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Comparing *in vitro* Embryonic Development of Bovine Oocytes Cultured in G1.3/G2.3 Sequential Culture Media and CR1aa Medium

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Abstract: *In vitro* embryo culture is an important step of *in vitro* production of bovine embryos. The aim of this study was to compare the *in vitro* development of bovine embryos in G1.3/G2.3 sequential culture media and CR1aa culture medium. Oocytes were obtained from ovaries of slaughtered cows and matured in TCM-199 medium in humidified air containing 5% CO_2 at 39°C. Matured oocytes were fertilized *in vitro* using frozen bull sperm prepared with Percoll separation. After fertilization, the presumptive zygotes were denuded from cumulus cells and randomly allotted for 2 treatments: Culture in CR1aa (n = 116) and G1.3/G2.3 (n = 125). The embryo culture was carried out in 50 μL droplets of the media that were placed in incubator containing 6% CO_2, 5% O_2 and 89% N_2. The embryos were evaluated on days 3, 5 and 7 following *in vitro* fertilization. The difference of cleavage rates between 2 treatments was not found significant ($p>0.05$). However, significant difference was observed between 2 treatments at the level of the obtaining rate of morula and blastocyst ($p<0.05$). The results showed that G1.3/G2.3 sequential media significantly favoured developing of morula and blastocysts in comparison to CR1aa medium (32.0% versus 10.40% versus 18.96 and 2.58%, respectively).

Key words: Bovine, IVF, embryo culture, G1.3/G2.3 sequential media, CR1aa

INTRODUCTION

In Vitro embryo Production (IVP) using slaughterhouses as a source of oocytes is of great importance for the mass production of cattle embryos for research purposes and the improvement of cattle populations (Pinyopummintr and Bavister, 1991; Sagirkaya, 1998). Competence of any blastocyst resulting after *In Vitro* Maturation (IVM), Fertilization (IVF) and Culture (IVC) would be affected by the factors in the culture environment during the production of embryos. A wide variety of epigenetic factors, including ions, energy substrates, amino acids, vitamins, growth factors, cytokines and hormones play important roles in early embryonic development (Abdoon *et al*., 2001; Kuran *et al*., 2002).

Recently, sequential culture media were formulated for *in vitro* development of mammalian embryos (Krisher *et al*., 1999; Swain *et al*., 2001). Studies showed that a medium cultured for long periods of time can rapidly deteriorate, resulting in its inability to support embryo development. A sequential media system overcomes this problem and also has the advantage of altering energy substrates and substrate concentrations, thus, mimicking the change in environment experienced by the developing embryo *in vivo* as it travels through the oviduct to the uterus (Steeves and Gardner, 1999; Swain *et al*., 2001).

CR1aa contains the basic minimum essential medium, which is a blend of amino acids, vitamins, inorganic salts, ribonucleosides and deoxyribonucleosides. Another major component of CR1aa is the basal medium eagle, which is a supplemental mixture of additional amino acids, vitamins and inorganic salts (Rosenkrans *et al*., 1993; Sagirkaya *et al*., 2004).

Serum-free culture systems have been proven to be beneficial for the production of good quality embryos from IVM-IVF bovine oocytes (Abe and Hoshi, 2003). One such serum-free culture system is the sequential media G1.2 and G2.2 system. These media were formulated specifically to prevent intracellular stress to the embryo, thereby maintaining embryo viability. Additionally, these media take into account the changing carbohydrate and

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amino acid requirements of the embryo. As a result, these media are able to support high rates of blastocyst development in culture of embryos from many species (Lane *et al*., 2003).

The objective of this study, was to compare the developmental capability of IVM/IVF bovine embryos from 1-cell stage to the blastocyst stage cultured in sequential culture system, G1.3/G2.3 sequential media and conventional CR1aa culture medium.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO USA), except where, otherwise indicated.

Bovine oocyte recovery: Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory in a thermos filled with physiological saline solution (0.9%, w v^{-1}, NaCl) at 34±2.0°C. Ovarian follicles measuring 2-8 mm in diameter were aspirated with an 18-gauge needle using vacuum suction (100 mmHg; 28 mL min^{-1}). Oocytes with homogenous cytoplasm surrounded by at least 3 layers of cumulus cells were selected.

***In vitro* maturation:** Selected Cumulus-Oocyte Complexes (COCs) were washed 3 times in TL-Hepes containing 10% FBS (fetal bovine serum) and 1% v:v penicillin streptomycin (10.000 U mL^{-1} penicillin G, 10.000 μg mL^{-1} streptomycin) and placed into 500 μL of maturation medium in 4-well dishes (Nunc, Roskilde, Denmark), 25-35 oocytes $well^{-1}$. Each well of maturation medium was covered with 400 μL mineral oil. The medium used for maturation of COCs was TCM-199 containing Earle salts, 2.2 g L^{-1} sodium bicarbonate, 10% FBS, 50 μg mL^{-1} sodium pyruvate, 1% v:v penicillin-streptomycin, 5.0 μg mL^{-1} LH (luteinizing hormone), 0.5 μg mL^{-1} FSH (follicle stimulating hormone) and 10 ng mL^{-1} EGF (epidermal growth factor). Oocytes were matured for 22-24 h in a humidified atmosphere composed of 5% CO_2 at 39°C.

Sperm preparation and *in vitro* fertilization: After 22-24 h of *in vitro* maturation, matured COCs were washed twice in TL-Hepes and then transferred to 44 μL fertilization drops (10-12 oocytes/drop) under mineral oil. Fertilization medium was modified Tyrode's based medium containing 0.2 mM Na-pyruvate, 6 mg mL^{-1} Fatty Acid Free Bovine Serum Albumin (BSA-FAF) and 25 μg mL^{-1} gentamycin (Parrish *et al*., 1986). Oocytes were cultured until the addition of spermatozoa.

Frozen-thawed semen from a single bull was used for the fertilization of oocytes. Percoll density gradient system was applied for the separation of motile fraction of the frozen-thawed semen (Parrish *et al*., 1986; 1995). Percoll (2 mL at 90% concentration) was transferred to the bottom of a 15 mL centrifuge tube and 2 mL of 45% Percoll was placed on top with a great caution. Frozen sperm was thawed at 36°C for 1 min and then carefully layered onto the Percoll gradient system. The tube loaded with sperm sample and Percoll layers was then centrifuged at 1200× g for 15 min at room temperature. The supernatant was removed carefully without disrupting the pellet containing live sperm cells. After the pellet was resuspended, sperm concentration was determined by using a haemocytometer. Sperm were then, diluted to 50×10^6 sperm cells mL^{-1} in TL-Hepes, which would produce a 2×10^6 spermatozoa mL^{-1} final concentration. The fertilization procedure was completed by adding 2 μL of diluted sperm, 2 μL of PHE solution (20μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine in final concentration) and 2 μL of 2 μg mL^{-1} heparin into the 44 μL fertilization drops containing oocytes, respectively. Oocytes and sperm were co-cultured together for 24 h in 5% CO_2 in humidified air at 39°C in the incubator (Keskintepe and Brackett, 1996; Parrish *et al*., 1986, 1995; Sagirkaya, 1998).

***In vitro* culture:** At 24 h after insemination, the cumulus cells were removed by placing the presumptive zygotes in a 1.5 mL eppendorf tube and vortexing at the highest speed for 3 min. Embryos were then randomly divided into 2 groups. The 1st group was placed in 50 μL CR1aa developmental medium (Rosenkrans *et al*., 1993; Rosenkrans and First, 1994), while the 2nd group was placed in G1.3/G2.3 sequential medium.

Group 1: The 1st culture system was CR1aa (Rosenkrans *et al*., 1993) containing 114.6 mM NaCl, 3.08 mM KCl, 26.18 mM $NaHCO_3$, 2.52 mM Na Lactate, 0.55 mM L-Lactate, 0.4 mM Na pyruvate, 1.5 mg mL^{-1} L Glutamine, 3 mg mL^{-1} BSA-FAF, 1% antibiotic antimycotic solution, 10 μL mL^{-1} MEM essential and 20 μL mL^{-1} BME nonessential amino acids.

Group 2: The 2nd system consisted of G1.3/G2.3 sequential culture media supplemented with 8 mg mL^{-1} fatty acid free-BSA. The embryos were cultured for the 1st 72 h in G1.3 and then embryos were transferred to the G2.3 until the end of the culture period (G1.3/G2.3, Version 3, Vitrolife, Englewood, Colorado, USA).

Culture was performed at 39°C in humidified atmosphere containing 6% CO_2, 5% O_2 and 89% N_2. Embryo culture in group 1 was continuously done in medium without any change for 9 days and in group 2 media were changed 72 h after IVF. Fertilization day was considered as day 0. Cleavage, morula and blastocyst development rates were evaluated from presumptive zygotes on days 3, 5 and 7 using a stereomicroscope. The experiment was replicated 3 times.

Statistical analysis: Statistical analysis of the data was done using graphpad software program (Version 2.02, LSU Medical Center, USA). Significant differences between the groups were compared with the chi-square (χ^2) and Fisher's exact tests. The p-value used to determine significance in all tests was 0.05.

RESULTS

The objective of this study was to compare the developmental ability of embryos in 2 different culture systems, CR1aa medium and sequential G1.3/G2.3 media. Cleavage, morula and blastocyst formation rates of *in vitro* matured oocytes in TCM-199 supplemented with FBS were evaluated. Experiments were replicated 3 times. As shown in Table 1, embryos developed in sequential G1.3/G2.3 had a greater ability to develop to different stages, beginning especially, form morula stage.

In group 1 (CR1aa) and 2 (G1.3/G2.3), a total of 116 and 125 oocytes were fertilized and 64.65% (75 of 116) and 60.8% (76 of 125) of oocytes cleaved within 72 h of *in vitro* culture, respectively. The differences between cleavage rates of groups were not significant ($p>0.05$) (Table 1). After culturing cleaved embryos in CR1aa medium and G1.3/G2.3 sequential media, development rates to the morula stage were 18.96% (22 of 116) and 32.00% (40 of 125), respectively. The difference between development rates to the morula stage was significant ($p<0.05$) (Table 1). Development rates to the blastocyst stage from *in vitro* fertilized embryos were 2.58% (3 of 116) and 10.40% (13 of 125) for the groups 1 and 2, respectively. There was a significant difference between 2 groups in the percentage of embryos successfully developing to the blastocyst stage ($p<0.05$) (Table 1). Furthermore, blastocyst development rates from cleaved embryos were determined as 4.00% (3 of 75) and 17.10% (13 of 76) for the groups 1 and 2, respectively. The difference between development rates to the blastocyst stage from cleaved embryos of the groups was also found significant ($p<0.05$).

DISCUSSION

The objective of this study was to compare the blastocyst formation capability between CR1aa and G1.3/G2.3 sequential media. This study suggested that the sequential G1.3/G2.3 medium has a higher rate of morula and blastocyst formation.

Competence of any blastocyst resulting after IVM, IVF and IVC could be affected by many factors in the culture environment during the production of embryos. A wide variety of epigenetic factors, including ions, energy substrates, amino acids, vitamins, growth factors, cytokines and hormones plays important roles in early embryonic development (Abdoon *et al*., 2001; Duque *et al*., 2003; Krisher *et al*., 1999; Kuran *et al*., 2002; Pinyopummintr and Bavister, 1991).

According to Lim *et al*. (2003), Nonessential Amino Acids (NEAA) have a stimulatory effect upon all developmental stages and Essential Amino Acids (EAA) enhance blastocyst formation and the hatching of blastocysts, but EAA were found to be toxic during the early developmental stages. Rosenkrans and First (1994) reported favourable effects of MEM or NEAA in CR1aa culture medium supplemented with BSA when used 65±3 h after insemination. In this study, the embryo development rates were similar to those of Rosenkrans and First (1994).

Recent developments have included the supplementation of media with amino acids (Steeves and Gardner, 1999) and the use of sequential media for the extended culture of pre-implantation embryos (Lane *et al*., 2003). Physiological sequential media are formulated to reflect the carbohydrate levels of the reproductive tract and reduce the cellular stress on the embryos (Lane *et al*., 2003). These sequential media would mimic the change in environment experienced by the developing embryo *in vivo*, enabling the biochemical and morphological changes

Table 1: Effect of CR1aa and G1.3/G2.3 sequential media on *in vitro* development of bovine embryos

Experiments	No. oocytes fertilized (n)	No. cleaved embryos (%)	No. morulae (%)	No. blastocyst (%)	No. blastocyst/ cleaved embryos (%)
Group 1					
CR1aa	116	64.65^a (75/116)	18.96^a (22/116)	2.58^a (3/116)	4.00^a (3/75)
Group 2					
G1.3/G2.3	125	60.80^a (76/125)	32.00^b (40/125)	10.40^b (13/125)	17.10^b (13/76)

Values with different superscripts (a and b) in the same column are significantly different ($p<0.05$)

such as the maternal zygotic transition, compaction, blastocoel formation and expansion (Swain *et al*., 2001). Studies based in version 2 of this media have been shown to support development of bovine (Lane *et al*., 2003), caprine (Bormann *et al*., 2003) and porcine (Swain *et al*., 2001) embryos. However, studies with Version 3 are limited (Arat *et al*., 2006; Garcia-Garcia *et al*., 2007).

In our study, the differences between the groups in terms of cleavage rates were not significant ($p>0.05$). However, the cleavage rate was lower than those reported by Mastromonaco *et al*. (2004), Krisher *et al*. (1999) and Keskintepe *et al*. (1995); similar to those of Gandhi *et al*. (2000), Swain *et al*. (2001) and Lane *et al*. (2003).

In this study, the differences between the rates of development to the morula and blastocyst stages were significant ($p<0.05$). The G1.3/G2.3 blastocysts from this study exhibited a higher development rate than blastocysts cultured in CR1aa. This may be a result of amino acids in the sequential media system. Swain *et al*. (2001) demonstrate that amino acids act as osmoregulators, osmoprotectants, pH regulators and energy sources for preimplantation embryos. The morula and blastocyst development rates in the G1.3/G2.3 medium was similar to that of Lane *et al*. (2003), was lower than that reported by Arat *et al*. (2006) and was higher than that for porcine reported by Swain *et al*. (2001).

During the 1st, 72 h of culture, embryos metabolize amino acids and produce ammonium ions as a result. Generation of high amounts of ammonium ions is extremely toxic to the mammalian embryos. The culture medium should be changed at intervals to protect embryos from this toxicity (Keskintepe and Brackett, 1996; Steeves and Gardner, 1999). In this study, low development rates of embryos in CR1aa medium might be resulted because of not changing media at 3rd or 4th days.

Krisher *et al*. (1999) demonstrate that culture medium can affect the metabolism of the resulting embryos, so it is unclear how much of what is currently known about the embryo metabolism, which may be an artefact of the culture conditions. Furthermore, several factors may influence the commercial embryo production rates, such as individual variation of the oocyte donor, semen, medium and culture conditions employed during each phase of *in vitro* embryo production.

CONCLUSION

Our study showed that the G1.3/G2.3 sequential culture system supports higher development rate to the blastocyst stage when compared to conventional CR1aa culture medium. This also, supports the idea that the requirements of embryos during developmental stages are changing. Thus, higher blastocyst development can be obtained from sequential culture system, which could provide better understanding of requirements of embryos during early embryonic development.

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