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Comparison of Two Different Serum-free Media for *In Vitro* Culture of Bovine Embryos

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ABSTRACT

The aim of the present study was to compare two different serum-free media, modified synthetic oviduct fluid (mSOF) and modified potassium simplex optimization medium (mKSOM) containing 20% RD (RPMI1640 + DMEM, 1:1 v/v) (RD-mKSOM), for *in vitro* culture (IVC) of bovine embryos. After *in vitro* maturation and fertilization, the presumptive zygotes were cultured in two different serum-free conditions for 7 days and 9 days to evaluate blastocyst formation and hatching, respectively. Serum supplemented conventional CR2 medium was used as control. After 7 day of culture, there was no significant difference in cleavage and blastocyst formation rates among three groups (mSOF, 59.3 and 30.1%; RD-mKSOM, 65.0 and 41.5%; control, 51.6 and 38.0%, respectively). Hatching rate was significantly higher in control (69.0%) than other experimental groups (mSOF, 22.0%; RD-mKSOM, 39.5%) (P<0.0001 and P<0.001, respectively). Although both serum-free conditions showed lower hatching rates than serum-added control, in serum-free groups, RD-mKSOM showed significantly higher hatching rate than mSOF (P<0.001). In addition, one-step using RD-mKSOM may facilitate IVC procedure than two-step culture system. In conclusion, the results indicate that one-step RD-mKSOM is more suitable defined culture system for IVC of bovine embryos than two-step mSOF.

(Key words : serum-free, *in vitro* culture, bovine embryo)

INTRODUCTION

There have been great improvement in *in vitro* production (IVP) system for bovine embryos, however, the rate of IVP-derived embryos to the blastocyst stage is limited to about 12~50% (Peterson and Lee, 2003). Bovine embryos are often cultured in serum-supplemented medium because serum contains beneficial substances for embryonic development such as growth factors and chelators of heavy metals. However, serum had a biphasic effect on bovine embryo development, including inhibition of the first embryonic cell division (Van Langendonckt *et al.*, 1997) and morphological deviations (Abe *et al.*, 1999). Furthermore, serum is believed to contribute to the large offspring syndrome in sheep and cattle (Holm *et al.*, 1996). Therefore, a chemically defined culture system should be provided to produce normal calves when the embryos are derived from IVP procedure. Although there are many options for *in vitro* culture (IVC) of bovine embryos, various chemically defined medium are still investigated for optimal conditions.

To obtain high quality embryos from IVP process, various culture systems has been developed such as synthetic oviductal fluid (SOF) (Tervit *et al.*, 1972; Krisher *et al.*, 1999), potassium simplex optimization medium (KSOM) (Erbach *et al.*, 1994; Liu and Foote, 1995) and *in vitro* development 101 (IVD101) (Abe and Hoshi, 2003) as one-step culture systems, and G1.2/G2.2 (Gardner, 1994), G1/G2 (Krisher *et al.*, 1999), eSOF-98/LSOF-98 (Thompson, 2000), Early-SOF/Late-SOF (Lim *et al.*, 2007), KSOM/SOF (Nedambale *et al.*, 2004, 2006) and SOF-A/SOF-B (Rho *et al.*, 2007) as two-step culture systems.

Since embryos *in vivo* move from the oviduct to the uterus where the secretions and gas atmosphere markedly differ, use of two-step culture system have been preferred to one-step system because the composition of the first and second steps in two-step system differs by the addition or omission of a

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particular substances (Chatot *et al.*, 1998; Gardner *et al.*, 1998). On the other hand, Momozawa and Fukuda (2011) claimed that modified KSOM with amino acids (mKSOM) with 20% RD (RPMI1640 + Dulbecco's MEM, 1:1 v/v), 70.2 μM myo-inositol and 1 mM N-acetylglucosamine (GlcNAc) (RD-mKSOM), could improve embryonic development and pregnancy rates in cattle.

In the present study, we compared two different serum-free defined culture systems, two-step (early-SOF and late-SOF as mSOF) and one-step (RD-mKSOM) culture systems to select optimal culture condition for IVP of bovine embryos.

MATERIALS AND METHODS

1. Chemicals

All inorganic and organic compounds were purchased from Sigma-Aldrich Korea (Yong-in, Korea) unless indicated in the text.

2. Oocyte Recovery and *In Vitro* Maturation (IVM)

Korean native, HanWoo cattle ovaries were collected at a local slaughterhouse and transported to the laboratory within 2 ~3 h in saline at 25~35℃. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3 to 8 mm follicles using an 18 gauge hypodermic needle attached to a 10 ml disposable syringe.

After washing three times in HEPES-buffered Tyrode's solution, the COCs that were enclosed by more than three layers of compact cumulus cells and an evenly granulated ooplasm were selected for IVM. Selected COCs were cultured in Nunc^{TM} 4-well culture dishes (Nunc, Roskilde, Denmark) containing 500 μl of IVMD101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) supplemented with 10 μg/ml follicle-stimulating hormone (FSH)-P (Folltrophin-V; Vertrepharm, London, UK) and 10% fetal bovine serum (FBS; Gibco-BRL, NY, USA) under warmed and gas-equilibrated mineral oil for 20~22 hat 38.5℃, 5% CO_2.

3. Sperm Preparation and *In Vitro* Fertilization (IVF)

After IVM, the matured oocytes were washed three times washing in IVF100 medium (Research Institute for the Functional Peptides, Yamagata, Japan), and placed into 45 μl drops of IVF100 medium under mineral oil. A frozen semen straw from the HanWoo cattle was rapidly thawed in a 38℃ water bath and the semen was diluted with Tyrode's albumin lactate pyruvate (TALP) solution and washed twice in a same medium by centrifugation at 503 × g for 5 min. The final sperm pellet was resuspended in IVF100 medium and the number of spermatozoa was counted using a hemocytometer then adjusted to 1.0×10^7/ml by further dilution. A 5 μl aliquot of the sperm suspension were introduced to a 45 μl droplet of IVF100 medium containing matured oocytes. Incubation was carried out at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 6 h.

4. IVC

Culture media used this experiment was that media was RD-mKSOM for one-step culture (Momozawa and Fukuda, 2011) and mSOF for two-step culture (Lim *et al.*, 2007). The components of culture media display in Table 1.

At the end of the insemination period, groups of 10 oocytes were stripped free from cumulus cells, and transferred into 50 μl of one of IVC media above. For two-step culture including control, the medium was replaced to second one after 3 day of IVC. The incubation was conducted at 38.5℃ under the 5% CO_2, 5% O_2, and 90% N_2 humidified at mosphere for 7 to 9 days. Following 24 h of culture, the presumptive zygotes which did not undergo cleavage were removed and at this time, the IVC medium was replaced with fresh medium according to each experimental group. The blastocyst formation and hatching rates were recorded, on 7 and 9 days after IVF, respectively.

5. Statistical Analysis

All the experiments of treated group were repeated 3~4 times. Results subjected to statistical analyses were expressed as mean ± SD. Data were subjected to one-way ANOVA (PRISM software version 4.0; GraphPad, San Diego, USA). Difference at $P<0.05$ was considered significant.

RESULTS

As shown in Fig. 1(A), (B), there was no significant difference in cleavage and blastocyst formation rates among three groups (mSOF, 59.3 ± 14.8 (166/280) and 30.1 ± 13.2 (50/166) %; RD-mKSOM, 65.0 ± 14.1 (195/300) and 41.5 ± 14.8 (81/195) %; control, 51.6 ± 15.1 (129/250) and 38.0 ± 17.4 (49/129) %, respectively).

As shown in Fig. 1(C), hatching rate was significantly higher

Table 1. Composition of media for bovine embryo culture

Components	Unit	Serum-added		Serum-free		
		Two-step CR2		One-step RD-mKSOM	Two-step mSOF	
		Day 0～3	Day 4～9		Day 0～3	Day 4～9
$CaCl_2 \cdot 2H_2O$	mM			1.71		
$CaCl_2$	mM				1.8	1.8
KCl	mM	3.1	3.1	2.5	6.55	6.55
KH_2PO_4	mM			0.35	0.24	0.24
$MgSO_4 \cdot 7H_2O$	mM			0.2		
NaCl	mM	114.7	114.7	95	107.63	107.63
$NaHCO_3$	mM	26.2	26.2	25	25	25
Na-lactate	mM			10	6.60	3.30
Na-pyruvate	mM	0.4	0.4	0.2	0.33	0.11
$MgCl_2$	mM				0.49	0.49
$MgSO_4$	mM				0.86	0.86
L-Glutamine	mM	1	1	1	0.5	
L-Cysteine	mM			0.03807		0.038
Glutathione	mM			0.00033		0.199
Glycine	mM			3	1.492	1.492
EDTA	mM			0.01		
HEPES	mM			10	5	5
Glucose	mM			1.0	1.50	2.775
ITS	μl/ml	0.02	0.02	0.02	0.02	0.02
Myo-Inositol	μM/ml			70.2	-	100
N-Acetylglucosamine	mM			1	1	1
Sodium citrate	mM			0.5	2.890	0.5
Hypotaurine	mM				0.5	
EAA	%	1	1	1	-	2
NEAA	%	0.5	0.5	2	1	1
PVA	mg/ml			0.1	0.1	0.1
RPMI1640 + DMEM	% (1:1 v/v)			20		
FBS	%		0.5			
BSA	mg/ml	0.3	0.15			

in control (69.0 ± 11.0 (33/49) %) than other experimental groups (mSOF, 22.0 ± 11.2 (11/50) %; RD-mKSOM, 39.5 ± 12.2 (32/81) %) (P<0.001 and P<0.01, respectively). Although both serum-free conditions showed lower hatching rates than serum-added control, in serum-free groups, RD-mKSOM showed significantly higher hatching rate than mSOF (P<0.01).

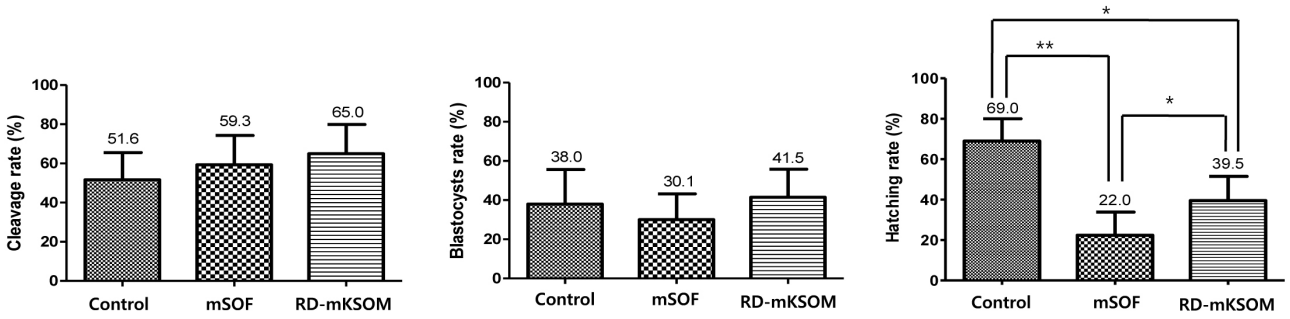

Fig. 1. Comparison of two different serum-free media (RD-mKSOM, serum-free one-step culture system; mSOF, serum-free two- step culture system) and serum-added control by evaluation of cleavage (A), blastocyst formation (B) and hatching (C) rates. Data are expressed as the mean ± SD. Five replicates. * P<0.01, ** P<0.001.

DISCUSSION

Analysis of embryonic physiology and metabolism shows that the requirements for exogenous substrates change with development. Therefore, two-step culture system may be theoretically more optimal for culture of developing embryos than single step one. However, in present study, one-step system (RD-mKSOM) showed higher capacity to support hatching of bovine blastocysts when compared to two-step mSOF system. RD-mKSOM one-step culture system was superior to mSOF two-step one in supporting the development of bovine embryos and was also advantageous to increase the hatching rate of blastocyst derived from IVP. These contradictory results can be thought to reflect optimal condition of RD-mKSOM for culturing bovine embryos in present study.

Momozawa and Fukuda (2011) reported that the composition (20% RD, 70.2 μM myo-inositol and 1 mM GlcNAc) of RD-mKSOM leads to beneficial effects on embryonic development. The key substance of 20% RD is vitamins. Vitamin C and vitamin E have protective effects on embryonic development. Experiments with supplementation of vitamin C to the medium in the presence or absence of vitamin E strongly suggest that vitamin C is active against oxidation reactions via the protection of vitamin E (Tareq *et al.*, 2012; Córdova *et al.*, 2010). Olson and Seidel (2000) reported that vitamin E improved the blastocyst rate of IVP bovine embryos. Similar results are also found in the reports of Long *et al.* (2000) and Xu *et al.* (2004). Myo-inositol in bovine embryo is incorporated into phosphoinositides and inositol phosphates, during the critical stage of blastocyst formation (Hynes *et al.*, 2000). In addition, GlcNAc widely exists as a component of glycoprotein and glycosaminoglycan in both animals and plants cells (Nancarrow and Hill, 1995). This glycoprotein possibly acts as a facilitator and its pure form may be beneficial for IVP of mammalian embryos. However, both factors of Myo-inositol and GlcNAc are contained in mSOF and RD-mKSOM, and they are not critical factors for showing superiority of RD-mKSOM. Glycine is the most abundant amino acids in bovine oviductal and uterine fluids (Moore and Bondioli, 1993). Takahashi and Kanagawa (1998) suggested that the concentration of glycine in mSOF might to be adjusted to 5 mM or less for optimal condition for bovine embryos when essential and non-essential amino acids are also combined in the medium, as in the similar condition of bovine oviductal fluid. Therefore, low concentration of glycine in mSOF (1.5 mM) may be one of the causes to decrease (or to delay) hatching rate of blastocysts than that of blastocysts cultured in RD-mKSOM (3 mM glycine) in the present study.

In present study, concentration of glucose was higher in late-mSOF media compared with RD-mKSOM. Pre-implantation embryos are known to gain their energy by oxidative phosphorylation, i.e. the oxidation of pyruvate and amino acids, and embryos in compaction stage only can switch to glycolysis (De La Torre-Sanchez *et al.*, 2006). High levels of glucose in culture medium have been considered as negative factors to developmental arrests in IVP embryos for generating reactive oxygen species (ROS) (Iwata *et al.*, 1998). Glucose inhibits hypoxanthine phosphoribosyl transferase (HPRT) activity, and resulted in the production of ROS via xanthine oxidase (Guérin *et al.*, 2001). IVP embryos using glucose inappropriately at

post-compaction stages showed improper developmental feature (Thompson and Peterson, 2000). Too high concentration of glucose at post-compaction stage induces excessive production of lactic acid, which give rise to metabolic block with poor entry into Krebs' cycle and block at glucose-6-phosphate isomerase leading to useless accumulation of glycogen, and finally lead to toxic effect on the embryos (De La Torre-Sanchez *et al.*, 2006). Although two-step mSOF system are convenient for adjust of the concentration of glucose at pre- and post-compaction stage, low level of glucose contained in RD-mKSOM may be beneficial for the development of bovine embryos *in vitro*. In addition, one-step using RD-mKSOM may facilitate IVC procedure than two-step culture system.

In conclusion, the present results demonstrated that one-step RD-mKSOM is more suitable defined culture system for IVC of bovine embryos than two-step mSOF.

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