—Original Article—

Establishment of An Advanced Chemically Defined Medium for Early Embryos Derived from *In Vitro* Matured and Fertilized Bovine Oocytes

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Abstract. A chemically defined medium would be useful for analyzing promoters or inhibitors in *in vitro* culture (IVC) of bovine embryos. However, an IVC system for bovine embryos in a chemically defined medium has not been fully established. The present study was carried out to establish an advanced chemically defined medium for bovine embryos that supports a high rate of embryo development to the blastocyst stage. In the first experiment, we examined the effects of addition of Medium RD (RPMI1640 and Dulbecco's MEM, 1:1 v/v) to mKSOM/aa on developmental competence. The addition of 10% RD to mKSOM/aa with BSA improved the rate of development to the blastocyst stage; however, 10% RD-mKSOM/aa with PVP, which is a chemically defined medium, caused a reduction in the percentage of hatching blastocysts. In the second experiment, embryos were cultured in the chemically defined medium of 10% RD-mKSOM/aa containing 11.7, 23.4, 46.8, 70.2 or 96.8 μ M inositol. Inositol at the concentration of 70.2 μ M improved the rate of development to the hatching blastocyst stage. In the third experiment, the optimal RD concentration in the IVC medium was evaluated. Embryos were cultured in the chemically defined medium supplemented with 10, 20 or 30% (v/v) RD. The rate of development to the blastocyst stage was highest with 20% RD. In the fourth experiment, the effects of N-acetylglucosamine (GlcNAc) as an IVC medium supplement on developmental competence were examined. The rate of development to the blastocyst stage with 1.0 mM GlcNAc was significantly higher than that without GlcNAc, but the rate of development with 1.2 mM GlcNAc was not different from that without GlcNAc. We also evaluated the ability of blastocysts produced in RD-mKSOM/aa to develop to normal calves after being transferred into recipients. Ten of the 16 recipients became pregnant, with 9 delivering normal calves. These results indicate that 20% RD-mKSOM/aa containing 70.2 μ M myo-inositol and 1 mM GlcNAc is useful as a chemically defined medium for IVC of bovine embryos.

Key words: Bovine embryos, Chemically defined medium, GlcNAc, Myo-inositol

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In vitro production (IVP) of bovine embryos by an *in vitro* maturation-fertilization culture system has been greatly improved. 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% [1]. One possible reason for this is that embryo culture conditions, particularly the culture medium used for embryos, are suboptimal. Optimal conditions of a culture medium for embryos are 1) a high rate of embryo development to the blastocyst stage with repeatable results and 2) production of high-quality blastocysts that have the competence to develop into competence to normal calves. In most studies on culture of mammalian embryos, the culture medium is supplemented with serum. A beneficial effect of serum on the development of bovine oocytes to the blastocyst stage has been reported [2–4]. However, serum contains many unknown factors, and the quality of serum can vary greatly depending on the source (animal and/or supplier) [5]. The use of serum also involves a risk of microbial contamination. Furthermore, serum is suspected of contributing to large offspring syndrome in sheep [6, 7] and calves [8]. Therefore, to support a high rate of

embryo development to the blastocyst stage with repeatable results and obtain high-quality blastocysts that have the competence to develop into normal calves, a culture system using a chemically defined medium must be established for IVP of bovine embryos. The use of a chemically defined medium would also provide consistent results and enable evaluation of factors that promote or prevent embryonic development. However, an IVC system for bovine embryos in a chemically defined medium has not been fully established. Various chemically defined media have been reported for bovine embryos, including SOF [9–11], CR1aa [12], BECM (bovine embryo culture medium) [2] and KSOM [13]. In mice, a simple medium named KSOM was used for successful culture of early embryos [14], but replacement of BSA in KSOM with PVA reduced the rate of blastocyst formation and severely inhibited hatching *in vitro*. The addition of amino acids to BSA-free KSOM was found to restore the rate of blastocyst formation and hatching [15, 16]. In cattle, we have reported that modified KSOM/aa (mKSOM/aa) containing BSA and modified TCM199 (glucose-free) containing BSA were embryo culture media supporting development to the blastocyst stage [17]. However, in our preliminary experiment, mKSOM/aa with polyvinylpyrrolidone (PVP), which is a chemically defined medium, was found to markedly depress the rate of blastocyst formation.

On the other hand, protein-free Medium RD (RPMI1640 and Dulbecco's MEM, 1:1 v/v) was shown to be superior to TCM199 for culture of rabbit embryos [18]. Therefore, we hypothesized

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Table 1. Experiment design and treatment groups^a

Experiment	Media		
	Sperm capacitation	IVF	IVC
1	FM + 0.1 μ M A23187 ^b + 1.5 U/ml heparin + 5 mM caffeine	FM + 1.5 U/ml heparin	10% RD-mKSOM/aa + 10 mg/ml BSA or mKSOM/aa + 10 mg/ml BSA or 10% RD-mKSOM/aa + 1 mg/ml PVP + 0.5 mM sodium citrate
2	FM + 0.1 μ M A23187 ^b + 1.5 U/ml heparin + 5 mM caffeine	FM + 1.5 U/ml heparin	10% RD-mKSOM/aa + 1 mg/ml PVP + 0.5 mM sodium citrate + 11.7, 23.4, 46.8, 70.2 or 96.8 μ M myo-inositol
3	BSA-free FM + 0.5 mg/ml D-penicillamine + 0.5 mM citrate + 0.25 mM methyl- β -cyclodextrin + 1 mg/ml PVP + 5 mM caffeine	BSA-free FM + 1 mg/ml PVP	10, 20 or 30% RD-mKSOM/aa + 1 mg/ml PVP + 0.5 mM sodium citrate + 70.2 μ M myo-inositol
4	BSA-free FM + 0.5 mg/ml D-penicillamine + 0.5 mM citrate + 0.25 mM methyl- β -cyclodextrin + 1 mg/ml PVP + 5 mM caffeine	BSA-free FM + 1 mg/ml PVP	20% RD-mKSOM/aa + 1 mg/ml PVP + 0.5 mM sodium citrate + 70.2 μ M myo-inositol + 0, 0.6, 1.0 or 1.2 mM N-acetylglucosamine

^a IVM was described in Materials and Methods. ^b Calcium ionophore A23187.

that Medium RD could be superior to TCM199 for culture of bovine embryos. However, the glucose concentration in Medium RD (8.3 mM) is too high for bovine embryos. Accordingly, we considered using RD as a supplement for mKSOM/aa (10%RD-mKSOM/aa, RD and mKSOM/aa, 1:9 v/v). Carney and Foote suggested that a high concentration (117 μ M) of inositol in RD promotes the development of rabbit embryos [18]. The original concentration of inositol in 10% RD-mKSOM/aa was 11.7 μ M. However, the optimal concentration of inositol in 10% RD-mKSOM/aa for bovine embryos has not been determined.

Furthermore, N-acetylglucosamine (GlcNAc) exists widely as a component of glycoprotein and glycosaminoglycan in both animals and plants cells. Nancarrow and Hill [19] reported that an oviduct-specific glycoprotein was secreted mainly from day -1 to day 3 of the ovarian cycle and that this ovine estrus-associated glycoprotein enhanced the proportion of sheep oocytes that cleaved *in vitro*. Additionally, a serum-free medium of chinese hamster ovary cells containing 1 mM GlcNAc markedly enhanced cell proliferation (Nakano, personal communication). Therefore, we considered the effect on IVC of supplementation of a chemically defined medium for bovine embryos with GlcNAc.

The objective of this study was to establish an advanced chemically defined medium for bovine embryos that supports a high rate of embryo development to the blastocyst stage. In the first experiment, we examined the effects of addition of Medium RD to mKSOM/aa on developmental competence. In the second experiment, embryos were cultured in the chemically defined medium of 10% RD-mKSOM/aa containing various concentrations of inositol. In the third experiment, we evaluated the optimal RD concentration in the IVC medium. In the fourth experiment, we examined the effects of GlcNAc as an IVC medium supplement on developmental competence. Moreover, we evaluated the ability of blastocysts pro-

duced in RD-mKSOM/aa to develop to normal calves after being transferred into recipients.

Materials and Methods

In vitro maturation (IVM) of oocytes

Cattle ovaries (mostly from crossbred cows) obtained at a local abattoir were wrapped in paper towels soaked in 0.9% saline and transported to the laboratory at about 30 C. Cumulus-oocyte complexes (COCs) were aspirated from 2–6-mm follicles of ovaries into a 5-ml disposable syringe with a 21-gauge needle and were placed in plastic Petri dishes. Fully grown oocytes with an unexpanded cumulus (more than 5 layers) and nonpolar ooplasm filling the perivitelline space were washed several times with maturation medium. COCs were divided into two groups by the appearance of their ooplasm, homogeneous (finely, evenly granulated) or heterogeneous (unevenly granulated to various degrees) [20], and cultured for 24 h in 50- μ l droplets of maturation medium under mineral oil (Bristol-Myers Squibb, Princeton, NJ, USA) in culture dishes (35 $\times$ 10 mm, Nunc, Roskilde, Denmark) in a CO₂ incubator (5% CO₂, 95% humidified air, 39 C). Each droplet contained about 10 COCs. The maturation medium was 10 mM HEPES-buffered TCM-199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.3 mM sodium pyruvate, 0.5 IU/ml hFSH (containing 2.31 IU/ml LH, Biogenesis, England, U.K.), 1 μ g/ml estradiol-17 β (Sigma-Aldrich, St. Louis, MO, USA), 3 mg/ml polyvinylpyrrolidone (PVP, M_r 40,000, Sigma), 65 μ g/ml dibekacin sulfate (Meiji-Seika, Tokyo, Japan) and 1.5% 2nd fraction of ultracentrifuged bovine follicular fluid (bFF). The 2nd fraction of bFF was prepared by ultracentrifugation of bFF at 220,000 $\times$ g for 48 h at 4 C [17].

Sperm preparation and in vitro fertilization (IVF)

The basic medium for sperm washing, capacitation and insemination was modified BO medium [21], hereafter referred to as fertilization medium (FM), which consisted of 113.45 mM NaCl, 4.02 mM KCl, 2.25 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.83 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.52 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 25 mM NaHCO_3 , 13.90 mM glucose, 12.5 mM HEPES (pH 7.4), 65 $\mu\text{g}/\text{ml}$ dibekacin sulfate, 10 mg/ml BSA (fraction V, Wako Pure Chemical Industries, Osaka, Japan) and $2 \times 10^{-4}\%$ (w/v) phenol red [20]. The methods used for sperm washing were the same in all experiments, but the methods used for sperm capacitation and insemination were different in the experiments (Table 1). One straw (0.5 ml/straw) of frozen semen of Japanese Black cattle was thawed in a water bath at 39 C for 20 sec, suspended in 5 ml of BSA-free FM supplemented with 10 mM caffeine (sodium benzoate, Sigma-Aldrich), and washed twice by centrifugation at $700 \times g$ for 5 min. In experiments 1 and 2, the method used for sperm capacitation was the same as that described previously [22]. Spermatozoa resuspended in 1 ml of BSA-free FM supplemented with 10 mM caffeine were treated with 0.1 μM Ca ionophore A23187 (Calbiochem-Behring, La Jolla, CA, USA) for 60 sec. After treatment, the sperm suspension was immediately diluted 1:1 with FM containing 20 mg/ml BSA supplemented with 3 units/ml heparin (heparin sodium salt, Wako). The spermatozoa were preincubated for 3 h in a CO_2 incubator (5% CO_2 , 95% humidified air, 39 C). The sperm concentration at preincubation was about 1×10^7 sperm/ml. After the 3-h preincubation, a small amount of sperm suspension was introduced into each 40- μl droplet of FM supplemented with 1.5 units/ml heparin to obtain a final concentration of 120 sperm/ μl . Subsequently, COCs were introduced into droplets of sperm suspension (about 10 oocytes/drop). In experiments 3 and 4, the method used for sperm capacitation was the same as that described previously [23] with some modifications. The washed spermatozoa were resuspended in 0.5 ml of BSA-free FM supplemented with 10 mM caffeine. The sperm suspension was diluted 1:1 with BSA-free FM supplemented with 1.0 mg/ml penicillamine (Sigma-Aldrich), 0.5 mM methyl- β -cyclodextrin (Sigma-Aldrich), 1.0 mM citrate (sodium citrate-trisodium salt, Kanto Chemical, Tokyo, Japan) and 1 mg/ml PVP. The spermatozoa were preincubated for 4 h in a CO_2 incubator (5% CO_2 , 95% humidified air, 39 C). The sperm concentration at preincubation was about 2×10^7 sperm/ml. After the 4-h preincubation, 5 μl of sperm suspension was introduced into each 40- μl droplet of BSA-free FM with 1.0 mg/ml PVP. Eventually, the sperm concentration at insemination was about 1,000 sperm/ μl . At 30 min after introduction of sperm, COCs at 24 h after IVM were introduced into the droplets of sperm suspension (about 10 oocytes/drop). In all experiments, spermatozoa and oocytes were coincubated for 6 h in a CO_2 incubator (5% CO_2 , 95% humidified air, 39 C).

In vitro embryo culture (IVC)

At 6 h after insemination, oocytes were freed from cumulus cells by repeated pipetting. Subsequently, presumptive zygotes were cultured for 240 h in 50- μl of culture medium covered with mineral oil under a gas-phase of 5% CO_2 , 5% O_2 and 90% N_2 with high humidity at 39 C. Each droplet contained approximately 10 presumptive zygotes. At 96 h after insemination, embryos at the

8-cell stage or a more advanced stage were transferred to a fresh culture medium supplemented with 10 μM β -mercaptoethanol (β -ME; Sigma-Aldrich). At 120–144 h after insemination, embryos at the morula stage or a more advanced stage were transferred to a fresh culture medium supplemented with 50 μM β -ME. Oocytes that did not progress to the next cleavage stage were removed from drops containing developing embryos. Cleavage and development to the 8-cell or more advanced stage, to the morula stage and to the blastocyst stage were observed at 48, 96, 120–144 and 144–216 h after insemination, respectively, under an inverted microscope.

Embryo freezing

Excellent or good blastocysts at Days 6–9 (Day 0 = day of insemination) were placed into a freezing medium at room temperature (20 C). The basic freezing medium was modified PB1 medium [24]. This medium, designated as mPB1, contained 1 mg/ml PVP instead of BSA. The freezing medium consisted of mPB1 supplemented with 14.8 mM L-proline [25], 0.2 M trehalose, 7% (v/v) ethylene glycol and 0.5% (v/v) glycerol. Embryos were loaded into 0.25-ml straws (IMV, France) and placed at -7°C in a methanol bath in a programmable freezer. Seeding was induced immediately. Samples were held for about 1 min at -7°C after seeding, cooled at $0.5^\circ\text{C}/\text{min}$ to -25°C , and then placed in liquid nitrogen gas, plunged and stored in liquid nitrogen at -196°C for 30–50 days.

Experimental design

The experimental design is presented in Table 1.

Experiment 1: Effects of addition of Medium RD to mKSOM/aa

The embryo culture medium used for a control was modified KSOM/aa [15], hereafter referred to as mKSOM/aa, which consisted of 98.6 mM NaCl; 2.5 mM KCl; 1.71 mM CaCl_2 ; 0.35 mM KH_2PO_4 ; 0.2 mM MgSO_4 ; 0.3 mM sodium pyruvate; 3.0 mM sodium lactate; 25 mM NaHCO_3 ; 0.68 mM L-glutamine; premixture solutions of 12 essential amino acids for basal medium Eagle (Sigma-Aldrich) and 7 nonessential amino acids for minimum essential medium (Sigma-Aldrich; final concentrations of L-leucine, L-methionine, L-tryptophan and glycine being modified to 0.4, 0.1, 0.05 and 3 mM, respectively); 10 mM HEPES; 5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{g}/\text{ml}$ transferrin and 5 ng/ml sodium selenite (ITS media supplement, Sigma-Aldrich); 10 μM EDTA; and 65 $\mu\text{g}/\text{ml}$ dibekacin sulfate [17]. The embryo culture medium used for treatment was mKSOM/aa supplemented with 10% (v/v) RD (RPMI1640 and Dulbecco's MEM, 1:1 v/v) [18], hereafter referred to as 10% RD-mKSOM/aa, which contained an increased concentration of glucose (1 mM). Presumptive zygotes were cultured in 10% RD-mKSOM/aa with 10 mg/ml BSA, 10% RD-mKSOM/aa with 1 mg/ml PVP and 0.5 mM sodium citrate or mKSOM/aa with 1 mM glucose and 10 mg/ml BSA. In all experiments, the glucose concentration in the culture medium was adjusted to 1 mM. The RPMI1640 used in experiment 1 was purchased from Life Technologies Corporation (Cat. no. 11875, Life Technologies, Grand Island, NY, USA), and the Dulbecco's MEM (low glucose) used in experiment 1 was purchased from Sigma-Aldrich (Cat. No. D6046). Experiments were performed at least three times.

Table 2. Effect of addition of Medium RD to mKSOM/aa on development to the blastocyst stage of bovine IVM-IVF oocytes

Type of oocytes	Culture media	Replacement with a chemically defined medium	No. of oocytes examined	No. (%) of oocytes developed to				Hatching rate (%) of blastocysts
				≥2 cells	≥8 cells	Morula	Blastocyst	
Hetero ¹	mKSOM/aa	No ^{a)}	115	94 (81.7)	78 (67.8) ^d	67 (58.3) ^d	62 (53.9) ^d	71.0 ^f
	10% RD-mKSOM/aa	No ^{b)}	114	103 (90.4)	94 (82.5) ^e	80 (70.2) ^e	73 (64.0) ^e	82.2 ^f
	10% RD-mKSOM/aa	Yes ^{c)}	111	91 (82.0)	81 (73.0) ^d	67 (60.4) ^d	56 (50.5) ^d	16.1 ^g
Homo ²	mKSOM/aa	No ^{a)}	69	55 (79.7)	39 (56.5)	30 (43.5)	28 (40.6)	67.9 ^f
	10% RD-mKSOM/aa	No ^{b)}	94	69 (73.4)	50 (53.2)	36 (38.3)	33 (35.1)	93.9 ^g
	10% RD-mKSOM/aa	Yes ^{c)}	30	24 (80.0)	21 (70.0)	16 (53.3)	10 (33.3)	20.0 ^h

¹ Heterogeneous ooplasm. ² Homogeneous ooplasm. ^{a)} The culture medium was mKSOM/aa with 10 mg/ml BSA. ^{b)} The culture medium was 10% RD-mKSOM/aa with 10 mg/ml BSA. ^{c)} The culture medium was 10% RD-mKSOM/aa with 1 mg/ml PVP and 0.5 mM citrate. ^{d-h} Values in the same column with different superscripts are significantly different for each type of oocyte ($P < 0.05$ for d and e, and $P < 0.01$ for f, g and h).

Experiment 2: Effects of various concentrations of myo-inositol in 10% RD-mKSOM/aa

Presumptive zygotes were cultured in 10% RD-mKSOM/aa with 1 mg/ml PVP, 0.5 mM citrate and myo-inositol at various concentrations. Inositol was used at concentrations of 11.7, 23.4, 46.8, 70.2 and 93.6 μ M. The original concentration of myo-inositol in 10% RD-mKSOM/aa was 11.7 μ M. The RPMI1640 used in experiment 2 was purchased from Life Technologies (Cat. no. 11875), and the Dulbecco's MEM (low glucose) used in experiment 2 was purchased from Sigma-Aldrich (Cat. No. D6046). Experiments were performed at least twice.

Experiment 3: Optimal RD concentrations in IVC medium

Presumptive zygotes were cultured in mKSOM/aa supplemented with 10, 20 or 30% (v/v) RD, 1 mg/ml PVP, 0.5 mM citrate and 70.2 μ M myo-inositol. The RPMI1640 (without glucose and phenol red) and Dulbecco's MEM (without glucose, sodium pyruvate and phenol red) in experiment 3 were prepared as a customized medium by Kojin-bio (Sakado, Japan). In this experiment, the concentrations of inorganic salts, sodium pyruvate, sodium lactate, NaHCO_3 , L-glutamine, glycine, HEPES, EDTA, ITS and dibekacin sulfate contained in the culture medium were the same as those in mKSOM/aa. Experiments were performed at least four times.

Experiment 4: Effects of supplementation of N-acetylglucosamine (GlcNAc)

Presumptive zygotes were cultured in 20% RD-mKSOM/aa with 0, 0.6, 1 or 1.2 mM GlcNAc, 1 mg/ml PVP, 0.5 mM citrate and 70.2 μ M myo-inositol. The RPMI1640 (without glucose and phenol red) used in experiment 4 was prepared as a customized medium by Kojin-bio, and the Dulbecco's MEM (low glucose) used in experiment 4 was purchased from Sigma-Aldrich (Cat. No. D6046). Experiments were performed at least four times.

Experiment 5: Cryopreservation ability of blastocysts produced with 10% RD-mKSOM/aa

In this experiment, blastocysts developed in 10% RD-mKSOM/aa with 1 mg/ml PVP, 0.5 mM citrate and 70.2 μ M myo-inositol were used for cryopreservation. In addition, frozen-thawed blastocysts were cultured in 10% RD-mKSOM/aa containing 10 mg/ml BSA and 100 μ M β -ME, which is not a chemically defined medi-

um, in order to observe hatching. For thawing, each straw containing frozen embryos was held in air for 12 sec and then in 35 C water for 8 sec. The embryo was removed from the straw and placed in a drop in PB1 supplemented with 20% fetal bovine serum for 10 min at 39 C. After thawing, the embryos were cultured for 168 h under a gas phase of 5% CO_2 , 5% O_2 and 90% N_2 with high humidity at 39 C. Survival of embryos (i.e., embryos that reexpanded and continued development) up to 48 h after thawing and hatching up to 168 h after thawing were assessed.

Experiment 6: Ability of blastocysts produced with mKSOM/aa supplemented with 10% or 20% RD to develop into live calves

In this experiment, 7 fresh blastocysts and 9 frozen-thawed blastocysts were used for embryo transfer. Fresh blastocysts were produced with 10% RD-mKSOM/aa containing 1 mg/ml PVP, 0.5 mM citrate and 70.2 μ M myo-inositol. Frozen-thawed blastocysts were produced with 20% RD-mKSOM/aa containing 1 mg/ml PVP, 0.5 mM citrate, 70.2 μ M myo-inositol and 0 or 1 mg/ml mM GlcNAc. Excellent or good blastocysts at Days 6–7 (Day = day of insemination) were subjected to cryopreservation by conventional freezing. Frozen-thawed blastocysts were cultured overnight by the same method as that used in experiment 5. Excellent blastocysts were selected for embryo transfer. The recipient cows were synchronized by treatment with a progesterone device (CIDR, InterAg, Hamilton, New Zealand) for 14 days. A single blastocyst was transferred nonsurgically into the uterine horn ipsilateral to the corpus luteum of each recipient on Day 6 (1 recipient), 8 (14 recipients) or 9 (1 recipient) of the estrous cycle (Day 0 = day of estrus).

Statistical analysis

Percentage data were subjected to an arcsine transformation and analyzed by one-way ANOVA. Treatment differences were determined using Tukey's multiple comparison test.

Results

Experiment 1: Effects of addition of Medium RD to mKSOM/aa

The results of experiment 1 are presented in Table 2. In the oocytes with heterogeneous ooplasm, the rates of development to the 8-cell or more advanced stage, morula stage and blastocyst stage

Table 3. Effect of various concentrations of myo-inositol in 10% RD-mKSOM/aa on development to the blastocyst stage of bovine IVM-IVF oocytes

Type of oocytes	Concentration of myo-inositol (μM)	No. of oocytes examined	No. (%) of oocytes developed to				Hatching rate (%) of blastocyst
			≥ 2 cells	≥ 8 cells	Morula	Blastocyst	
Hetero	11.7 ^a	111	91 (82.0) ^{bc}	81 (73.0) ^b	67 (60.4)	56 (50.5)	16.1 ^b
	23.4	120	103 (85.8) ^b	95 (79.2) ^b	75 (62.5)	66 (55.0)	36.4 ^c
	46.8	108	91 (84.3) ^b	84 (77.8) ^b	69 (63.9)	54 (50.0)	42.6 ^{cd}
	70.2	116	91 (78.4) ^{bc}	80 (69.0) ^{bc}	71 (61.2)	66 (56.9)	45.5 ^{cd}
	93.6	74	53 (71.6) ^c	43 (53.1) ^c	40 (54.1)	36 (48.6)	33.3 ^{bc}
Homo	11.7 ^a	30	24 (80.0)	21 (70.0)	16 (53.3)	10 (33.3)	20.0 ^{bc}
	23.4	48	35 (72.9)	27 (56.3)	17 (35.4)	14 (29.2)	14.3 ^{bc}
	46.8	88	63 (71.6)	50 (56.8)	41 (46.6)	30 (34.1)	10.0 ^b
	70.2	38	27 (71.1)	23 (60.5)	18 (47.4)	14 (36.8)	42.9 ^c
	93.6	20	13 (65.0)	7 (35.0)	6 (30.0)	4 (20.0)	0.0 ^{bc}

^a The original concentration in 10% RD-mKSOM/aa. ^{b,c,d} Values in the same column with different superscripts are significantly different for each type of oocyte ($P < 0.05$ for b vs. c, and $P < 0.01$ for b vs. d).

Table 4. Effect of various concentrations of RD in *in vitro* culture medium on development to the blastocyst stage of bovine IVM-IVF oocytes

Type of oocytes	Concentration of RD (%)	No. of oocytes examined	No. (%) of oocytes developed to			
			≥ 2 cells	≥ 8 cells	Morula	Blastocyst
Hetero	10	102	81 (79.4)	68 (66.7)	59 (57.8)	44 (43.1)
	20	97	88 (90.7)	76 (78.4)	62 (63.9)	47 (48.5)
	30	94	78 (83.0)	65 (69.1)	48 (51.1)	34 (36.2)
Homo	10	50	40 (80.0)	33 (66.0)	26 (52.0) ^a	18 (36.0) ^{ab}
	20	57	46 (80.7)	39 (68.4)	37 (64.9) ^a	25 (43.9) ^a
	30	57	42 (73.7)	33 (57.9)	16 (28.1) ^b	12 (21.1) ^b

^{a,b} Values in the same column with different superscripts are significantly different for each type of oocyte ($P < 0.05$).

with 10% RD-mKSOM/aa containing BSA were significantly higher than those with mKSOM/aa containing BSA. On the other hand, the rates of development to the 8-cell or more advanced stage, morula stage, blastocyst stage and hatching blastocyst stage with 10% RD-mKSOM/aa containing PVP, which is a chemically defined medium, were lower than those with 10% RD-mKSOM/aa containing BSA. There were no differences in the rate of development to the blastocyst stage between 10% RD-mKSOM/aa containing PVP and mKSOM/aa containing BSA. However, 10% RD-mKSOM/aa containing PVP caused a reduction in the percentage of hatching blastocysts compared with that obtained with mKSOM/aa containing BSA. On the other hand, in the oocytes with homogeneous ooplasm, the rates of cleavage and development to the 8-cell or more advanced stage, morula stage and blastocyst stage were not different among the groups. However, the rate of development to the hatching blastocyst stage with 10% RD-mKSOM/aa containing PVP was significantly lower than the rates obtained with mKSOM/aa containing BSA and 10% RD-mKSOM/aa containing BSA.

Experiment 2: Effects of various concentrations of myo-inositol in a chemically defined medium of 10% RD-mKSOM/aa

The results of experiment 2 are presented in Table 3. In the oocytes with heterogeneous ooplasm, the rates of development to the blastocyst stage were not different among the groups. While not significant, the rate of development to the blastocyst stage with inositol at the concentration of 70.2 μM was the highest among the groups. The percentage of hatching blastocysts was increased dose-dependently by inositol at concentrations from 23.4 to 70.2 μM . The percentages of hatching blastocysts with 23.4, 46.8 and 70.2 μM inositol were significantly higher than that with 11.7 μM inositol. On the other hand, in the oocytes with homogeneous ooplasm, the rates of development to the blastocyst stage were not different among the groups. However, the percentage of hatching blastocysts with 70.2 μM inositol was the highest among the groups.

Experiment 3: Optimal RD concentrations in IVC medium

The results of experiment 3 are presented in Table 4. In the oocytes with heterogeneous ooplasm, the rates of development to the blastocyst stage were not different among the groups. While not significant, the rate of development to the blastocyst stage with 20% RD was highest among the groups. On the other hand, in the

Table 5. Effects of supplementation of N-acetylglucosamine (GlcNAc) in 20% RD-mKSOM/aa on development to the blastocyst stage of bovine IVM-IVF oocytes

Type of oocytes	Concentration of GlcNAc (mM)	No. of oocytes examined	No. (%) of oocytes developed to			
			≥2 cells	≥8 cells	Morula	Blastocyst
Hetero	0	78	68 (87.2)	59 (75.6)	53 (67.9)	37 (47.4) ^a
	0.6	61	55 (90.2)	50 (82.0)	45 (73.8)	35 (57.4) ^{ab}
	1.0	73	68 (93.2)	65 (89.0)	58 (79.5)	48 (65.8) ^b
	1.2	79	70 (88.6)	63 (79.7)	50 (63.8)	37 (46.8) ^a
Homo	0	76	57 (75.0)	42 (55.3)	33 (43.4)	22 (28.9)
	1.0	56	41 (73.2)	29 (51.8)	24 (42.9)	18 (32.1)

^{a,b} Values in the same column with different superscripts are significantly different for each type of oocyte ($P < 0.05$).

Table 6. Ability to develop into live calves of blastocysts produced with mKSOM/aa supplemented with 10% or 20% RD

Group of embryos	Experiment no.	Quality of blastocyst	Estrous cycle of recipient (Day) ^a	Pregnancy	Birth of live calves
Fresh ^b	1	Excellent	8	-	(Returned to estrus on Day 81)
	2	Excellent	8	+	+ Live calf (♂40 kg, Day 310)
	3	Excellent	9	+	+ Live calf (♀30 kg, Day 287)
	4	Excellent	8	-	(Returned to estrus on Day 25)
	5	Excellent	8	+	+ Live calf (♂38 kg, Day 285)
	6	Excellent	8	+	+ Live calf (♀43 kg, Day 295)
	7	Excellent	8	-	(Returned to estrus on Day 61)
Frozen-thawed A ^c	1	Excellent	8	+	+ Live calf (♀35 kg, Day 295)
	2	Excellent	8	-	(Returned to estrus on Day 49)
	3	Excellent	6	+	+ Live calf (♀27 kg, Day 303)
B ^d	1	Excellent	8	+	- Stillbirth (♀, Day 286)
	2	Excellent	8	+	+ Live calf (♂26 kg, Day 289)
	3	Excellent	8	+	+ Live calf (♂28 kg, Day 307)
	4	Excellent	8	+	+ Live calf (♂40 kg, Day 288)
	5	Excellent	8	-	(Returned to estrus on Day 49)
	6	Excellent	8	-	-

^a Day of estrus=Day 0. ^b The blastocysts were produced with 10% RD-mKSOM/aa containing 1 mg/ml PVP, 0.5 mM citrate and 70.2 µM myo-inositol (experiment 1). ^c The blastocysts were produced with 20% RD-mKSOM/aa containing 1 mg/ml PVP, 0.5 mM citrate and 70.2 µM myo-inositol (experiment 2). ^d The blastocysts were produced with 20% RD-mKSOM/aa containing 1 mg/ml PVP, 0.5 mM citrate, 70.2 µM myo-inositol and 1 mM GlcNAc (experiment 4).

oocytes with homogeneous ooplasm, the rates of development to the morula and blastocyst stages with 30% RD were significantly lower than those with 20% RD.

Experiment 4: Effects of supplementation of N-acetylglucosamine (GlcNAc)

The results of experiment 4 are presented in Table 5. In the oocytes with heterogeneous ooplasm, there were no differences in the rates of cleavage and development to the 8-cell or more advanced stages and morula stage among the groups with different concentrations of GlcNAc. However, the rate of development to the blastocyst stage with 1 mM GlcNAc was significantly higher than that without GlcNAc, but the rate of development with 1.2 mM GlcNAc was not different from that without GlcNAc. On the other hand, in the oocytes with homogeneous ooplasm, there were no differences in the developmental competences at GlcNAc concentrations be-

tween 0 mM and 1 mM.

Experiment 5: Cryopreservation ability of blastocysts produced with 10% RD-mKSOM/aa

After freezing and thawing, the survival rate and rate of development to hatching blastocysts of blastocysts produced in 10% RD-mKSOM/aa containing 1 mg/ml PVP, 0.5 mM citrate and 70.2 µM inositol were 85.0% (34/40) and 80.0% (32/40), respectively.

Experiment 6: Ability to develop into live calves of blastocysts produced with mKSOM/aa supplemented with 10% or 20% RD

The results of experiment 6 are presented in Table 6. Seven fresh blastocysts produced in 10% RD-mKSOM/aa containing 1 mg/ml PVP, 0.5 mM citrate and 70.2 µM inositol were transferred to 7 recipients. Four of the seven recipients became pregnant and delivered 4 normal calves. Nine frozen-thawed blastocysts produced

in 20% RD-mKSOM/aa containing 1 mg/ml PVP, 0.5 mM citrate, 70.2 μ M inositol and 0 or 1 mM GlcNAc (3 blastocysts with 0 mM GlcNAc and 6 blastocysts with 1 mM GlcNAc) were transferred to 9 recipients. Six of the 9 recipients became pregnant. Of these, 5 delivered live calves; there was 1 stillbirth.

Discussion

The basic embryo culture medium used in this study was mK-SOMaa. In mice, the addition of amino acids to BSA-free KSOM (KSOM/AA) was found to restore the rates of blastocyst formation and hatching [15, 16]. The mKSOM/aa in the present study was a modified form of KSOM/AA medium, and the modified ingredients were 0.3 mM sodium pyruvate [9], 3.0 mM sodium lactate [9], 1.0 mM glucose (in our preliminary experiment), BME amino acids [9] (final concentrations of L-leucine, L-methionine and L-tryptophan being modified to 0.4, 0.1 and 0.05 mM, respectively), MEM nonessential amino acids [9, 26] (concentration of glycine adjusted to 3 mM [27]) and ITS (5 μ g/ml insulin [28], 5 μ g/ml transferrin, 5 ng/ml sodium selenite). We have reported that mK-SOM/aa containing BSA without glucose was an embryo culture medium supporting development to the blastocyst stage [17]. However, in our preliminary experiment, mKSOM/aa with PVP, which is a chemically defined medium, was found to markedly depress the rate of blastocyst formation (data not shown). This result suggests that mKSOM/aa is still not sufficient as a chemically defined medium.

On the other hand, protein-free Medium RD (RPMI1640 and Dulbecco's MEM, 1:1 v/v) was shown to be superior to TCM199 for culture of rabbit embryos [18]. We have also reported that modified TCM199 (glucose-free) containing BSA was an embryo culture medium supporting development to the blastocyst stage [17]. Therefore, we hypothesized that Medium RD could be superior to TCM199 for culture of bovine embryos. However, the glucose concentration in Medium RD (8.3 mM) is too high for bovine embryos. Accordingly, we considered using RD as a supplement to mKSOM/aa (10%RD-mKSOM/aa, RD and mKSOM/aa, 1:9 v/v). The results of experiment 1 demonstrated that addition of Medium RD to the m-KSOM/aa medium resulted in good embryonic development *in vitro* for a semidefined IVC medium. However, replacement of BSA in this medium with PVP and citrate resulted in decreases in the rates of both blastocyst formation and hatching (Table 2). Although citrate is purified from a commercial BSA as an embryotrophic factor [29], this result shows that BSA cannot be replaced with only citrate and PVP.

As shown in Table 3, the presence of 70.2 μ M myo-inositol in 10% RD-mKSOM/aa with citrate and PVP improved the hatching rate of blastocysts, but more than 70.2 μ M inositol did not improve the hatching rate of blastocysts. Carney and Foote also suggested that a high concentration (117 μ M) of inositol in RD medium promotes the development of rabbit embryos [18]. Inositol is essential for rabbit blastocyst expansion [30, 31] and stimulates hatching of hamster blastocysts [32]. Furthermore, in bovine embryos, inositol is incorporated into phosphoinositides and inositol phosphates, inducing a very large change in the pattern of second messenger formation at the critical stage of blastocyst formation [33]. Holm *et al.*

[4] reported that the addition of a high myo-inositol concentration (2.77 mM) to SOF medium containing citrate and PVA enhanced bovine embryo development. In the case of a simple culture medium such as SOF, the addition of a high myo-inositol concentration may be required to enhance embryo development *in vitro*.

As shown in Table 4, the optimal RD concentration in RD-mK-SOM/aa with citrate, PVP and inositol was 20%. In addition, RD at a concentration of 30% caused a reduction in the rate of development to the blastocyst stage. In experiment 3, on the other hand, the RD concentration in the IVC medium was different among the groups, and the concentrations of inorganic salts, sodium pyruvate, sodium lactate, glucose, NaHCO₃, L-glutamine, glycine, myo-inositol, HEPES, citrate, EDTA, ITS and dibekacin sulfate in the IVC medium were the same. The concentrations of vitamins in RD-mKSOM/aa changed in a manner dependent on the RD concentration, and key substances in RD might be vitamins, except myo-inositol. In cattle, culture of bovine embryos with vitamin E resulted in development of more embryos to early and expanded blastocysts [34]. In hamsters, pantothenate significantly stimulated blastocyst development compared with the effects of the vitamin-free control and other single vitamins except thiamine [35]. In experiment 3, the concentration of vitamins in 20% RD-mKSOM/aa may have been suitable for early embryonic development.

As shown in Table 5, GlcNAc at the concentration of 1 mM in 20% RD-mKSOM/aa containing citrate, PVP and myo-inositol improved the rate of development to the blastocyst stage. GlcNAc exists widely as a component of glycoprotein and glycosaminoglycan in both animals and plants cells. Nancarrow and Hill [19] reported that an oviduct-specific glycoprotein was secreted mainly from day -1 to day 3 of the ovarian cycle and that this ovine estrus-associated glycoprotein enhanced the proportion of sheep oocytes cleaved *in vitro*. They also suggested that this glycoprotein may have a facilitatory role and in its pure form be a useful component of embryo production media *in vitro*. The results of this study demonstrate that GlcNAc is beneficial for *in vitro* embryonic development when added as a supplement to a chemically defined medium for bovine embryos.

Recently, an advanced chemically defined medium for IVC of porcine zygotes named PZM-5 has been developed [36, 37]. Interestingly, PZM-5 is a comparatively simple culture medium. In contrast, the chemically defined medium of 20% RD-mKSOM/aa used in the present study is a comparatively complicated culture medium. In our preliminary experiment, mKSOM/aa with PVP, which is a comparatively simple culture medium, reduced the rate of development to the blastocyst stage of bovine embryos (data not shown). These results show that the involvement of many substances that exist in an IVC medium is necessary for the development of bovine embryos.

In this study, before IVM, COCs were divided into two groups by the appearance of their ooplasm, homogeneous and heterogeneous. The reason for this was that the appearance of the ooplasm reflects the *in vitro* developmental competence of the oocytes. We have reported that developmental competence to the blastocyst stage was significantly greater in oocytes with heterogeneous ooplasm than in oocytes with homogeneous ooplasm [17]. Nagano *et al.* [38] reported that a heterogeneous ooplasm indicated an ac-

Table 7. Composition of a 20% RD*-mKSOM/aa chemically defined medium

Component	mM	Component	mM	Component	mM
Inorganic salts		Amino acids		Vitamines	
NaCl	98.60	L-Alanine	0.08	Biotin	0.00008
KCl	2.50	L-Arginine	0.19	D-Ca Pantothenate	0.00006
CaCl ₂ •2H ₂ O	1.71	L-Arginine•HCl	0.04	D-Pantothenic acid (hemi Ca)	0.0016
Ca(NO ₃) ₂ •4H ₂ O	0.04	L-Asparagine	0.12	Choline chloride	0.0050
MgSO ₄	0.20	L-Aspartic acid	0.10	Folic acid	0.0012
NaHCO ₃	25.00	L-Cystine•2HCl	0.12	Myo-Inositol	0.0702
Na ₂ HPO ₄	0.56	L-Glutamic acid	0.09	Niacinamide	0.0040
NaH ₂ PO ₄	0.09	L-Glutamine	0.68	Para-aminobenzonic acid	0.0008
Fe(NO ₃) ₃ •9H ₂ O	0.000024	Glycine	3.00	Pyridoxine HCl	0.0024
KH ₂ PO ₄	0.35	L-Histidine	0.05	Riboflavin	0.00016
Other components		L-Histidine•HCl•2H ₂ O	0.02	Thiamine HCl	0.0014
D-glucose	1.00	L-Hydroxyproline	0.02	Vitamine B ₁₂	0.0004
Sodium pyruvate	0.30	L-Isoleucine	0.28		
Sodium lactate	3.00	L-Leucine	0.44		
EDTA (4Na)	0.01	L-Lysine•HCl	0.26		
Glutathione	0.00033	L-Methionine	0.11		
Sodium citrate	0.50	L-Phenylalanine	0.13		
N-acetylglucosamine	1.00	L-Proline	0.10		
Insulin	5 µg/ml	L-Serine	0.15		
Transferrin	5 µg/ml	L-Threonine	0.26		
Sodium selenite	5 ng/ml	L-Tryptophan	0.05		
PVP	1 mg/ml	L-Tyrosine•2Na•2H ₂ O	0.13		
HEPES (pH 7.4)	10.00	L-Valine	0.26		
Dibekacin sulfate	65 µg/ml				
Phenol red	0.026				

* RD: Glucose-free RPMI1640 and Dulbecco's MEM (low glucose), 1:1 (v/v).

cumulation of lipids and good developmental potential, whereas a homogeneous ooplasm indicated aging and low developmental potential. In the present study, the effects of RD, inositol and GlcNAc were marked in oocytes with heterogeneous ooplasm. Although the reason why there is a difference in reactivity to RD, inositol and GlcNAc between heterogeneous ooplasm and homogeneous ooplasm is unknown, a possible reason is that oocytes with heterogeneous ooplasm are developmentally more competent and that this developmental competence is induced by supplementation of RD, inositol and GlcNAc.

In experiment 5, blastocysts produced with the 10% RD-mKSOM/aa chemically defined medium showed high ability of cryopreservation. In cattle, *in vivo* cultured embryos are superior in terms of cryosurvival to their *in vitro* cultured counterparts [39, 40]. At least, the results of experiment 5 indicate that advancement of the IVC system contributes to improvement of blastocyst quality measured in terms of survival after cryopreservation.

The results of experiment 6 showed that blastocysts produced in mKSOM/aa supplemented with 10% or 20% RD had higher ability to develop into live calves (Table 6). Large offspring syndrome has been observed in bovine and ovine offspring following transfer of IVP or cloned embryos [6–8, 41, 42]. Lazzari *et al.* [43] reported that birth weights of calves derived from bovine embryos produced with SOF medium supplemented with serum or BSA were significantly larger than those of calves derived from sheep oviduct cul-

ture, superovulation or artificial insemination. In the present study, the birth weights of calves were normal, indicating that it is important to establish an IVC system with a chemically defined medium.

In conclusion, 20% RD-mKSOM/aa with 70.2 µM myo-inositol, 1 mg/ml PVP, 0.5 mM sodium citrate and 1 mM GlcNAc (Table 7) is an advanced chemically defined medium for bovine embryos. The results of this study should contribute to the improvement of an IVC system for bovine embryos.

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