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Theriogenology 67 (2007) 293–302
Theriogenology
www.theriojournal.com

Improved in vitro bovine embryo development and increased efficiency in producing viable calves using defined media

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Received 6 March 2006; accepted 22 July 2006

Abstract

In this study, we developed a defined culture medium that supported improved in vitro bovine embryo development and calving rate after embryo transfer (ET). In vitro-matured bovine oocytes from abbatoir-derived ovaries from Korean native, HanWoo cattle were fertilized with frozen–thawed spermatozoa and embryos were cultured in two-step culture media. In Experiment 1, embryos were cultured in media supplemented with 8 mg/mL BSA, or 0.1 mg/mL PVA and 8 mg/mL BSA + 2.77 mM myo-inositol or 0.1 mg/mL PVA + 2.77 mM myo-inositol. Although defined culture media containing PVA supported lower developmental competence compared to undefined media (containing BSA; 8% versus 34%, respectively), defined culture media containing 2.77 mM myo-inositol increased rates of blastocyst formation up to 28%. In Experiment 2, the effect of energy substrate (1.5 mM glucose or 1.2 mM phosphate) in PVA–myo-inositol defined culture medium on in vitro embryo development was investigated. Defined culture media containing PVA, myo-inositol and phosphate supported better embryo development to blastocysts compared to medium supplemented with both glucose and phosphate (43% versus 31%). In Experiment 3, the effect of epidermal growth factor (EGF) in PVA + myo-inositol–phosphate two-step culture medium on in vitro embryo development was investigated. Among 0, 1, 10 and 100 ng/mL EGF concentrations, the maximal effect was observed with 10 ng/mL EGF (52% blastocyst formation). In Experiment 4, total cell number and calving rate were compared between defined PVA–myo-inositol–phosphate–EGF medium and undefined medium containing BSA, glucose and phosphate. No differences in total cell number of blastocysts obtained from the two groups were observed, however, the rate of viable offspring production was increased using the defined culture medium, compared to the undefined culture medium. In Experiment 5, the relative abundance of mRNA transcripts [interferon-tau (If-τ), glucose transporter-1 (glut-1) and insulin like growth factor 2 receptor (Igf2r)] were analyzed in blastocysts derived from undefined or defined culture media. Gene expression of If-τ, glut-1 was significantly increased in defined culture medium compared to undefined medium. In conclusion, chemically defined culture media without BSA or FBS improved developmental competence of in vitro cultured bovine embryos and delivery of viable calves after ET.
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Keywords: Culture media; PVA; Embryo development; Embryo transfer; Calving rate

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doi:10.1016/j.theriogenology.2006.07.011

1. Introduction

In order to improve in vitro culture systems for bovine blastocyst production, culture media have been supplemented with a variety of antioxidants, growth factors and/or macromolecules. Among the latter, bovine serum albumin (BSA) and fetal bovine serum (FBS), which are widely used as protein sources for embryo culture media, are complex and undefined mixtures of proteins, growth factors, peptides, etc. Serum and BSA can have a stimulatory effect on embryo growth [1–3]. Thompson [4] clearly demonstrated that BSA played a substantial nutritive role during blastocyst development, especially post-compaction. This is perhaps not surprising given that albumin is the most prevalent extracellular protein in the mammalian reproductive tract. Although BSA had a beneficial effect on embryo development, it is difficult to elucidate specific functions of growth factors or other stimulants because BSA is an undefined mixture of compounds. Chemically defined conditions without serum or serum-derived proteins allow more precise observation of the effects of growth or other embryotrophic factors in a given medium [5–7]. It was demonstrated that chemically defined medium supported bovine zygote development to blastocysts, with varying rates of success [8,9].

In a large field study with in vitro produced (IVP) bovine preimplantation embryos, pregnancy rates following transfer of IVP embryos ranged from about 12 to 50% [10]. Factors shown to influence the maintenance of pregnancy following transfer of IVP embryos include the embryo culture system, embryo quality, embryo evaluator, number of embryos transferred per recipient, synchrony of embryo development with the recipient's day of estrous cycle, transfer technician, fresh versus frozen embryos and heat stress effects on the embryo or recipient [11–15]. Among these factors, embryo quality produced in vitro culture system was considered a critical factor.

Accordingly, the present study was performed to: (1) optimize a BSA- and FBS-free chemically defined culture medium by supplementing it with myo-inositol, EGF, or both, and modifying energy substrates; (2) produce offspring by transferring preimplantation stage embryo obtained using the improved chemically defined culture medium; (3) compare the gene expression patterns of preimplantation embryos produced with the chemically defined and undefined media. The genes were interferon tau (If-τ), insulin-like growth factor 2 receptor (Igf2r), glucose transporter-1 (glut-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2. Materials and methods

2.1. Oocyte collection and in vitro maturation (IVM)

Korean native, *HanWoo* cattle ovaries were collected from a local abbatoir into saline at 35 °C and transported to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) from follicles 2–8 mm in diameter were aspirated using an 18 gauge needle attached to a 10 mL disposal syringe. The COCs with evenly-granulated cytoplasm and enclosed by more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Invitrogen, Carlsbed, CA, USA), supplemented with 10% FBS, 2 mM $NaHCO_3$ (Sigma–Aldrich Corp., St. Louis, MO, USA), and 1% penicillin–streptomycin (v/v). For IVM, COCs were cultured in four-well dishes (30–40 oocytes per well; Falcon, Becton-Dickinson Ltd., Plymouth, UK) for 22 h in 450 μL TCM-199 supplemented with 10% FBS, 0.005 AU/mL FSH (Antrin, Teikoku, Japan), and 1 μg/mL 17β-estradiol (Sigma–Aldrich Corp.) at 39 °C in a humidified atmosphere of 5% CO_2.

2.2. Sperm preparation and in vitro culture of embryos

Motile spermatozoa were selected by a swim-up technique [16]. At 22 h of IVM, oocytes were inseminated (day 0) with 1×10^6 spermatozoa/mL for 18 h in 50 μL/well of tyrode's albumin-lactate-pyruvate (TALP)-IVF medium on a four-well plate. Groups of six or seven zygotes were cultured in 25 μL microdrops of the two-step defined culture medium, as described in Table 1 [17], overlaid with mineral oil (Sigma–Aldrich Corp.). Presumptive zygotes were cultured in early stage medium for the first 5 days post-insemination, and then moved into later stage medium. All incubations were done at 39 °C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Cleavage and blastocyst formation were recorded on days 2 and 7, respectively. Hatching of blastocysts was recorded on day 9.

2.3. Evaluation of blastocyst quality

Randomly selected blastocysts at day 8 were used for differential staining. The cell numbers of blastomeres, inner cell mass (ICM) and trophectoderm (TE) cells in blastocysts were counted after staining, as described [18]. Blastocysts were incubated in 500 μL of BSA-free, HEPES-buffered TCM-199 supplemented with 1% (v/v) Triton X-100 and 100 μg/mL propidium

Table 1
Composition of two-step culture medium for culturing bovine embryos

Componet	Units	Early stage medium	Later stage medium
NaCl	mM	107.70	107.70
KCl	mM	7.16	7.16
$NaHCO_3$	mM	25.07	25.07
KH_2PO_4*	mM	1.19	1.19
Na-lactate	mM	6.60	3.30
Na-pyruvate	mM	0.33	0.11
$CaCl_2$	mM	1.71	1.71
$MgCl_2$	mM	0.49	0.49
HEPES[a]	mM	5	5
Glucose*	mM	1.50	1.50
EAA[b]	%	–	2
NEAA[c]	%	1	1
PVA	mg/mL	0.1	0.1

Supplementation was dependent upon experimental design.
[a] *N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid].
[b] MEM essential amino acids (Gibco 11130-051).
[c] MEM non-essential amino acids (Gibco 11140-050).

iodide for 30 s. When the TE color visibly changed to red and embryos shrank slightly during treatment, blastocysts were incubated at 4 °C overnight in 500 μL of fixative solution (25 μg/mL bisbenzimide in absolute ethanol). The blastocysts were then treated with 99% (v/v) glycerol, mounted onto a glass microscope slide in a droplet of glycerol solution, and cell numbers for each parameter were counted using epifluorescence microscopy. The ICM cell nuclei labeled with bisbenzimide appeared blue, whereas TE cell nuclei labeled with both bisbenzimide and propidium iodide appeared pink.

2.4. Embryo transfer

Embryos were removed from culture medium on day 7 and one or two embryos were transferred nonsurgically to the uterine horn ipsilateral to the corpus luteum of HanWoo and Holstein heifers that had been observed in estrus between day 2 before (+2) and the same day as the IVF of the embryos (0 synchrony). For pregnancy diagnosis, cows with non-returns to estrus after embryo transfer were initially selected, and subsequently confirmed by transrectal palpation at approximately days 35 and 60 of gestation. Pregnant cows were monitored by transrectal palpation at regular intervals thereafter.

2.5. Determination of the relative abundance of gene transcripts in bovine preimplantation embryos derived from the chemically defined and undefined culture conditions

Five blastocysts were washed in three changes of PBS and transferred into 0.2 mL of 4 M guanidium isothiocyanate lysis solution containing 1% β-mercaptoethanol. Total RNA was extracted by thiocyanate extraction and dissolved in 10 μL RNase-free water, as described by Szafranska et al. [19] and was subjected to reverse transcription-polymerase chain reaction (RT-PCR). Reverse transcription was carried out at 37 °C for 60 min. Individual RT reactions (15 μL each) consisted of 5 mM $MgCl_2$, 1× RT buffer, 2.5 μM oligo(dT), 1 mM dNTP, and 50 IU murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotechnologies, Oakville, ON, Canada). Primer sequences, annealing temperature and the approximate sizes of the amplified fragments are listed in Table 2. All primers (If-τ, Igf2r, glut-1 and GAPDH gene) were selected based on the bovine sequence of the respective genes available in the gene database at the National Center for Biotechnology Information. The cDNA (1 μL) was amplified in a 50-μL PCR reaction containing 1.25 units hot start *Taq* polymerase (Qiagen, Hilden, Germany) and its buffer, 1.5 mM $MgCl_2$, 2 mM dNTP, and 25 pmol specific primers. The PCR amplification was carried out for one cycle with denaturing at 95 °C for 15 min, and 35 subsequent cycles with denaturing at 95 °C for 30 s, annealing for 30 s, extension at

Table 2
Details of primers used for reverse transcription-polymerase reaction

Gene	Primer sequences	Annealing temperature (°C)	Cycle number	Fragment size (bp)
GAPDH	5′-CATCACCATCTTCCAGGAGCGAGA, 3′-CCTGCTTCACCACCTTCTTGATGT	55	30	573
If-τ	5′-GCTATCTCTGTGCTCCATGAGATG, 3′-AGTGAGTTCAGATCTCCACCCATC	55	30	359
Igf2r	5′-CGCCTACAGCGAGAAGGGGTTAGTC, 3′-AGAAAAGCGTGCACGTGCGCTTGTC	60	50	293
Glut-1	5′-AGGAGCTGTTCCACCCCCTGGGAG, 3′-TGTGGGTGAAGGAGACTCTGGCTG	59	35	327

72 °C for 30 s and a final extension at 72 °C for 10 min. Ten microliters of PCR products were fractionated on a 1.5% agarose gel, and stained with ethidium bromide. For each pair of gene-specific primers, semilog plots of the fragment intensity as a function of cycle number were used to determine the range of cycle number over which linear amplification occurred, and the number of PCR cycles was kept within this range. The expression level for each gene was determined by densitometric analysis using Gel Doc software (Bio-Rad, Hercules, CA, USA). Relative expression levels of each gene were represented as the ratio of each gene to the GAPDH gene.

2.6. Experimental designs

In Experiment 1, in order to compare embryo developmental competence between undefined media containing BSA or FBS and defined media containing PVA, presumptive zygotes after in vitro fertilization were cultured in two-step culture medium containing either 8 mg/mL BSA, 10% FBS or 0.1 mg/mL PVA. In addition, in order to increase the rate of blastocyst formation in the defined media up to the level expected in FBS- or BSA-containing media, the effect of 2.77 mM myo-inositol on embryo production was evaluated in an undefined medium containing 8 mg/mL BSA or a defined medium containing 0.1 mg/mL PVA.

In Experiment 2, after evaluating the effect of myo-inositol and macromolecules in the defined media, IVP embryos were cultured in defined culture media (containing 0.1 mg/mL PVA and 2.77 mM myo-inositol) with or without 1.5 mM glucose and 1.2 mM phosphate as energy sources.

In Experiment 3, based on the results of Experiment 2, developmental competence of the IVP embryos was evaluated after including 0, 1, 10 or 100 ng/mL of EGF in the defined media (containing 0.1 mg/mL PVA + 2.77 mM inositol + 1.2 mM phosphate).

In Experiment 4, developmental competence between undefined medium (containing 8 mg/mL BSA) and optimized defined medium as determined in Experiment 3 (containing 0.1 mg/mL PVA + 2.77 mM inositol + 1.2 mM phosphate + 10 ng/mL EGF) was compared and at day 8, randomly selected blastocysts produced in the optimized defined medium as determined in Experiment 3 were differentially stained to count the numbers of ICM and TE cells. The cell numbers were compared with those in blastocysts cultured in undefined medium (containing 8 mg/mL BSA) as described in Experiment 1. The efficiency of pregnancy and calving rates was compared following transfer of the embryos produced in the undefined (BSA and FBS) media, the optimized defined media and in vivo.

In Experiment 5, to assess the quality of preimplantation embryos cultured in the optimized defined media as determined in Experiment 4, in terms of gene expression related preimplantation embryo development, the relative abundance of gene expression (GAPDH, If-τ, glut-1 and Igf2r) was evaluated.

2.7. Statistical analysis

Each experiment was replicated at least 10 times and IVF embryos were randomly allocated into each treatment group. The percent values of each experiment were normalized by arcsine transformation prior to any statistical analysis. However, untransformed data are presented in tables and figures. Each value of experimental parameter was subjected to analysis of variance (ANOVA) and protected least significant different (LSD) test using general linear models to determine differences among experimental groups. When a significant treatment effect was found in each experimental parameter, data were compared by the least squares method. Statistical significance was determined where the *P*-value was less than 0.05.

3. Results

3.1. Experiment 1: effect of BSA, FBS, PVA or myo-inositol supplementation of the culture media

The rates of blastocyst (32.4%, 33.6% versus 8.4%, respectively) or hatched blastocyst formation (57.3%, 48.1% versus 18.5%) in the IVP embryos cultured in undefined media (BSA or FBS) were significantly higher than in defined media containing PVA (Table 3). However, no differences in the number of cleaved embryos were observed among the experimental groups. In order to improve the developmental competence of embryos cultured in defined culture medium, the effect of myo-inositol was evaluated (Table 4). Although zygotes developed in chemically defined (PVA) medium showed the least developmental competence, supplementing the defined medium with myo-inositol improved the rates of blastocyst (28.3% versus 9.4%) or hatched blastocyst formation (34.7% versus 23.5%) compared to the defined medium without myo-inositol. Myo-inositol did not improve the rates of blastocyst (32.9% versus 35.8%) or hatched blastocyst formation (47.3% versus 50.8%) in the BSA-containing undefined medium, compared to the control defined medium with PVA.

Table 3
Developmental competence of in vitro produced bovine embryos cultured in 8 mg/mL BSA- or 10% FBS-containing undefined media or 0.1 mg/mL PVA-containing defined media (Experiment 1)

Macromolecules	No. of oocytes cultured	No. of embryos cleaved (%)	No. of blastocysts (%)	Hatched blastocysts/ total blastocysts (%)
FBS	341	253 (74.2)	82 (32.4)[a]	47 (57.3)[a]
BSA	392	321 (81.9)	108 (33.6)[a]	52 (48.1)[a]
PVA	388	321 (82.7)	27 (8.4)[b]	5 (18.5)[b]

Within a column, values without a common superscript letter (a, b) differ ($P < 0.05$). The base medium was the two-step cultured medium described by Lim et al. [17]. Embryos were randomly allocated to each experimental group and the experiments were replicated at least 10 times.

Table 4
Effect of 2.77 mM myo-inositol on developmental competence of in vitro fertilized bovine embryos produced in 8 mg/mL BSA-containing undefined or 0.1 mg/mL PVA-containing defined media (Experiment 1)

Macromolecules	Myo-inositol	No. of oocytes fertilized	No. of embryos cleaved (%)	No. of blastocysts (%)	Hatched blastocysts/ Total blastocysts (%)
BSA	–	206	165 (80.1)	59 (35.8)[a]	30 (50.8)[a]
BSA	+	213	167 (78.4)	55 (32.9)[a]	26 (47.3)[ab]
PVA	–	218	181 (83.0)	17 (9.4)[b]	4 (23.5)[b]
PVA	+	220	173 (78.6)	49 (28.3)[a]	17 (34.7)[ab]

Within a column, values without a common superscript letter (a, b) differ ($P < 0.05$). The base medium was the two-step cultured medium as described by Lim et al. [17]. Embryos were randomly allocated to each experimental group and the experiments were replicated at least 10 times.

3.2. Experiment 2: effect of energy substrate (glucose or phosphate) in chemically defined media

Defined culture medium with both glucose (1.5 mM) and phosphate (1.2 mM) had significantly lower developmental competence compared to that in phosphate-containing defined medium (30.6% versus 43.0%, Table 5). However, developmental competence was not significantly different between the glucose- or phosphate-containing or glucose- and phosphate-free defined media. Regardless of energy substrate modification, the rates of hatched blastocyst formation did not differ.

3.3. Experiment 3: effect of EGF in chemically defined media improved from Experiment 2

Supplementing the defined medium with 10 ng/mL EGF (51.7%) stimulated blastocyst formation compared to 1 ng/mL EGF (40.2%), 100 ng/mL EGF (38.8%) or the control (39.8%, Table 6). Hatched blastocysts rates in 10 ng/mL (63.9%) and 100 ng/mL (62.3%) EGF were significantly higher than in the other groups. However, the number of cleaved embryos did not differ among the experimental groups.

Table 5
Effect of supplementation with or without glucose and/or phosphate on the in vitro developmental competence of bovine embryos cultured in chemically defined medium (Experiment 2)

Media	Energy substrate		No. of oocytes fertilized	No. of embryos cleaved (%)	No. of blastocysts (%)	Hatched blastocysts/ total blastocysts (%)
	Glucose (1.5 mM)	Phosphate (1.2 mM)				
Defined*	+	+	260	196 (75.4)	60 (30.6)[a]	19 (31.7)
	+	–	273	217 (80.1)	82 (37.8)[ab]	34 (41.5)
	–	+	271	214 (78.4)	92 (43.0)[b]	35 (38.0)
	–	–	262	208 (79.4)	81 (38.9)[ab]	26 (32.1)

Within a column, values without a common superscript letter (a, b) differ ($P < 0.05$). Embryos were randomly allocated to each experimental group and the experiments were replicated at least 10 times.

* The base two-step medium [17] was supplemented with 0.1 mg/mL PVA and 2.77 mM myo-inositol.

Table 6
Effect of various concentrations of epidermal growth factor (EGF) on preimplantation development of bovine embryos derived from in vitro fertilization (IVF) (Experiment 3)

Media	EGF (ng/mL)	No. of oocytes fertilized	No. of embryos cleaved (%)	No. of blastocysts (%)	Hatched blastocysts/ total blastocysts (%)
Defined*	0	216	166 (76.8)	66 (39.8)[a]	29 (43.9)[a]
	1	213	174 (81.7)	70 (40.2)[a]	34 (48.6)[a]
	10	261	209 (80.1)	108 (51.7)[b]	69 (63.9)[b]
	100	228	178 (78.1)	69 (38.8)[a]	43 (62.3)[b]

Within a column, values without a common superscript letter (a, b) differ ($P < 0.05$). Embryos were randomly allocated to each experimental group and the experiments were replicated at least 10 times.
* The base two-step medium [17] was supplemented with 0.1 mg/mL PVA + 2.77 mM myo-inositol + 1.2 mM phosphate.

Table 7
Comparison of preimplantation developmental competence and cell number (ICM and TE) of bovine embryos cultured in undefined (PVA) or defined (BSA) media (Experiment 4)

Media	No. of oocytes cultured	No. of embryos cleaved (%)	Percentage		ICM	TE	Total	ICM/ total
			Blastocysts/ cultured oocytes	Blastocysts/ cleaved embryos				
Undefined[a]	589	445 (75.6)[c]	24.4[c]	32.4[c]	27.8 ± 13.0	115.5 ± 59.8	143.3 ± 65.6	0.21 ± 0.1
Defined[b]	594	504 (84.8)[d]	40.7[d]	48.0[d]	28. 1 ± 9.6	114.3 ± 54.9	142.4 ± 56.1	0.21 ± 0.09

The data represent mean ± S.E.M. of cell counts on 30 (undefined) or 35 (defined) blastocysts. Within a column, values without a common superscript letter (c, d) differ ($P < 0.05$).
[a] The base two-step medium [17] was supplemented with 8 mg/mL BSA as described in Table 1.
[b] The base two-step medium was supplemented with 0.1 mg/mL PVA + 2.77 mM myo-inositol + 1.2 mM phosphate + 10 ng/mL EGF.

3.4. Experiment 4: assessment of embryo quality by blastocysts formation, cell allocation to ICM and TE, and monitoring pregnancy rate after embryo transfer

As a result of comparing developmental competence on the blastocysts formation, in improved chemically defined medium it was significantly increased (Table 7). In order to evaluate embryo quality as an embryo viability indicator, differential staining was performed to count the numbers of total cells, ICM and TE cells in blastocysts cultured in undefined or defined culture media determined in Experiments 1–3. In the two groups of media, the numbers of total cells, ICM and TE cells did not differ significantly (Table 7). Following transfer of embryos derived from use of the improved defined medium, the proportions of pregnant recipients (at day 35, 55.4% versus 27.0% or 37.1%; at day 60, 48.2% versus 24.3% or 22.8%) and calving rates (44.6% versus 18.9% or 17.4%) were higher than those for undefined (FBS or BSA) media. Pregnancy rates at days 35 (55.4% versus 61.5%) and 60 (48.2% versus 58.6%)

Table 8
Pregnancy and calving rates with IVP bovine embryos derived from undefined (BSA and FBS) media, defined (PVA) medium and in vivo (Experiment 4)

Media	No. of embryos transferred	No. of pregnant cows (%)		No. of calves (%)
		Day 35	Day 60	
Undefined (FBS)[a]	37	10 (27.0)[d]	9 (24.3)[d]	7 (18.9)[d]
Undefined (BSA)[b]	35	13 (37.1)[d]	8 (22.8)[d]	6 (17.4)[d]
Defined[c]	56	31 (55.4)[e]	27 (48.2)[e]	25 (44.6)[e]
In vivo	174	107 (61.5)[e]	102 (58.6)[d]	97 (55.7)[e]

Within a column, values without a common superscript letter (d, e) differ ($P < 0.05$).
[a] The base two-step medium [17] was supplemented with 10% FBS as described in Table 2.
[b] The base two-step medium was supplemented with 8 mg/mL BSA as described in Table 2.
[c] The base two-step medium was supplemented with 0.1 mg/mL PVA + 2.77 mM myo-inositol + 1.2 mM phosphate + 10 ng/mL EGF.

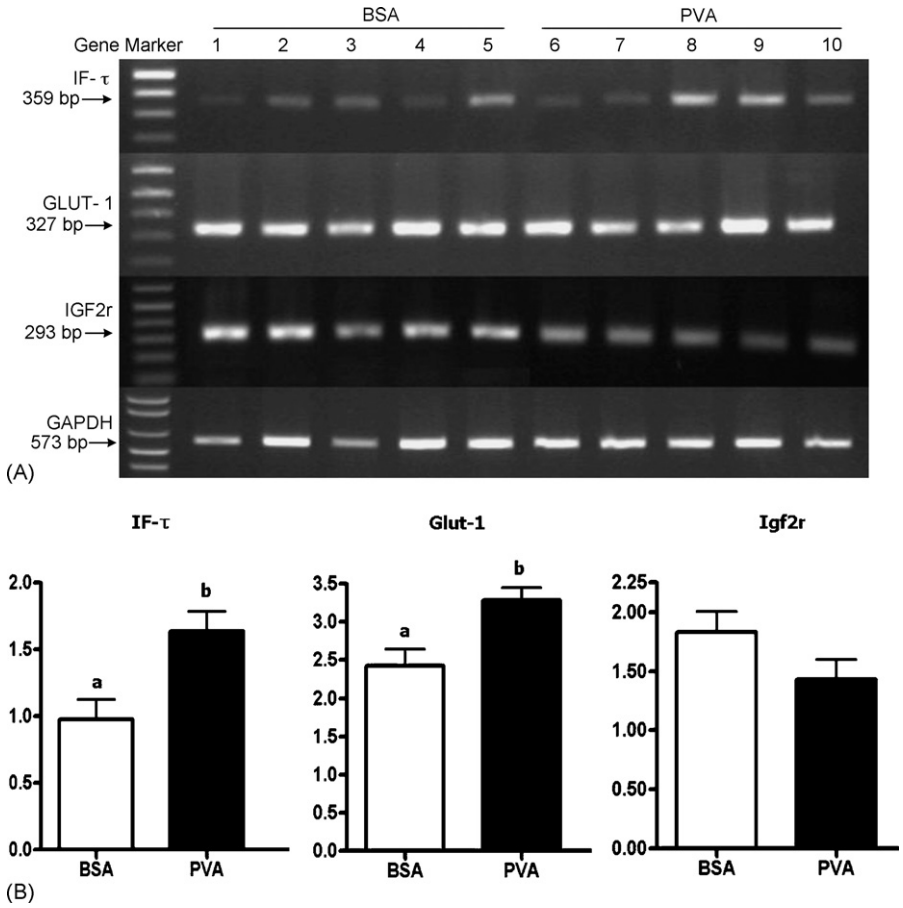

Fig. 1. Representative gel photographs (A) and relative mRNA abundance graph (B) of the mRNA expression of GAPDH, Interferon-tau (If-τ), glucose transporter-1 (glut-1), insulin-like growth factor 2 receptor (Igf2r) in preimplantation bovine embryos derived from in vitro fertilized. The undefined medium contains 8 mg/mL BSA (lanes 1–5) and the optimized defined medium contains 0.1 mg/mL PVA + 2.77 mM myo-inositol + 1.2 mM phosphate + 10 ng/mL EGF (lanes 6–10). Day 8 blastocysts were chosen for analyzing mRNA transcripts for each gene, If-τ, glut-1, and Igf2r. [a,b] Difference between the two groups ($P < 0.05$).

and calving rates (44.6% versus 55.7%) after transfer of IVP embryos cultured in the defined medium were similar compared to the rates after transfer of in vivo embryo (Table 8).

3.5. Experiment 5: relative abundance of mRNA transcripts in blastocysts derived from undefined and defined media

Representative gel photographs of a semi-quantitative RT-PCR assay of the analyzed gene transcripts in day 8 blastocysts are shown in Fig. 1. Relative abundance of transcripts of If-τ and glut-1 was significantly higher in blastocysts derived from the improved chemically defined media. The level of Igf2r transcript expression was in between the expression levels in undefined and defined media.

4. Discussion

Undefined culture media have been used for embryo culture because protein supplementation is critical for embryo development and because better embryo development was frequently observed in undefined media compared to defined media. However, undefined in vitro culture media that were supplemented with BSA and FBS contained a potential source of viral or other infectious pathogenic agents that have infected embryos [20,21]. To overcome this problem, in this study we showed that by modifying and supplementing defined media, embryo developmental competency and calving rates were improved compared to undefined media.

Compared to undefined culture media, the defined media containing PVA had the least ability to support embryo development (Table 3). In order to increase

blastocyst formation rates in defined media, myo-inositol, an isomer of a C6 sugar alcohol that belongs to the Vitamin B complex group, was added. Some studies have shown that myo-inositol is incorporated into phospho-inositides and inositol phosphates in rabbit embryos [22] and can enhance bovine in vitro embryo developmental competence [23]. In the present study, there was higher developmental ability of embryos cultured in chemically defined medium with myo-inositol. However, supplementation of myo-inositol in undefined media containing BSA did not increase embryo developmental competence. It is likely to be present in commercial BSA preparations in minute concentrations but sufficient for biological action, thus accounting for the lack of an additional effect in medium containing BSA.

Supplementing culture media with myo-inositol improved embryo development slightly up to preimplantation stages, but development was still lower than in undefined media containing BSA. To increase development in defined media, by modifying energy substrate composition, i.e., adding glucose and phosphate, blastocyst rates were improved. Glucose used to be routinely used in embryo culture systems. Embryo metabolism is biphasic; at the time of embryonic activation, energy metabolism switches from the use of lactate and pyruvate via the Krebs cycle and oxidative phosphorylation to the primary use of glucose via the Embden–Meyerhof pathway [24,25]. In that regard, the absence of glucose in later preimplantation stages caused a decrease in the blastocyst total cell number [25]. Hence, our results can be explained by the availability of glucose for late preimplantation embryo development.

It was noteoworthy that glucose also inhibited embryo development. These contradictory results are explained by a biphasic effect of glucose: lower concentrations (<3 mM) stimulated early cleavage stage embryo development, whereas high concentrations (3–5 mM) inhibited early embryo development. The situation is complicated by observations that phosphate exacerbates the inhibitory effects of glucose on in vitro embryo development. Phosphate stimulates the activity of the glycolytic pathway, and as a result causes a decrease in ATP production via mitochondria respiration (TCA cycle and oxidase phosphorylation). Kim et al. [26] reported that without phosphate, glucose alone had no detrimental effect on early embryonic development. In contrast, we showed that in culture media containing glucose, phosphate inhibited embryo development to the blastocyst stage (Table 5). This result resembles that obtained by Barnett and Bavister [27] and Biggers and McGinnis [28]. However, according to this study, culture media which contained the only glucose, phosphate, or neither, did not have any significant difference.

Growth factors such as growth hormone (GH) and EGF may be viewed as local regulators involved in the subtle coordination of cellular proliferation and differentiation. In that regard, EGF stimulates distinct cellular functions, which suggests a possible effect on early development of mammalian embryos [29]. In our study, 10 ng/mL EGF was more beneficial on the IVP embryo development than other concentrations examined (Table 6). Mtango et al. [30] reported that addition of EGF to the culture medium resulted in higher developmental capacities than did other growth factors used, after the blastocysts were vitrified, thawed and cultured. Consequently, by this study, bovine embryos derived IVF had higher cleavage and blastocysts formation rates in the chemically defined medium which was improved thorough Experiments 1–3 (Table 7).

Prior to transfer of embryos into recipients, embryo cell numbers are considered the most important markers of embryo development competence. Cell numbers were evaluated in preimplantation stage embryos derived using undefined (BSA) and improved defined media. The cell numbers of preimplantation stage embryos derived using improved chemically defined media in our study should be compared with those from embryos in undefined media containing BSA as used in many countries and guaranteed validated as an IVP system. The cell numbers of ICM and TE from both media groups were not significantly different (Table 7), indicating that preimplantation stage embryos produced using defined media in this study were not inferior to those from undefined media. Transfer of blastocysts produced in defined medium into the uterine tubes of recipients showed increased pregnancy and calving rates similar to those resulting from transfer of in vivo produced embryos (Table 8).

For further comparison of preimplantation stage embryos derived from undefined and defined media, we analyzed the pattern of mRNA expression. In that regard, GAPDH, selected as a control, had a similar pattern to β-actin and tubulin in all preimplantation embryos. The relative patterns of expression of component genes of the IGF system, GLUT, and If-τ, a gene critical for the establishment of pregnancy in cattle, have been investigated in preimplantation stage bovine embryos [31–35]. Expression of If-τ and glut-1 was increased in preimplantation stage embryos derived from defined culture medium compared to undefined medium. It is well known that If-τ is secreted

by trophectoderm and is related to the implantation success of bovine blastocysts by inhibiting Cox. Inhibition and deficiency of cyclooxygenase (Cox) which generates prostaglandin via the Cox pathway, causes abnormalities in ovulation, fertilization, implantation and decidualization [36,37]. A lower expression of glut-1, which is important for glucose uptake for placental utilization, may affect glucose uptake and metabolism and embryo development. Morita et al. [38] verified that glucose incorporation and glut-1 transcripts were reduced in IVP mouse blastocysts. In this study, expression of Igf2r transcripts was not significantly different in preimplantation stage embryos derived from undefined versus defined culture media. Both Igf2 and Igf2r are imprinted genes that play important roles in preimplantation development and Igf2r is the receptor corresponding to the signal pathway for growth factor regulation of fetal growth. It was shown that levels of Igf2 mRNA were significantly elevated in bovine fetuses originating from IVP embryos compared with their in vivo counterparts [39]. As relative amounts of various transcripts are associated with protein supplementation of the culture medium [32], we may postulate that this differential mRNA expression pattern in the two culture media has an influence on embryo implantation, pregnancy maintenance and fetal growth.

In conclusion, we inferred that lower developmental competence of IVP embryo in chemically defined media was improved by supplementation with myo-inositol and EGF, and modification of energy substrate composition. Although the cell numbers of ICM and TE did not differ, after embryo transfer the preimplantation stage embryos developed in defined medium supported higher pregnancy and calving rates. Moreover, the relative abundance of mRNA transcripts levels for If-τ, glut-1, and Igf2r were different in the blastocysts derived from undefined versus defined media, which might have accounted for the differences in embryo implantation, fetal development and delivery of calves.

Acknowledgements

This study was supported by grants from the Ministry of Science and Technology (Biodiscovery and Top Scientist Fellowship), and the Biogreen 21-1000520030100000. We thank Dr. Barry D. Bavister, University of New Orleans, for his great editing of the manuscript. The authors are grateful for a graduate fellowship provided by the Ministry of Education, through the BK21 program.

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