have since been further improved and allowed the development of superior cell-free media. Furthermore, new sequential media have been developed to address specifically the nutritional needs of the developing embryos at different stages of development. These differences have been designed to meet the changing nutritive requirement of the embryo as a function of their stage of development; therefore, culture should take place in different media according to developmental stage. However, more research is still needed for a more effective sequential culture system for *in vitro* produced bovine embryos. Hence, the study was aimed to develop a more effective culture system for the *in vitro* production of bovine embryos.

The study was conducted to establish a better protocol for the *in vitro* culture of bovine embryos which will yield higher blastocyst rate as well as improve the quality of the produced embryo. Furthermore, it will provide future researchers a medium that can be used for better and productive *in vitro* culture of bovine embryos. The improvement of the current *in vitro* culture system by the use of sequential culture medium is the premise of the study.

Materials and Methods

Collection of ovaries and oocyte aspiration

Swamp buffalo ovaries were obtained at local slaughterhouse and transported to the laboratory in normal saline solution (85% NaCl). Cumulus-oocyte complexes were aspirated from 2 to 8 mm diameter follicles using an 18-gauge needle fitted to a 10 ml syringe. Collected cumulus-oocyte complexes were held and washed 3 times with HEPES-buffered modified Tyrode's medium (Bavister et al., 1983), supplemented with 3 mg/ml bovine serum albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, MO, USA), 0.2 mM sodium pyruvate (Sigma) and 50 µg/ml gentamicin sulfate (Sigma). Only oocytes with compact layers of cumulus cells and evenly granulated cytoplasm were used for maturation.

In vitro maturation

The in vitro maturation was performed following the procedure described by Atabay et al. (2006). Briefly, the selected cumulus oocyte complexes were placed in a maturation media containing HEPES-buffered TCM 199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% calf serum (CS, Gibco), 0.2 mM sodium pyruvate, 0.02 units/ml FSH (Sigma), 1µg/mm estradiol-17 β (Sigma), 10 ng/ml EGF (Sigma), and 50µg/ml gentamicin sulfate (Atabay et al., 2006). Ten to twelve oocytes were cultured in the maturation medium for 22 to 24 hr at 38.5ºC under 5% CO_2.

In vitro fertilization

In order to increase the percentage of live sperm for fertilization, dead and alive spermatozoa were separated using the discontinuous percoll gradient method. In brief, two 0.5 ml straws of frozen buffalo semen were thawed at 37ºC for 1 min. The thawed semen was layered on top of the percoll gradients. The tube was then centrifuged at 2000 rpm for 20 min and the top layer was removed immediately after centrifugation with sterile micropipette. The washed sperm pellet was again washed with 6 ml Brackett and Oliphant Defined Medium and centrifuged at 1000 rpm for 5 min. Supernatant was removed until 200 µl of sperm pellet was left in the tube. Semen concentration was determined using a haemocytometer and adjusted to 4 x 10^6 sperm/ml concentration with Brackett and Oliphant Defined Medium. Fifty µl of the semen suspension was added to pre-equilibrated fertilization drop consisting of Brackett and Oliphant Defined Medium supplemented with 3 mg/ml bovine serum albumin and 2.5 mM theophylline, to have a final concentration of 2 x 10^6 sperm/ml. Ten to twelve *in vitro* matured oocytes was transferred to the fertilization drop after 3 times of washing in sperm free Brackett and Oliphant Defined Medium-Theophylline bovine serum albumin drops. The spermatozoa and oocytes were co-incubated for 16 to 18 h at 38.5ºC under 5% CO_2.

In vitro culture and assessment of embryonic development

After 18 hr sperm cells and oocytes co-incubation, presumptive zygotes were washed by centrifugation to remove the cumulus cells and spermatozoa. *In vitro*

culture was done using six different culture media with three replicates conditions as follows:

All treatments were cultured at 38.5ºC under a 5% carbon dioxide, 5% oxygen and 90% nitrogen gas system. The rates of cleavage and subsequent development to blastocyst stage were taken on the 2nd and 7th day of *in vitro* culture, respectively. Blastocyst cell number was determined using the Hoechst-PI differential staining.

Hoechst-Propidium Iodide (PI) Differential Staining of Blastocyst

In vitro produced blastocysts were washed using phosphate buffered solution containing 0.2% polyvinyl alcohol (PBS-PVA) and were incubated for 30 sec in PBS-PVA containing 0.2% (v/v) Triton X and 0.1 mg Propidium Iodide (PI). It was transferred to a 4-well dish containing 25ug/ml Hoechst 33342 dissolved in absolute ethanol and incubated 30 mins at 37°C. After staining, blastocysts were washed in PBS-PVA and mounted on a glass slide. Mounting was done by adding glycerol droplets on the side of the embryo and then be flattened with glass slip. The embryo was viewed in a fluorescence microscope (Nikon Inverted Microscope Ti) and focused using UV light.

The nuclei of tropectoderm cells labelled by both Hoechst and PI stain appeared blue, whereas the nuclei of inner cell mass cells labelled only by Hoechst appeared red (Muenthaisong, 2005). The number of tropectoderm and of inner cell mass cells were counted separately and recorded according to its age and developmental stage. Images were taken using Nikon Imaging Software-Basic Research.

Statistical analysis

The data were analyzed using Duncan's multiple range tests with the help of SAS (version 9.9). All values were expressed as the Mean ± SD. A probability of $P<0.05$ was considered statistically significant.

Results and Discussion

All treatments have the same *in vitro* maturation and *in vitro* fertilization media used along with their corresponding conditions. When immature bovine oocytes are released from their follicles and are cultured in standard maturation medium, they resume the first meiotic division (Edwards, 1965). Although it may appear difficult to influence the quality of an oocyte during the maturational period, the alteration of basic maturation conditions can affect oocyte competence significantly, as reflected by the morula and blastocyst yield after *in vitro* fertilization (Rose and Bavister, 1992).

Table 2 shows the comparison for each treatment's percentage of oocytes that cleaved to 2-4 cell stage, percentage development to blastocysts stage and the number of cells/blastocyst. Statistical analysis revealed no significant difference on percentage cleavage among treatments. The cleavage to 2-4 cells after *in vitro* fertilization was considerably the same for all treatments, whether in modified Synthetic Oviductal or modified Potassium Simplex Optimization Medium and bovine serum albumin or 5% fetal calf serum; however, Treatment 1 (65.96±2.54) tended to have the highest average of cleavage rate among the treatments.

The blastocyst developments in each treatment are also presented in Table 2. Results showed that the rate of blastocyst development in Treatment 6 (45.04±3.39) was significantly higher than Treatment 1 (30.06±4.05), Treatment 2 (28.87±3.42) and Treatment 5 (35.42±2.41), but showed no significant difference with Treatment 3 (40.58±3.61) and Treatment 4 (39. 40.81±2.58), respectively.

In several *in vitro* studies, modified synthetic oviductal fluid (mSOF) as a sole *in vitro* culture media demonstrated inconsistent results and blastocysts produced had higher percentage of apoptotic blastomeres. The previous findings led to the improvement of the previous formulation thus resulted in a new culture media such as Synthetic Oviductal Fluid supplemented with amino acids, vitamins and bovine serum albumin (Gardner et al., 1994) and Charles Rosenkrans Medium (Rosenkrans and First, 1994). However, even the new formulations failed to produce consistent results and still require the addition of serum to achieve reliable results (Pinyopunmintr and Bavister, 1994; Carolan et al., 1995; Thompson et al., 1998). The result of the present study is consistent with the previous findings that supplementation of foetal calf serum especially on the later part of culture increases the blastocyst development. Another media such as modified Potassium Simplex Optimization Medium sustained not only mouse embryos but also bovine oocytes culture *in vitro*.

Aside from culture media used, supplementation of amino acids is beneficial for embryo development. Although serum or bovine serum albumin typically are added to the medium as a protein supplement to improve culture efficiency (Leibfried-Rutledge et al., 1986), different lots of this protein with different purity levels can produce highly variable effects during the period of culture ((Mckiernan and Bavister, 1992)). In the study, treatments with 5% fetal calf serum after initial 3 days in bovine serum albumin resulted in higher blastocyst development, which means that embryos may need more protein source to sustain its development *in vitro*.

Previous research results revealed that the embryo quality classification carries a superior significance for obtaining pregnancy in embryo transfer (Callesen et al., 1995). Blastocyst cell count after the Hoechst-PI differential staining in the present study is presented in

Table 1: Treatments used in *in vitro* culture of bovine presumptive zygotes

Treatments	Component
Treatment 1	mSOF + 3mg/ml BSA for 1-7 days
Treatment 2	mKSOM + 3mg BSA for 1-7 days
Treatment 3	mSOF + 3mg/ml BSA for 1-3 days and mSOF with 5% FCS for 4-7 days
Treatment 4	mKSOM + 3mg/ml BSA for 1-3 days and mKSOM + 5% FCS for 4-7 days
Treatment 5	mKSOM + 3mg/ml BSA for 1-3 days and mSOF + 5% FCS for 4-7 days
Treatment 6	mSOF + 3mg/ml BSA for 1-3 days and mKSOM + 5% FCS for 4-7 days

Table 2: Development of *in vitro* fertilized bovine embryos cultured in different culture media

Treatment	Number of oocytes cultured	Cleavage rate (2-4 cells)	% Blastocyst development	Blastocyct Cell Numbers (n)
T1-Control	50	65.96±2.54	30.06±4.05 ^{c}	154.40±3.05^{bc} (6)
T2	43	65.08±4.27	28.87±3.42 ^{c}	153.60±3.51^{c} (5)
T3	50	64.04±2.11	40.58±3.61 ^{ab}	153.20±5.89 ^{c} (6)
T4	42	64.42±3.81	40.81±2.58 ^{ab}	162.00±4.30 ^{b} (5)
T5	43	64.26±3.17	35.42±2.41^{bc}	154.60±2.97 ^{bc} (5)
T6	49	63.22±3.52	45.04±3.39 ^{a}	170.20±3.96 ^{a} (6)

Percentages and values represent means ± SD of 4 replicates. Within columns, values with different superscripts (a,b,c) are significantly (P<0.05) different.

Table 2. Result showed that the blastocysts derived in Treatment 6 had significantly higher cell count (170.20±3.96) compared with all the treatments. The result indicates that sequential culture of embryos in modified Synthetic Oviductal + 3mg/ml bovine serum albumin for 1-3 days and modified Potassium Simplex Optimization Medium + 5% fetal calf serum for 4-7 days is essential to produce good quality embryos.

Conclusion

The sequential culture system of modified Synthetic Oviductal and modified Potassium Simplex Optimization Medium is better than modified Synthetic Oviductal or modified Potassium Simplex Optimization Medium alone based on blastocyst production and quality. In sequential culture system, the use of modified Potassium Simplex Optimization Medium in the later part of *in vitro* culture is beneficial in embryo development and that bovine serum albumin is suitable during the early *in vitro* culture and foetal calf serum is required during the later part of embryonic development.

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