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Development of *in vitro* produced bovine embryos in four different culture media

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ABSTRACT

The objective of this study was to compare the developmental ability of embryos in 4 different media, CR1aa, modified CR2, BCEM-g and TCM-199. Bovine oocytes were matured and then fertilized *in vitro*. A total of 915 oocytes were used for fertilization, 681 (74%) of which cleaved. The 2-cell embryos were divided into 4 groups, and cultured in 1 of the 4 different culture media. Percentage of development to morula-blastocyst stage from the 2 cell stage were determined as 46, 31, 30 and 28% for CR1 aa, CR2, BCEM-g and TCM-199 culture media respectively. The development rate obtained with CR1aa was significantly higher ($P<0.01$) compared to that with the other media.

Key words: Bovine, Embryo, Fertilization

In vitro embryo production (IVP) is very useful and important tool employed for mass production of embryos for both commercial and research purposes. Culturing embryos with a consistent rate of blastocyst development is very important for laboratories. However, the number of blastocyst producted through an IVP system changes from one laboratory to another, even the same technique or media is used by different laboratories (Leibfried-Rutledge *et al.* 1997, Gardner 1998).

Embryo culture media can be classified into 2 groups, simple and complex media. Simple media are well-defined salt solutions i.e. CR1aa, CZB Menezo's B2. These media can be easily prepared in the laboratory and they are generally supplemented with protein sources such as serum or BSA. Despite its beneficial effects on embryo development, protein supplementation makes media poorly defined. Simple media are especially useful to elucidate factors affecting early embryo development. Nevertheless, better understanding of nutritional requirements of embryos can only be achieved by using completely defined, protein-free media, which makes new culture media formulations highly effective (Krisher *et al.* 1990).

Complex media i.e., TCM–199 and Ham's F10, containing mixtures of amino acids and other ingredients, may not always support the optimal conditions for embryo development. These media are usually supplemented with serum when used for embryo culture, which caused undefined conditions. Therefore, examination of the nutritional requirements of embryos becomes difficult or impossible, and this is partly responsible for variability in the development rate of embryos. In addition, it has been proved that hypoxanthine present in complex media at levels of 6-30 µl/ml is a significant factor in the encouragement of blocking development (Gordon 1994).

Energy sources are important in embryo culture. Pyruvate, lactate and amino acids are the preferred energy substrates of the early cleavage stage embryos and glucose has inhibitory effect during this time. Around the compaction event, glucose uptake and utilization by embryos increase, and blastocyst stage embryos have the ability to metabolize glucose both oxidatively and by aerobic glycolysis. However, glucose is required for the synthesis of phospholipids and triacylglycerols and to supply precursor for complex sugars of mucopolysaccharides and glycoproteins. Also, glucose plays an important role in the production of nucleic acids and NADPH. Therefore, complete removal of glucose from culture media is unlikely to benefit the embryos (Gardner 1998, Thompson 2000).

Understanding and improvements in conditions for the *vitro* culture of embryos can be expected to increase the yield of viable transferable stage blastocysts. This study was designed to compare the efficacy of 4 different *in vitro* culture media, viz. CR1aa (Rosenkrans *et al.* 1993), modified CR2 (Wang *et al.* 1997), BCEM-g (Park *et al.* 1997) and TCM-199 (Wang *et al.* 1997) to support embryo development from 2-cell to blastocyst stage.

MATERIALS AND METHODS

Ovaries were obtained from a local slaughterhouse and transported in saline (0.9% NaCl; 30±2°C) within 3-4hr.

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Oocytes were aspirated from 2-6 mm follicles by means of an 18-g needle attached to a 10 ml syringe. Aspirated follicular contents were placed in a 100 mm plates and searched for oocytes under a stereomicroscope in a warm room (30±2°C). Oocytes were first washed 3-times in TL-HEPES (Bavister *et al.* 1983), and then transferred to maturation plates. Groups of 10 oocytes were cultured in 50 µl drops of maturation medium consisting of TCM-199+10% FCS+2mM glutamine+0.25 mM Na-pyruvate+0.5µg/ml FSH+5µg/ml FSH+5µg/ml LH+gentamycin under mineral oil.

A gradient system was utilized to isolate motile sperm. Five hundred µl of 90% Percoll was pipetted to the bottom of a 1.5 ml centrifuge tube, and 500µl of 45% Percoll was carefully situated on top. Two straws of frozen sperm were first thawed at 35°C for 1 min and then layered onto the Percoll gradient. After that, the tube including the Percoll was centrifuged at 700×g for 15 min at room temperature. The pellet having live sperm was recovered, and sperm concentration was figured out using a haemocytometer. Sperm was then diluted to 50×10^6 spermatoza/ml in TL-HEPES. Fertilization of oocytes was carried out as described by Parrish *et al.* (1988). Briefly, after 20-24 hr incubation in maturation medium at 39°C in a humidified atmosphere of 5% CO_2 in air, the oocytes were washed twice in TL-HEPES and then placed in 44 µl fertilization drops under mineral oil. Each drop contained 10 oocytes. Fertilization medium used in this study was modified Tyrode's based medium including 0.2 mM Na-pyruvate, 6 mg/ml fatty acid free-bovine serum albumin (BSA-FAF) and 25 µg/ml gentamycin. A 2 µl aliquot of sperm suspension was added into each fertilization drop to obtain final concentration of 1.0×10^6 sperm cells/ml. Following sperm addition, 2 µl of PHE (20 µM penicillamine, 10 µM hypotaurine and 1 µM epinephrine) and 2 µl of 2 µg/ml heparin were also added. Oocytes and sperm cells were co-cultured for 48 hr (Parrish *et al.* 1988).

After this, the cumulus cells were removed by transferring the embryos to a 1.5 ml eppendorf tube, and vortexing a high speed for approximately 3 min. Two-cell stage embryos were then randomly divided into 4 groups and cultured in CR1aa, modified CR2, BCEM-g and TCM-199 media. CR1aa contained 114.6 mM NaCl, 3.1 mM KCl, 26.1 mM $NaHCO_3$, 5 mM hemicalcium lactate, 1 mM L-glutamine, 0.4 mM Na-pyruvate, 3 mg/ml BSA-FAF, 10 µl/ml 100×MEM, 20 µl/ml 50×BME and 25 µg/ml gentamycin. Modified CR2 was used with slight modification and composed of 108.3 mM NaCl, 2.9 mM KCl, 24.9 mM $NaHCO_3$, 2.5 mM hemicalcium lactate, 0.5 mM Na-pyruvate, 1 mM L-glutamine, 10 µl/ml 100×MEM, 20 µl/ml 50×BME and 25 µg/ml gentamycin. BCEM-g medium consisted of 89 mM NaCl, 3.2 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 0.35 mM NaH_2PO_4, 10 mM Na-lactate, 0.5 mM Na-pyruvate, 1 mM L-glutamine, 10 µl/ml 100×MEM, 20 µl/ml 50×BME, 1 mg/ml polyvinylalcohol (PVA) and 25 µg/ml gentamycin. Finally, TCM-199 was supplemented with 2 mM L-glutamine, 0.25 mM Na-pyruvate, 10% FCS and 25 µg/ml gentamycin.

To culture the 2-cell embryos, 50 µl drops of each culture medium were prepared and covered with mineral oil. CR1aa and BCEM-g media were supplemented with 10% FCS at day 4 of culture. The day of fertilization was considered as day 0. The embryos were examined daily and the number of those reaching the morula-blastocyst stage was recorded.

Experiments were replicated 6 times. For statistical analyses, χ^2 test was used.

RESULTS AND DISCUSSION

Numbers and percentages of cleaved and the morula-blastocyst stage embryos were shown in Table 1. In the present study, a total of 915 oocytes were fertilized and 681 (74%) of them cleaved. This cleavage rate was comparable to that of earlier studies (Hawk and Wall 1994, Wang *et al.* 1997, Sagirkaya 1998, Thompson *et al.* 1998). Percentages of development to the morula-blastocyst stage were found 46, 31, 30, 28% for CR1aa, modified CR2, BCEM-g and TCM-199 culture media respectively. The difference between CR1aa and the other media was significantly important ($P<0.01$). Only TCM-199 was a complex medium and the others were simple medium. TCM-199 was supplemented with 10% FCS from the beginning of culture period. It has been shown that FCS has a biphasic effect on bovine embryo development during culture. The presence of FCS inhibits development during early cleavage, but development is advanced when FCS is present from the start of compaction (Thompson *et al.* 1998). FCS has a concentration of 5.56 mM glucose, and it was suggested that this level of glucose is detrimental to early embryo development (Takahasi and First 1992). In addition, the presence of hypoxanthine in TCM-199 affects development negatively (Gordon 1994). These effects might be responsible for lower development rate observed in TCM-199.

Among simple culture media, CR1aa contained BSA from the beginning of culture period, and BCEM-g included PVA instead of BSA. Both media were supplemented with 10%

Table 1. Fertilization of bovine oocytes and their development to the morula-blastocyst stage in CR1aa, CR2, BCEM-g and TCM-199 culture media

No. of fertilized oocytes	No. of 2-cell embryos (%)	Culture media	No. of cultured two-cell embryos	No. of morula-blastocyst (%)
915	681 (74)	CR1aa	190	116 (46)[a]
		CR2	198	83 (31)[b]
		BCEM-g	142	55 (30)[b]
		TCM-199	151	58 (28)[b]

Different superscripts in the same column differ significantly ($P<0.01$).

FCS on day 4. Supplementation of media with protein is beneficial for embryo development. FCS and BSA are 2 commonly used protein sources (Wang *et al.* 1997). Beneficial effect of protein supplementation might be responsible for higher development rate in CR1aa. In most studies, it was reported that development rates of embryos are generally lower following *in-vitro* development in PVA-supplemented medium than in BSA-supplemented medium (Krisher *et al.* 1999, Lonergan *et al.* 1999). This could be the reason of low development rate obtained from BCEM-g medium.

In conclusion, it is an important task to attempt and start to optimize a medium that will support early life in IVF laboratories. Our results suggested that CR1aa medium is better choice than the other media. However, further studies are important to evaluate the nutritional requirements of embryos druing early embryonic development *in-vitro* and to increase the yield of good quality blastocyst for transfer.

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REFERENCES

Bavister B D, Leibfried-Rutledge M L and Lieberman G. 1983. Development of preimplantation embryos of golden hamster in a defined culture medium. *Biology of Reproduction* **28**: 235-47.

Gardner D K. 1998. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology* **49**: 83-102.

Gordon I. 1994. *Laboratory Production of Cattle Embryos.* pp. 242-61. CAB International, Wallingford.

Hawk H W and Wall R J. 1994. Improved yields of bovine blastocysts from *in vitro*-produced oocytes. II. Media and co-culture cells. *Theriogenology* **41**: 1585-94.

Krisher R L, Lane M and Bavister B D. 1999. Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. *Biology of Reproduction* **60**: 1345-52.

Leibfried-Rutledge M L, Dominko T, Critser E S and Critser J K. 1997. Tissue maturation *in vivo* and *in vitro*: Gamete and early embryo ontogeny. *Reproductive Tissue Banking.* (Ed.) Karow A. pp. 23-138. Academic Press, New York.

Lonergan P, O'Kearney-Flynn M and Boland M P. 1999. Effect of protein supplementation and the presence of an antioxidant on the development of bovine zygotes in synthetic oviduct fluid medium under high or low oxygen concentration. *Theriogenology* **51**: 1565-76.

Park K W, Iga K and Niwa K. 1997. Exposure of bovine oocytes to EGF during maturation allows them to develop to blastocysts in a chemically-defined medium. *Theriogenology* **48**: 1127-35.

Parrish J J, Susko- Parrish J L, Winer M A and First N L. 1988. Capacitation of bovine sperm by heparin. *Biology of Reproduction* **38**: 1171-80.

Rosenkrans C F Jr, Zeng G Q, McNamara GT, Schoff P K and First N L. 1993. Development of bovine embryos *in-vitro* is affected by energy substrates. *Biology of Reproduction* **49**: 459-62.

Sagirkaya H. 1998. Effects of CR1aa and TCM-199 media on *in vitro* embryonic development until blastocyst stage in cattle. *The Journal of the Faculty of Veterinary Medicine University of Uludag* **17**: 55-63.

Takahashi Y and First N L. 1992. *In vitro* development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37**: 963-78.

Thompson J G, Allen N W, McGowan L T, Bell A C S, Lambert M G and Tervit H R. 1998. Effect of delayed supplementation of fetal calf serum to culture medium on bovine *in vitro* and following transfer. *Theriogenology* **49**: 1239-49.

Thompson J G. 2000. *In vitro* culture and embryo metabolism of cattle and sheep embryos-a decade of achievement. *Animal Reproduction Science* **60-61**: 263-75.

Wang S, Liu Y, Holyoak G R and Bunch T D. 1997. The effects of bovine serum albumin and fetal bovine serum on the development of pre-and post cleavage-stage bovine embryos cultured in modified CR2 and M199 media. *Animal Reproduction Science* **48**: 37-45.