韓國受精卵移植學會誌(2004) 第 19 卷 第 2 號
Korean J. Emb. Trans. (2004) Vol. 19, No. 2, pp. 133~145

Transfer, Cryopreservation and Production of Bovine Embryos Cultured in Serum-Free System

Y. J. Im[1,2], J. H. Kim[1], H. B. Song[3] and Y. G. Jung[2]
[1]*Division of Applied Life Science, Gyeongsang National University*

Serum-Free Medium에서 배양한 한우 배의 내동성과 이식

임여정[1,2] · 김진회[1] · 송해범[3] · 정연길[2]
[1]국립경상대학교 응용생명과학부

SUMMARY

난자의 체외성숙 및 체외배양에는 일반적으로 동물의 혈청을 사용하고 있다. 그러나, 채취한 소의 상태에 따라서 혈청의 질에 차이가 있어 실험데이터가 일정하지 않을 수 있고, 그것으로부터 바이러스, 세균, 마이코플라즈마 등에 오염될 가능성이 있다. 따라서, 본 실험에서는 완전 무 혈청 배양액에서 난자의 성숙, 배 발생율, 세포 수, 동결성을 검토하였다.

다음으로, 근래 혈청배지로 생산한 체외 배양 수정란은 과체중의 산자 생산, 초기 산자 사망률, caesarean section, dystocia 같은 발병으로 문제가 지적되고 있다. 그러나 무 혈청 배지로 생산한 체외 배양 수정란은 이러한 현상을 개선할 수 있다는 보고가 있어 본 실험에서는 완전 무혈청 배양액에서 생산된 동결란과 신선란을 젖소에 이식하여 그 결과를 검토하였다.

무혈청 배양액에는 에너지원과 세포성장인자가 첨가된 IVD101과 IVMD101, 대조군인 혈청 배양액에는 TCM199 + 10% FBS가 사용되었다. IVMD101에 의해 체외성숙이 이루어 졌고, 혈청이 첨가된 TCM199 + 10% FBS에서는 12.4% 배 발생율을 보인데 반해, 무혈청 배양액 IVD101과 IVMD101이 각 32.4%, 34.5%로 훨씬 높은 배 발생율을 보였다. 더욱이, 세포 수에 있어서도 무혈청 배양액에서 발생된 배반포의 세포수가 혈청이 첨가된 배양액에서 발생된 배반포의 세포수보다 월등하였으며, 이는 체내란과 비슷한 세포 수를 보이고 있다.

수정란의 내동성을 보면, 융해하고 24시간 배양기 정치 후에 IVD101과 IVMD101은 94.5%, 95.8%의 생존율을 보인 반면 TCM199 + 10% FBS는 52.5%에 불과하다. 융해하고 72시간후 탈출 배반포율이 IVD101과 IVMD101는 78.4%, 83.7%였는데 반해 TCM199 + 10% FBS는 32.0%였다. 마지막으로, 무혈청 배양액에서 발생한 동결란과 신선란을 젖소에 이식한 후에 임신율에 있어서는 유의적 차이는 없었지만, 무혈청 배지에서 생산된 수정란의 내동성의 탁월함이 증명되었다.

이러한 결과들로 종합해 보면, 무혈청 배양액 (IVD101, IVMD101)에서 발생된 수정란이 혈청 배양액에서 발생된 수정란보다 배발생율, 세포수, 동결성, 임신율에 있어서 우수함을 알 수 있었다. 이러한 무혈청 배양액은 배발생 과정의 연구뿐만 아니라 수정란 이식을 위한 고품질의 체외 수정란의 대량 생산, 복제, 형질전환 동물 생산 등에도 유익할 것으로 사료된다.

(Key words : serum-free medium, bovine embryo)

[2] 이티바이오텍(ET Biotech)
[3] 대구대학교 생명자원학부(Division of Life Resources, Daegu University)
† Correspondence : E-mail : im3104@hotmail.com

INTRODUCTION

Bovine immature oocytes can be developed to the blastocyst stage by *in vitro*-maturation(IVM) and *in vitro*-fertilization(IVF) techniques. The techniques for producing bovine preimplantation embryos by *in virto* maturation, *in vitro* fertilization, and *in vitro* culture have been markedly developed in recent years. The techniques are used nuclear cloning, gene manipulation for the production of transgenic cows, and basic research on the mechanisms of oocyte maturation, fertilization, and early embryonic development. It is well known that there are substantial differences in the quality of blastocysts developed under various culture conditions from IVM-IVF bovine oocytes when morphology, cell numbers, and viability after cryopreservation are examined (Takahashi and First, 1992; Voelkel and Hu, 1992; Shamsuddin and Larsson, 1993).

Serum has been usually used for the culture of somatic cell *in vitro*, *in vitro* maturation of bovine oocytes and *in vitro* culture of the preimplantation embryos. Serum contains a variety of components such as amino acids, carbohydrates, lipids, inorganic salts, vitamines, hormones and growth factors that may stimulate and/or inhibit oocyte maturation and embryonic development. Bovine embryos have been generally arrested at the stages of th 8- to 16-cell embryos when they were cultured in medium supplemented with only serum(Wright and Bondioli, 1981). To overcome the developmental failure of bovine embryos *in vitro*, coculture systems with a variety of somatic cells have been successfuliy tired to achieve the full development of embryos at the blastocyst stage(Eyestone and First, 1989; Fukui, 1989; Rexroad, 1989). However, the presence of undefined components such as serum and somatic cells makes it difficult to characterize the precise roles of growth factors and/or secretory products from either somatic cells or embryos which are capable of promoting early embryonic development.

Successful cryopreservation of *in vitro*-derived embryos and subsequent pregnancies of these embryos after thawing has been reported(Fukuda et al., 1990; Hochi et al., 1996). However, survial rates of frozen *in vitro*-derived embryos, as measured either by post-thaw development in culture or by pregnancies following embryo transfer, have been lower than those reported for *in vivo*-derived embryos(Massip et al., 1995). Viability of frozen thawed *in vitro*-derived embryos could be affected by the culture conditions during the embryo development(Semple et al., 1995).

This study were to develop serum-free culture systems which could production of good quality embryos with a higher survival rate after freezing-thawing, compared to the existing culture system with a serum-containing medium.

MATERIALS AND METHODS

1. Culture Media

Medium was prepared to volume with water purified by reverse osmosis and filtraion through a Milli-Q system(Japan Millipore Co., Tokyo, Japan). All the media were sterilized by filtering through a 0.22 μm membrane filter(Gelman Science, Ann Arbor, MI). Media were stored at 4°C and fresh media were prepared every 2 weeks. Serum-free media used for maturation of oocytes and embryo culture were the IVMD101 and IVD101 media. The formulations of these serum-free media reported(Yamashita et al.,1999). The formulation of complete serum-free media(IVMD 101 and IVD101) are shown in Table 1. The basal medium of IVMD101 and IVD101 was prepared from specially ordered TCM199 medium(DM199; Kyokuto Pharmaceutical Co., Tokyo, Japan) which excludes glucose, Tween-80 and para-aminobenzoic acid from the original components of TCM199. Both serum-free media contained Earle's salts buffered with 25 mM

Table 1. Composition of media for *in vitro* maturation (IVM) and *in vitro* embryo culture (IVC)

Components	IVD101	IVMD101
D-glucose(mM)	2.22	5.56
Sodium pyruvate(mM)	0.27	0.91
Sodium lactate(mM)	2.48	-
L-cysteine(mM)	0.05	-
GSH(μm)	200	-
Taurine(mM)	5	5
Selenium(nM)	5	5
Insulin(μg/mL)	-	5
TGF-α(ng/mL)	-	10
Apo-transferrin(μg/mL)	10	10
bFGF(ng/mL)	10	-
TGF-β1(ng/mL)	1	-
TIMP-1(μg/mL)	0.5	-
Aprotinin(μg/mL)	0.5	-
BSA(mg/mL)	1	1
HEPES(mM)	5	5
Gentamicin sulfate(μg/mL)	10	10

Basal medium of IVD101, IVMD101 and DM199.
*DM199 medium dose not contain glucose. Tween-80 and paraaminobenzoic acid from original components of TCM199 (Yamashita et al.,1999).

HEPES (Sigma Chemical Co. St. Louis, MO). The concentration of glucose in IVD101 medium was reduced to 2.22 mM compared to 5.56 mM in TCM199 and IVMD101 media. The concentration of reduced glutathione(GSH) in IVD101 medium was in creased to 200 μM compared to 5.56 mM in TCM199 and IVMD101 media. Complete IVMD101 was composed of DM199 basal medium supplemented with 5.56 mM glucose, 0.91 mM pyruvate, 5 mM taurine, 5 nM Se, 5 μg/mL bovine insulin, 10 ng/mL human recombinant transforming growth factor-α(TGF-α), 10 μg/mL bovine apo-transferrin, 10 μg/mL gentamycin, and 25 mM HEPES(Sigma Chemicals, St. Louis, MO). Complete IVD101 was composed of DM199 basal medium supplemented with 2.22 mM glucose, 0.27 mM pyruvate, 2.48 mM lactate, 0.05 mM cysteine, 200 μM GSH, 10 ng/mL human recombinant basic fibroblast growth factor (bFGF), 1 ng/mL human recombinant transforming growth factor-β_1 (TGF-β_1), 0.5 μg/mL bovine tissue inhibitor of metalloproteinase-1(TIMP-1), 0.5 μg/mL bovine aprotinin. The IVD101 medium contained the same concentrations of taurine, Se, apotransferrin and gentamycin as in IVMD101 medium, but no insulin or TGF-α. Serum-supplemented medium used in this study was tissue culture medium199 with Earle's salts(TCM199; GIBCO BRL, Grand IIsland, NY) buffered with 25 mM HEPES and containing 10%(v/v) heat inactivated FBS(GIBCO Laboratories, Grand NY).

2. Oocyte Collection and Maturation *In Vitro*

Hanwoo ovaries were collected at a local meat processing plant and transported to the laboratory in sterile 0.9% NaCl at 15～20℃ within 2 hr. After washing several times with with calcium, magnesium-free Dulbecco's phosphate buffered saline (D-PBS), cumulus-oocyte complexes(COCs) were obtained by aspirationof small ovarian follicles (2～7 mm diameter) with a 18-gauge needle attached to a syringe. Oocytes with an intact and unexpanded cumulus oophorus were selected using a stereomicroscope and washed thrice in oocyte collection medium(OCM; Research Institute for the Functional Peptides, Yamagata, Japan) which consists of D-PBS supplemented with 5.56 mM glucose, 1.25 mM sodium pyruvate, 15 mg/mL sodium heparin(Sigma Chemicals, St. Louis, MO), 8 mg/mL phenol red, and 10 mg/mL gentamycin. Oocytes with 3～5 layers of compact cumulus cells and evenly granulated cytoplasm were used in the following experiments. For serum-free culture, the selected COCs were aliquoted into 6-well Repro C-1(Research Institute for the Functional Peptides) culture plate in 230 μL maturation medium (IVMD-101) and overlaid with 100 μL of paraffi liquid (Merck, Darmstadt, Germany). For serum-supple-

mented cultures, the COCs were handled in the same manner, except that TCM199á+ 10% FBS medium was used. The COCs were matured in either IVMD101 or TCM199 +á10%FBS supplemented with follicle-stimulating hormone(0.5 ㎍/mL), and human chorionic gonadotropin(5IU/ mL) for 22～24 hr at 38.5℃ in a humidified atmosphere of 5% CO_2 in air.

3. Sperm Preparation and *In Vitro* Fertilization

The method used for *in vitro* fertilization (IVF) was performed as described by Yamashita et al. (1999). Cryopreserved semen of a single Hanwoo was rapidly thawed at 37℃ and washed twice with 4 mL IVF100 medium (Research Institute for the Functional Peptides). The IVF medium consists of a modified BO medium (Brackett and Oliphant, 1975) supplemented with 25 mM sodium pyruvate, 0.5 mM cysteine, 5 mg/mL BSA, 5 mM caffeine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 7.5 mg/mL heparin. The spermatozoa were washed twice by centrifugation at 700g for 7 min and resuspended in the same medium. The concentration of spermatozoa was determined using a hemocytometer and then adjusted to 1.0×10^7/mL by further dilution. A 50 μL aliquot of the sperm suspension was combined with a 50 μL droplet of IVF100. After *in vitro* maturation, COCs were transferred to the sperm suspension(5.0×10^6/mL spermatozoa) and incubated for 5～6 hr at 38.5℃ in a humidified atmosphere of 5% CO_2 in air.

4. Embryo Culture

Bovine cumulus/granulosa cells(BCGCs) used for coculture were prepared by gentle pipetting of the COCs in either IVMD101 or TCM199 + 10% FBS. BCGCs were then cultured with the same medium in multiwell culture plates. Media were changed every 3～4 days, and confluent BCGC monolayers were obtained 8～9 days after seeding, which were subsequently used for the *in vitro* embryo culture (IVC). After *in vitro* fertilization (IVF), 25～30 inseminated oocytes were placed in 6-well Repro C-1 culture plates in 230 μL of either IVMD101 or TCM199 + 10% FBS, overlaid with 100 μL of paraffin liquid and incubated for 22～24 hr at 38.5℃ in a humidified atmosphere of 5% CO_2 in air. After these embryos were denuded by careful pipetting with capillary pipettes to completely remove the BCGCs, they were transferred to the previously prepared conØuent BCGC cultures which had been grown in IVMD101 or TCM199 + 10% FBS. The cocultures were incubated in a humidified atmosphere of 5% CO_2 in air at 38.5℃. The same number of denuded embryos were transferred to 6-well Repro C-1 plates in 230 μL serum-free medium (IVD101) without BCGC coculture and incubated in humidied atmosphere of 5% CO_2 / 5% O_2 / 90% N_2 at 38.5 ℃. Media in serum-free cultures were changed at 5 days after insemination, while those in serum-supplemented culture were changed every 2days. Embryo cultures were continued for up to 9days. Cultured embryos were observed daily using a stereomicroscope. The embryos of 2-cell, 8-cell, morula, and early blastocyst stages were collected at 22hr, 48hr, 5～6, and 6～7 days after insemination, respectively. Blastocysts, expanded blastocysts, and hatched blastocysts were collected at 7～9 days after insemination.

5. Freezing and Thawing Embryos

Freezing of embryos was carried out as reported by Takagi et al. (1994). The embryos (early blastocysts, blastocysts, and expaned blastocysts) were suspended in D-PBS supplemented with 1.8 M ethylene glycol (Amresco, Solon, OH), 5.56 mM glucose, 1.25 mM sodium pyruvate, 5 mg/mL BSA, and 10 ㎍/m gentamycin. After a 10 min equilibration period, the embryos were loaded into 0.25 mL plastic straw, with a 2～3 embryos per

straw, placed directly into a 0°C alcohol bath chamber, and held for 2 min. Then, the embryos were cooled from 0 to −6°C at 1°C/min and seeded at −6°C. After seeding, the straws were held for 10 min at −6°C, and cooled to −30°C at the rate of 0.3°C/min the straws were held for 10 min at −30°C. Finally, the straws were plunged into and stored in liquid nitrogen. After storage in liquid nitrogen for 2~3 weeks, frozen embryos were immediately thawed by placing the straws in a 35°C water bath and the contents drained into a sterile Petri dish. The embryos were then placed into the culture medium for rehydration and washed thrice. Embryos were cultured on feeder layers of BCGC in TCM199 supplemented with 10% FBS in 5% CO_2 in air at 38.5°C. Embryos were evaluated microscopically at 24 hr intervals up to 72 hr. Survival rates were assessed by the following parameters: (1) re-appearance of the blastocoele in embryos freezed at early blastocyst and blastocyst stages after 24 hr post-thaw culture; and (2) the ability to hatch *in vitro* after 72 hr post-thaw culture. (3) we examine the tolerance of cryopreservation of embryos developed from IVM-IVF oocytes cultured in serum-free media after embryo tranfer by Pregnancy diagnose.

6. Counting of Cell Numbers of Expanded Blastocysts by Giemsa Stain

At 190hr after IVF, blastocysts were examined to determine the cell number accordine to the procedure previously reported (Evans 1987). Brefly, a blastocyst was placed in 0.1%(w/v) trisodium citrate solution(Sigma, USA) for 15 min at room temperature and fixed in a solution of methanol : acetic acid (1:1, one embryo/1~2 drops). The blastocysts was placed on a glass slide, dried, and then stained with 20%(v/v) Giemsa's solution (MERCR, Germay) for 15min. The cell number of a blastocysts was determined by counting stained nuclei under a phase contrast microscope. (×400)

7. Embroy Transfer

After 7~8 days of embryo culture, fresh and frozen blstocysts(2~3 blstocysts per straw) were transferred into the uterus of a recipient Holstein cow with synchronized estrous cycles to evalute its viability. After transfer, each recipient cow was checked for return to estrus. Pregnancy was determined by ultrasonic echography on Day 40. Confirmed pregnancies were maintained until calving.

8. Statistical Analysis

All experimental data are presented as mean SD. Each experiment was performed at least three times and subjected to statistical analysis. For the statistical analyses, one-way analysis of variance (ANOVA) was performed to determine whether there were differences between all groups($p<0.05$), and the Fishers post-test was performed to determine significance between pairs of groups. A p value below 0.05 was considered significant. The statistical tests were performed on Statview software version 5.0(SAS Institute Inc, Cary, NC).

RESULTS AND DISSCUSION

1. *In Vitro* Raturation of Oocytes in Serum-free Medium

Before oocytes maturation, mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase. During a process of *in vitro* oocyte maturation, a preovulatory gonadotrophin surge initiates meiotic resumption characterized by germinal vesicle breakdown, chromosome condensation, formation of the first meiotic spindle, extrusion of the first polar body and arrest in metaphase of the second meiotic division.

These are increasing evidences that many factors are involved in mammalian oocyte maturation *in vitro*. Cumulus cells are potentially important in the acquisition of full developmental competence during oocytes maturation(Staigmillar and Moor, 1984; Vanderhyden and Armstrong, 1998). Serum

supplementations of maturation media have been commonly used in *in vitro* maturation of mammalian oocytes(Leibfried-Rutledge et al., 1986; Moor et al, 1998). Determined the postive effects of growth factors on the bovine cumulus expansion, oocyte maturation and the potential for subsequent embryonic development in a serum-free medium after *in vitro* fertilization by numerous researcher.

Transforming growth factor-α(TGF-α) or epidermal growth factor (EGF) dramatically enhanced cumulus expansion and rates of cleaved ova when cultured in a defined medium (Kobayashi et al., 1994a). The result demonstrates that TGF-α and EGF are beneficial to obtain mature oocytes with high quality for *in vitro* fertilization. In addition, LH or FSH also stimulated cumulus expansion and oocytes fertilization (Brackett et al., 1989, Kobayashi et al., 1994a), In summary, an imporved serum-free medium (IVMD101) was developed for bovine oocytes maturation *in vitro*.

2. Improved Serum-Free Media (IVD101 and IVMD101) for IVC

Table 1. showed formulation two serum-free media (IVD101 and IVMD101) for *in vitro* culture of bovine preimplantation embryos. The major characteristics of IVD101 medium are the supplementation with a low concentration of glucose (2.22 mM), lactate, protein factors (bFGF, TGF-β_1, TIMP-1, aprotinin, BSA) and antioxidant-related components (increased concentration of GSH, taurine, selenium, apotransferrin, cysteine). IVMD101 medium contains protein factors (TGF-α, insulin, BSA) and antioxidant-related components (taurine, selenium, apotransferrin). IVD101 medium was desighned for embryo culture in the absence of somatic cell cocultures under a low oxygen condition(5%), while IVMD101 medium was used in the presence of somatic cell coculture of BGC under an atmospheric oxygen concentration (20% O_2). Table 2 showns proportions of embryos developing to the blastocyst stage cultured in both IVD101(28.6%) and IVMD101(27.8%) serum-free media were higher than in TCM199 + 10% FBS (5.3%) serum-containing medium. Furthermore, the cell numbers per blastocyst obtained in the serum-free media were superior to those of blastocysts developed in serum-supplemented medium(Table 4). The mean cell numbers of Day 7 blastocysts developed in IVMD101(173.2 cells) and in IVD101 (169.7 cells) media were greater than that in the serum-supplemented medium (123.5 cells)(Table 4). Also, cell numbers of blastocysts obtained in the serum-free media were similar with blastocysts derived *in vivo*.

Serum contains beneficial substances for em-

Table 2. Development of *in vitro* matured and fertilized bovine emdryos on three different media

Culture medium	No. of inseminated oocytes	No. and (%) of oocytes developed to		
		2-cell	Blastocysts	Hatched blastocysts
Serum-free medium				
IVMD101	136	124(91.2)	47(34.5)[a]	38(27.8)[a]
IVD101	142	128(90.1)	46(32.4)[a]	41(28.6)[a]
Serum-supplemented medium				
TCM-199 + 10% FBS	113	89(86.7)	14(12.4)[b]	6(5.3)[b]

a,b : Values with different superscripts in each column differ significantly ($P<0.05$).

bryonic development such as growth factors and chelators of heavy metals. For this reason, bovine embryos are frequently cultured in serum-supplemented media with or without somatic cell cocultures. However, serum has a biphasic effect on bovine embryo development, inhibiting the first cleavage division but stimulating blastocyst development(Pinyopummintr and Bavister, 1991; Van Langendonckt et al., 1997). Recent studies have shown that the addition of serum to culture medium can cause morphological deviations(Gardner, 1994; Gardner et al., 1994; Thompson et al., 1995; Abe et al., 1999b). Furthermore, serum is suspected of contributing to the large offspring syndrome in sheep(Thompson et al., 1995; Holm et al., 1996; Sinclair et al., 1998).

3. Cryosurvival Rates of Blastocysts Cultured in Serum-Free Media (IVD101 and IVMD101) Higher than in Serum-Containing Medium (TCM199 + 10% FBS)

Examined the tolerance of cryopreservation of blastocysts developed from IVM-IVF oocytes cultured in the serum-free media(IVD101, IVMD101) and in the serum-contain medium (TCM199 + 10% FBS) (Table 3). Bovine embryos were frozen using the conventional slow freezing method in a ethylence glycol and cultured on fedder layers of BCGC in TCM199 supplemented with 10% FBS in 5% O_2 in air at 38.5℃ after thawing(Takagi M et al., 1994). Survival rate blastocysts after 24 hr incubation after thawing, the blastocysts cultured in both IVD101(94.5%) and IVMD101(95.8%) serum-free media were higher than in TCM199 + 10% FBS(52.5%) serum-contain medium.

After 72 hr incubation after thawing, hatching rates of blastocysts deveioped in IVD101(78.4%) and IVMD101(83.7%) were sighnificantly higher than that developed in the serum-supplemented medium(32.0%).

A comparative study of *in vivo* and *in vitro* produced embryos showed remarkable differences in their morphology, metabolism, and embryo qua-

Table 3. Post-thaw survival of bovine embryos developed in serum-free media (IVMD101 and IVD101) and serum-supplemented medium (TCM-199 + 10% FBS)

Culture medium	No. of embryos thawed	No. of embryos surviving after 24 hr(%)	No. of embryos hatching after 72 hr(%)
IVMD101	49	47(95.8)[a]	41(83.7)[a]
IVD101	37	35(94.5)[a]	29(78.4)[a]
TCM-199 + 10% FBS	25	13(52.5)[b]	8(32.0)[b]

[a,b] : Values with differnet superscripts in each column differ significantly (P<0.05).

Table 4. Mean cell numbers of bovine blastocysts cultured in three different media

Culture medium	No. of blastocysts examined	Mean±S.D.cell per blastocysts	Range(min-max)
IVMD101	23	173.2±59.2[a]	93~279
IVD101	17	169.7±47.3[a]	88~287
TCM-199 + 10% FBS	12	123.5±46.7[b]	63~273

[a,b] : Values with differnet superscripts in each column differ significantly (P<0.05).

lity with regard to cell numbers and frozen embryo viability (Shamsuddin M et al., 1993; Abe H et al., 1999; Greve T et al., 1993; Massip A et al., 1995). Although bovine embryos produced by IVM-IVF have yielded live calves after transfer of frozen-thawed blastocysts (Fukuda et al., 1990; Zhang et al., 1993), standard cryopreservation methods yielding high survival rates for *in vivo* derived bovine embryos usually result in low survival rates for *in vitro* developed embryos, suggesting that *in vitro*-produced bovine embryos are more sensitive to chilling and cryopreservation than *in vivo*-derived embryos(Leibo and Loskutoff, 1993; Pollard and Leibo, 1993; Hasler et al., 1995). Viability of frozen thawed *in vitro*-derived embryos could be affected by the culture conditions during the embryos development(Semple et al., 1995). Recently, it has been reported that tolerance to cryopreservation of bovine embryos developed from IVMIVF oocytes cultured in a serum-supplemented medium could be increased after removal of cytoplasmic lipid droplets(LD) by centrifugation, suggesting that cytoplasmic LD may directly affect the sensitivity of embryos to chilling and freezing (Ushijima et al., 1999). Bovine embryos cultured in serum-supplemented medium contain numerous cytoplasmic lipid droplets and immature mitochondoria compared to those cultured in serum-free medium. The accumulation of cytoplasmic lipids in embryos developed in serum- containing medium may be a result of incorporation on lipoproteins from the serum and may result in impaired function of mitochondria.(Hiroyuki ABE et al., 2003). Under the microscopic observations, blastocysts cultured in serum- supplemented medium in the presence of BGC cocultures had a dark, granular morphology with inclusion of abundant lipid-like vesicles, while blastocysts in the serum-free media(IVD101 and IVMD101) were more translucent and had less lipid-like droplets (Abe et al., 1999; Abe et al., 2001). In addition, the serum-derived blastocysts contained high levels of unsaturated fatty acids such as palmitoleic and oleic acids (Sata et al., 1999).

These results suggest that bovine embryos with high lipid content are more sensitive to cryopreservation procedures. In summary, sensitivity to cryopreservation of bovine embryos developed from IVM-IVF oocytes could be improved by serum-free cultures. The serum-free media are useful for the production of high quality bovine embryos for cryopreservation.

4. Pregnancy Rates after Tansfer Bovine Fresh Blastocysts and Frozen Blastocysts Derived in Serum-free Medium

A camparative study of *in vivo*- and *in vitro*-produced embryos revealed remarkable differences in their morphology, metabolism and quality (Shamsuddin et al., 1993; Massip et al., 1995). Thompson et al (1995) have reported that mean body weight of lambs from embryos cultured in serum-supplemented medium, that possessed abundant cytoplasmic lipid droplets, was sighnificantly heavier than that of lambs from embryos in serum-free medium. In study, only pregnancy rates were evaluated after embryo transfer of these embryos(fresh and frozen blastocysts) were obtained in serum-free medium in IVMD101 medium (Table 5). The pregnancy rates almost not different between fresh blastocysts(38.2%) and frozen blastocysts(34.9%). Although no data, it is nomal appearance and range birth weights that of all most

Table 5. Rates of pregnancy of bovine fresh blastocysts and frozen blastocysts derived in serum-free medium (IVMD101)

Condition	No. of embryo transferred	No. of pregnant recipients(%)
Fresh embryo	387	148(38.2)
Frozen embryo	758	265(34.9)

calves produced in serum-free medium (IVMD101), either fresh or frozen state. The study was examined the tolerance of cryopreservation of embryos developed from IVM-IVF oocytes cultured in either serum-free media. The sruvival rates of fresh and frozen blastocysts produced in IVMD101 medium were similar but frozen blastocysts produced in IVMD101 medium were excellent in pregnancy rates. These results strongly suggest that the serum-free media are useful for the production of high quality bovine embryos for cryopreservation.

It is well known that embryo transfer of *in vitro*-derived bovine embryos often results in the production of bovine calves with abnormalities such as heavier than normal birth weight and a high incidence of dystocia(Farin P et al., 1997; Behboodi E et al., 1995; Kruip TAM et al., 1997; Schmidt M et al., 1996). In a recent review, pregnancy, parturition and calf mortality rates of Japanese Black calves were compared after transfer of embryos developed in either serum-free media (IVMD101 and IVD101) or serum-supplemented media (TCM199 and HPM199 + 5% CS) (Hoshi H et al., 2003). The pregnancy rate of recipients receiving embryos from serum-free cultures(39.6 %, n=154) was slightly higher than those receiving embryos from serum-supplemented cultures(32.8%, n=67). Although the calving rate and abortion rate for embryos in serum-free and serum-supplemented cultures were not different(85.2% vs 86.4%; 14.8 % vs 13.6%, respectively), calf mortality for embryos from serum-supplemented cultures was greater than that for embryos from serum-free cultures (13.6% vs 4.9%).

The developmental rates at the blastocysts stage of nuclear tansferred embryos derived in both IVD101 and IVMD101 media were similar with that of nuclear tansfer embryos in serum-derived medium and normal calves were born from the blastocysts developed in the serum-free medium after nuclear transfer (Aoyagi et al., 1999).

SUMMARY

Serum-contain medium is commonly used for the production of *in vitro*-derived bovine embryos. However, were biological activity of serum varies from lot to lot, time consuming to choose better serum with good quality and risks of virus, bacteria and mycoplasma infection. This study established serum-free culture systems of *in vitro* embryo development to efficiently obtain a large number of blastocysts from ovaries of Hanwoo and oocytes maturation, cell number, tloerance of cryopreservation. Secondly, serum-contain medium is suspected of contributing to the large calf size, dystocia, cersarean sections, calf mortality and confirmed these blastocysts are high quality in terms of cyotolerance, high rates of pregancy and normal birth. For these reasons, Culture media (IVMD101 and IVD101) designed specifically for the preimplantation bovine embryo are rather simplistic, being based on salt solutions with additional energy substrates and growth factors.

An imporved serum-free medium (IVMD101) was developed for bovine oocytes maturation *in vitro*. Proportions of embryos developing to the blastocyst stage cultured in both IVD101(32.4%) and IVMD101(34.5%) serum-free media were higher than in TCM199+10% FBS(12.4%) serum-containing medium. Furthermore, the cell numbers per blastocyst obtained in the serum-free media were superior to those of blastocysts developed in serum-supplemented medium. Also, cell numbers of blastocysts obtained in the serum-free media were similar with blastocysts derived *in vivo*. Survival rate blastocysts after 24 hr incubation after thawing, the blastocysts cultured in both IVD101(94.5%) and IVMD101(95.8%) serum-free media were higher than in TCM199+10% FBS (52.5%) serum-containing medium. After 72 hr incubation after thawing, hatching rates of blastocysts developed in IVD101(78.4%) and IVMD101

(83.7%) were sighnificantly higher than that developed in the serum-supplemented medium(32.0%). The pregnancy rates almost not different between fresh blastocysts(38.2%) and frozen blastocysts (34.9%).

The results suggested that the improved serum-free media(IVMD101 and IVD101) offer several advantages over culture in serum-cotaining medium, including increased rates of blastocyst formation and high cell numbers. Addtitionally, the survival and hatching rates of embryos produced in serum-free media after post-thaw culture were superior to those of embryos produced in the serum-containg medium and useful for the production of high quality bovine embryos for cryopreservation.

These improved serum-free media are beneficial not only for the study of the mechanisms of early embryogenesis but also for mass production of good quality embryos for embryo tansfer, cloning and transgenesis.

REFERENCES

Abe H, Yamashita S, Satoh T and Hoshi H. 2002. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. Mol. Reprod. Dev., 61:57-66.

Abe H, Yamashita S, Satoh T and Hoshi H. 1999. Ultrastructure of bovine embryos developed from *in vitro*-matured and -fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. Mol. Reprod. Dev., 53:325-335.

Abe H, Otoi T, Tachikawa S, Yamashita S, Satoh T and Hoshi H. 1999. Fine structure of bovine morulae and blastocysts *in vivo* and *in vitro*. Anat Embryol., 199:519-527.

Aoyagi K, Ito H, Saitou M, Kanauchi T, Kobayashi M and Hoshi H. 1999. *In vitro* development and production of offspring from boving nuclear transfer embryos cultured in a serum-free medium. J. Reprod. Dev., 45:129-134.

Bavister BD. 1995. Culture of preimplantation embryos:facts and artifacts. Human Reprod. Update, 1:91-143.

Behboodi E, Anderson GB, BonDurant RH, Cargill SL, Kreuscher BR, Medrano JF and Murray JD. 1995. Birth of large calves that developed from *in vitro*-derived bovine embryos. Theriogenology, 44:227-232.

Brackett BG, Younis AI and Fayrer-Hosken RA. 1989. Enhanced viability after *in vitro* fertilization of bovine oocytes matured *in vivo* with high concentration of lutenizing hormone. Fertil. Steril., 52:319-324.

Brackett BG and Oliphant G. 1975. Capacitation of rabbit spermatopza *in vitro*. Biol. Reprod., 12: 260-274.

Evans EP. 1987. Karyotyping and sexing of gametes, embryos and fetuses and *in situ* hybridization to chromosomes. In. Mammalian Development.(Ed. H. Monk.) pp. 93-114.(IRL Press: City).

Eyestone WH and First NL. 1989. Co-culture of early cattle embruos to the blastocyst stage with oviductal tissue or in conditioned medium. J. Reprod. Fertil., 85:715-720.

Farin P and Farin C. 1997. Perspectives on large calves following transfer of embryos produced *in vitro*. Embryo Transfer Newslett, 15:15-19.

Farin PW and Farin CE. 1995. Transfer of bovine embryos produced *in vivo* or *in vitro* : survival and fetal development. Biol. Reprod., 52:676-682.

Fukuda Y, Ichikawa M, Naito K and Toyoda Y. 1990. Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells *in vitro* up to the blasto-

cyst stage. Biol. Reprod., 42:114-119.

Fukui Y. 1989. Effect of sera and steroid hormones on development of bovine oocytes matured and fertilized *in vitro* and co-culture with bovine oviduct epithelial cells. J. Anim. Sci., 67:1318-1323.

Gandolfi F. 1994. Autocrine, paracrine and environmental factors influencing embryonic development from zygote to blastocyst. Theriogenology, 41:95-100.

Greve T, Avery B and Callesen H. 1993. Viability of *in-vivo* and *in-vitro* produced bovine embryos. Reprod Dom Anim., 28:164-169.

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE and Trimmer SA. 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology, 43:141-152.

Hayakawa T, Yamashita K, Tanzawa K, Uchijima E and Iwata K. 1992. Growth-promoting activity of tissue inhibitor of metalloproteinase-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. FEBS Lett., 298:29-32.

Hayakawa T, Yamashita K, Ohuchi E and Shinagawa A. 1994. Cell growth-promoting activity of tissue inhibitor of metalloproteinase-2 (TIMP-2). J Cell. Sci., 107:2373-2379.

Hiroyuki ABE and Hoshi H. 2003. Evaluation of bovine embryos produced in high performance serum-free media, 49:193-202.

Hochi S, Semple E and Leibo SP. 1996. Effect of cooling and warning rates during cryopreservation on survival of *in vitro*-produced bovine embryos. Theriogenology, 45:837-847.

Holm P, Walker SK and Seamark RF. 1996. Embryo viability, duration of gestation and birth weight in sheep after transfer of *in vitro* matured and *in vitro* fertilized zygotes cultured *in vitro* or *in vivo*. J Reprod Fertil., 107:175-181.

Hoshi H. 1996. *In vitro* production of bovine preimplantation embryos in a serum-free medium. In: Gene expression and nutrition in animals: From cells to whole body (Ed. T. Muramatsu). Research Singpost, Trivandrum. India. pp. 59-70.

Hoshi H, Satoh T, Yamashita S, Abe H, Sata R, Tsujii H and Goto T. 1999. Production and embryo trasfer of preimplantation bovine embryos cultured in serum-free medium-comparative evaluation of developmental ability and quality of embryos obtained in serum-free and serum-supplemented medium. Tiss. Cult. Res. Commun., 18:163-172.

Hoshi H. 2003. *In vitro* production of bovine embryos and the application for embryo transfer. Theriogenology, 59:675-685.

Kikuchi M, Sendai Y, Yamashita S, Satoh T and Hoshi H. 1996. Expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) and collagenase genes in preimplantation bovine embryos. J. Reprod. Dev., 42:55-60.

Kobayashi K, Takagi Y, Satoh T, Hoshi H and Oikawa T. 1992. Development of early bovine embryos to the blastocyst stage in serum-free conditioned medium from bovine granulosa cells. *In vitro* Cell & Dev. Biol., 28A:255-259.

Kobayashi K, Yamashita S and Hoshi H. 1994a. Influence of epidermal growth factor and transforming growth factor-α on *in vitro* maturation of cumulus cell-enclosed bovine oocytes in a defined medium. J. Reprod. Fertil., 160:439-446.

Kobayashi K, Yamashita S, Stoh T and Hoshi H. 1994b. low oxygen amd glucose improves the development of fertilized bovine oocyte in defined mediun without somatic cells. *In vitro* Cell. Cev. Biol., 30A:556-558.

Kruip TAM and den Daas JHG. 1997. *In vitro* produced and cloned embryos: effects on preg-

nancy, parturition and offspring. Theriogenology, 47:43-52.

Leibfried-Rutledge ML, Crister ES and First NL. 1986. Effects of fetal calf serum and bovine serum albumin on *in vitro* maturation and fertilization of bovine and hamster oocyte complexes. Biol. Reprod., 35:850-857.

Leibo SP and Loskutoff NM. 1993. Cryobiology of *in vitro*-derived bovine embryos. Theriogenology, 39:81-94.

Martin KL, Hardy K, Winston RML and Leese HJ 1993. Activity of enzymes of energy metabolism in single human preimplantation embryos. J. Reprod. Fertil., 99:259-266.

Massip A, Mermillod P and Dinnyes A. 1995. Morphology and biochemistry of *in vitro* produced bovine embtyos : implications for their cryopreservation. Human Reprod., 10: 3004-3011.

Moor RM, Dai Y, Lee C and Fulka J. 1998. Oocyte maturation and embryonic failure. Human Reprod. Update, 4:223-236.

Pinyopummintr T and Bavister BD. 1991. *In vitro*-matured/*in vitro*-fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined protein-free culture media. Biol. Reprod., 45:736-742.

Pollard JW and Leibo SP. 1993. Comparative cryobiology of *in vitro* and *in vivo* derived bovine embryos. Theriogenology, 39:287(abstract).

Rieger D, Loskutoff NM and Betteridge KJ. 1992. Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced *in vitro*. Reprod Fertil Dev., 4:547-557.

Rexroad CE. 1989. Co-culture of domestic animal embryos. Theriogenology, 31:105-114.

Sata R, Tsujii H, Abe H, Yamashita S and Hoshi H. 1999. Fatty acid composition of bovine embryos cultured in serum-free and serum-containing medium during early embryonic development. J. Reprod. Dev., 45:97-103.

Satoh T, Kobayashi K, Yamashita S, Kikuchi M, Sendai Y and Hoshi H. 1994a. Tissue inhibitor of metalloproteinases (TIMP-1) produced by granulosa cells and oviduct cells enhances *in vitro* development of bovine embryo. Biol. Reprod., 50:835-844.

Satoh T, Kobayashi K, Yamashita S and Hoshi H. 1994b. Bovine tissue inhibitor of metalloproteinase-1(b TIMP-1) improves the frequency and quality of bovine embryo in serum-free medium. Cytotechnology. 14(suppl-1)(Abstr. 7. 37).

Schmidt M, Greve T, Avery B, Beckers JF, Sulon J and Hansen HB. 1996. Pregnancies, calves and calf viability after transfer of *in vitro* produced bovine embryos. Theriogenology, 46: 527-539.

Schultz GA and Heyner S. 1993. Growth factors in preimplantation embryos. Oxf. Rev. Reprod. Biol., 15:43-81.

Semple ME, Betteridge KJ and Leibo SP. 1995. Cryopreservation of *in vitro*-derived bovine embryos produced in a serum-free culture system. Theriogenology, 43:320(Abstr.).

Shamsuddin M, Lsrsson B, Gustafsson H and Rodriguez-Martinez H. 1993. *In vitro* development up to hatching of bovine *in vitro*- matured and fertilized oocytes with or without support from somatic cells. Theriogenology, 39:1067-1079.

Sinclair KD, McKvoy TG, Carolan C, Maxfield EK, Maltin CA, Young LE, Wilmut I, Robinson JJ and Broadbent PJ. 1998. Conceptus growth and development following *in vitro* culture of ovine embryos in media supplemented with bovine sera. Theriogenology, 49: 218(abstract).

Staigmiller RM and Moor RM. 1984. Effect of follicle cells on the maturation and deve-

lopmental competence of ovine oocytes matured outside the follicle. Gamete Res., 9: 221-229.

Takagi M, Otoi T, Boediono A, Saha S and Suzuki T. 1994. Viability of frozen-thawed bovine IVM/IVF embryos in relation to aging using various cryoprotectants. Theriogenology, 41: 915-921.

Takahashi Y and First NL. 1992. *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids, and vitamins. Theriogenology, 37:963-978.

Tervit HR, Wittingham DG and Rowson LEA. 1972. Successful culture *in vitro* of seep and cattle ova. J. Reprod. Fertil., 30:493-497.

Thompson JGE and Simpson AC, Pugh PA, Donnelly PE and Tervit H. R. 1990. Effect of oxygen concentration on *in-vitro* development of preimplantation sheep and cattle embtyos. J. Reprod. Fertil., 89:573-578.

Thompson JG, Gardner DK, Pugh PA, McMillan WH and Tervit HR. 1995. Lamb birth weight is affected by culture system utilized during *in vitro* pre-elongation development of ovine embryos. Biol. Reprod., 53:1385-1391.

Ushijima H, Yamakawa H and Nagashima H. 1999. Cryopreservation of bovine pre-morula-stage *in vitro* matured/*in vitro* fertilized embryos after delipidation and before use in nucleus transfer. Biol Reprod., 60:535-539.

Van Langendonckt A, Donnay I, Schuurbiers N, Auquier P, Carilan C, Massip A and Dessy F. 1997. Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium, 109: 87-93.

Vanderhyden BC and Armstrong DT. 1989. Role of cumulus cells and serum on the *in vitro* maturatuion, fertilization, and subsequent development of rat oocytes. Biol. Reprod., 40: 720-728.

Voelkel SA and Hu YX. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. Theriogenology, 37:1117-1131.

Wright RW and Bondioli KR. 1981. Aspects of *in vitro* fertilization and embryo culture in domestic animals. J. Anim. Sci., 53:702-729.

Walker SK, Hartwich KM and Seamark RF. 1996. The prodution of unusually large offspring following embryos manipultion: concepts and challenge. Theriogenology, 45:111-120.

Yamashita S, Abe H, Itoh T, Satoh T and Hoshi H. 1999. A serum-free culture system for efficient *in vitro* production of bovine blastocysts with improved viability after freezing and thawing. Cytotechnology, 31:1-9.

Yazawa S, Aoyagi Y, Konishi M and Takedomi T. 1997. Characterization and cytogenetic analysis of Japanese Black calves produced by nuclear trasnfer. Theriogenology, 48:641-650.

Zhang L and Barry DM. Denniston RS, Bunch TD, Godke RA. 1993. Birth of live calves after transfer of frozen-thawed bovine embryos fertilized *in vitro*. Vet. Rec, 132:247-279.

(접수일: 2004. 4. 20/ 채택일: 2004. 8. 10)