

Effects of Fractions of Bovine Follicular Fluid and Fetal Bovine Serum as Supplements to Maturation Medium on In Vitro Development of In Vitro Fertilized Bovine Embryos

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Abstract: The purpose of this study was to examine the effects of different fractions of bovine follicular fluid (bFF) and fetal bovine serum (FBS) as maturation medium supplements on development to the blastocyst stage of bovine oocytes matured and fertilized *in vitro*. Three bFF fractions obtained by ultracentrifugation were designated as the 1st, 2nd and 3rd fractions, and four FBS fractions, also obtained by ultracentrifugation, were designated as the 1st, 2nd, 3rd and 4th fractions. Cumulus-oocyte complexes (COCs) were divided into two groups: those with homogeneous ooplasm and those with heterogeneous ooplasm. COCs were cultured in basic maturation medium with a bFF fraction or FBS fraction. In oocytes with homogeneous ooplasm, the rates of development to the blastocyst stage with bFF-1st and bFF-2nd were significantly higher than that with bFF. In oocytes with heterogeneous ooplasm, the rate of development to the blastocyst stage with bFF-2nd was significantly higher than that with bFF. In oocytes with homogeneous ooplasm, the rate of development to the blastocyst stage with FBS-1st was significantly lower than that with FBS. In oocytes with heterogeneous ooplasm, the rate of development to the blastocyst stage with FBS-3rd was significantly higher than that with FBS. The results indicate that bFF-2nd and FBS-3rd, obtained by ultracentrifugation, are effective maturation medium supplements, as they promoted the development of high-quality matured bovine oocytes.

Key words: Bovine oocyte, In vitro maturation, Fraction, Follicular fluid, Fetal bovine serum

Introduction

Oocytes acquire normal fertilizability and developmental ability during maturation. The developmental capacity of embryos derived from *in vitro* matured *in vitro* fertilized oocytes is a much more definitive way to assess the normality of maturation [1]. In cattle, 10–20% (v/v) fetal bovine serum (FBS) is recommended as an oocyte maturation medium supplement [2]. Bovine follicular fluid (bFF) is also an effective maturation medium supplement [3–5]. However, the rates of development to the blastocyst stage of bovine oocytes matured and fertilized *in vitro* are about 30–40% [6–10]. The developmental ability of *in vitro* matured oocytes is lower than that of oocytes matured *in vivo* [11–13]. Therefore, the conditions of *in vitro* maturation (IVM), especially the maturation medium and its supplements, needs to be improved. In maturation *in vitro* of porcine oocytes, some studies have indicated that supplementation of bFF [14] or porcine follicular fluid (pFF) [14–16] to the maturation medium improved cumulus expansion, nuclear maturation, fertilization and development. In addition, Dean *et al.* [17] found that only the ultracentrifuged pFF fraction 1 (top fraction) contained a substance(s) which promoted cumulus expansion and male pronucleus formation in porcine oocytes matured and fertilized *in vitro*. Isolation of the promoting factor(s) in bFF or FBS would contribute to improvement of the IVM system for bovine oocytes. Therefore, we examined the effects of fractions of bFF and FBS obtained by ultracentrifugation maturation medium supplements on the nuclear and cytoplasmic maturation of bovine oocytes *in vitro*. The objective of this study was to improve the

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developmental competence of bovine oocytes to obtain a high frequency of embryo development to the blastocyst stage.

Materials and Methods

Ultracentrifugation

Cattle ovaries obtained at a local abattoir were wrapped in a paper towel soaked in 0.9% saline and transported to the laboratory at about 30°C. Bovine follicular fluid was aspirated from 2–5 mm follicles of ovaries into a 5-ml disposable syringe with a 21-gauge needle. Pooled follicular fluid was then centrifuged at $1,500 \times g$ for 20 min at room temperature to remove debris and blood cells. The supernatant of bFF was collected and stored at –20°C. Fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah, USA, cat. No. A-1115-L) stored at –28°C was heat-treated at 56°C for 30 min just before ultracentrifugation. Ultracentrifugation (KONTRON, Fixed-Angle Rotor TFT 80.13) at $220,000 \times g$ for 48 h at 4°C was used to fractionate 10.5 ml of bFF or FBS. Three bFF fractions were designated as the 1st (top, about 81%), 2nd (about 12%) and 3rd (bottom, about 7%) fractions. Four FBS fractions were also designated as the 1st (top, about 85.7%), 2nd (about 5.7%), 3rd (about 5.7%) and 4th (bottom, about 2.9%) fractions.

IVM of oocytes

Ovaries obtained at a local abattoir were wrapped in a paper towel 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 non-polar 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) and heterogeneous (unevenly granulated to various degrees) [18], and cultured for 24 h in 50- μ l droplets of maturation medium under mineral oil (Squibb and Son, Inc., 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 basic maturation medium was 12.5 mM HEPES-buffered TCM-199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.3 mM Na pyruvate, 0.5 IU/ml hFSH (containing 2.31 IU/ml

LH, Biogenesis, England, UK), 1 μ g/ml estradiol-17 β (Sigma, Chemical Co., St. Louis, MO, USA), 3 mg/ml polyvinylpyrrolidone (PVP, Mr. 40,000, Sigma) and 130 μ g/ml dibekasin sulfate (Meiji-Seika, Tokyo, Japan).

Sperm preparation and in vitro fertilization

The basic fertilization medium was modified BO medium [19], hereafter referred to as Fertilization Medium (FM), which consisted of 113.45 mM NaCl, 4.02 mM KCl, 2.25 mM CaCl₂·2H₂O, 0.83 mM NaH₂PO₄·2H₂O, 0.52 mM MgCl₂·6H₂O, 25 mM NaHCO₃, 13.90 mM glucose, 12.5 mM HEPES (pH 7.4), 130 μ g/ml dibekasin sulfate, 10 mg/ml BSA (fraction V, Wako Pure Chemical Inc., Osaka, Japan) and 2×10^{-4} % (w/v) phenol red [18]. BSA-free FM supplemented with 10 mM caffeine (caffeine sodium benzoate, Sigma) was used for sperm washing and sperm treatment with Ca ionophore A23187. One straw (0.5 ml/straw) of frozen bull semen was thawed in a water bath at 39°C for 20 sec, suspended in 3 or 5 ml of sperm-washing medium in a test tube, washed twice by centrifugation at $700 \times g$ for 5 min, and resuspended in 1.5 or 2 ml of sperm-washing medium. The spermatozoa were treated with 0.1 μ M Ca ionophore A23187 (Calbiochem-Behring, La Jolla, CA) for 60 sec [20]. The sperm suspension was immediately diluted 1:1 with FM containing 20 mg/ml BSA supplemented with 2 units/ml heparin (heparin sodium salt, Wako). The spermatozoa were preincubated for 3 h in a CO₂ incubator (5% CO₂, 95% humidified air, 39°C). After the 3-h preincubation, the concentration of progressively motile sperm was determined in a Makler counting chamber. In experiment 1, a small amount of sperm suspension was introduced into a 50- μ l droplet of FM supplemented with 1 unit/ml heparin under mineral oil in culture dishes to obtain 120 sperm/ μ l (for the oocytes with homogeneous ooplasm) and 100 sperm/ μ l (for the oocytes with heterogeneous ooplasm). In experiment 2, a small amount of sperm suspension was introduced into a 200- μ l droplet of FM supplemented with 1 unit/ml heparin under mineral oil in a culture dish to obtain 160 sperm/ μ l (for the oocytes with homogeneous ooplasm) and 120 sperm/ μ l (for the oocytes with heterogeneous ooplasm). Subsequently, COCs at 24 h after IVM were introduced into the fertilization droplets containing the spermatozoa and the oocytes and spermatozoa were co-incubated for 6 h in a CO₂ incubator (5% CO₂, 95% humidified air, 39°C).

In vitro embryo culture (IVC)

The embryo culture medium used in experiment 1

was 12.5 mM HEPES-buffered modified TCM-199 (glucose-free) supplemented with 0.3 mM sodium pyruvate, 3.0 mM sodium lactate, 5 μ g/ml insulin-5 μ g/ml transferrin-5 ng/ml sodium selenite (ITS, media supplement, Sigma), 130 μ g/ml dibekasin sulfate and 10 mg/ml BSA [21]. In experiment 2, the embryo culture medium was modified KSOM/aa [22], hereafter referred to as mKSOM/aa, which consisted of 98.6 mM NaCl, 2.5 mM KCl, 1.71 mM CaCl₂, 0.35 mM KH₂PO₄, 0.2 mM MgSO₄, 0.3 mM sodium pyruvate, 3.0 mM sodium lactate, 25 mM NaHCO₃, 0.68 mM L-glutamine, premixture solutions of 12 essential amino acids for basal medium Eagle (Sigma) and 7 nonessential amino acids for minimum essential medium (Sigma) (the final concentrations of L-leucine, L-methionine, L-tryptophan and glycine were modified to 0.4, 0.1, 0.05 and 3 mM, respectively), 10 mM HEPES, 5 μ g/ml insulin-5 μ g/ml transferrin-5 ng/ml sodium selenite (ITS, media supplement), 10 μ M EDTA, 65 μ g/ml dibekasin sulfate, and 10 mg/ml BSA. At 6 h after insemination, the oocytes were freed from cumulus cells by repeated pipetting. Subsequently, presumptive zygotes were cultured for 240 h in 50 μ l of culture medium covered with mineral oil under the gas phase of 5% CO₂, 5% O₂, 90% N₂ with high humidity at 39°C. Each droplet contained approximately 10 presumptive zygotes. At 96 h post insemination, embryos at the 8-cell stage or higher were transferred to a fresh culture medium supplemented with 10 μ M β -mercaptoethanol (β -ME) (Sigma). At 120–144 h post insemination, embryos at the morula stage or higher were transferred to a fresh culture medium supplemented with 50 μ M β -ME. Oocytes that did not progress to the next cleavage stage were removed from drops containing developing embryos. Cleavage, development to the 8-cell stage or higher and the blastocyst stage were observed at 48, 96 and 144–216 h post insemination, respectively, under an inverted microscope.

Experimental design

Experiment 1: effect of fractions of bFF on IVM

The objective of experiment 1 was to determine the effect of fractions of bFF obtained by ultracentrifugation on maturation of bovine oocytes *in vitro*. Oocytes were matured in basic maturation medium without a supplement or supplemented with 10% (v/v) bFF, 8.5% (v/v) bFF-1st, 1.5% (v/v) bFF-2nd or 0.5% (v/v) bFF-3rd.

Experiment 2: effect of fractions of FBS on IVM

The objective of experiment 2 was to determine the effect of fractions of FBS obtained by ultracentrifugation on maturation of bovine oocytes *in vitro*. Oocytes were

matured in a basic maturation medium supplemented with 10% (v/v) FBS, 8.5% (v/v) FBS-1st, 1.5% (v/v) FBS-2nd or 1.5% (v/v) FBS-3rd.

Experiment 3: comparison of developmental competence to the blastocyst stage of homogeneous and heterogeneous oocytes

In this experiment, we compared the developmental competence of oocytes with homogeneous ooplasm and oocytes with heterogeneous ooplasm matured in the medium supplemented with 1.5% (v/v) bFF-2nd.

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.

Results

Experiment 1: effect of fractions of bFF on IVM

The results of experiment 1 are presented in Table 1. In the oocytes with homogeneous ooplasm, the cleavage rate with bFF-2nd was significantly higher than that with no addition or with bFF or bFF-3rd ($P < 0.05$), and the rate of development to the 8-cell stage or higher with bFF-1st was significantly higher than that with no addition or with bFF or bFF-3rd. In addition, the rates of development to the blastocyst stage with bFF-1st and bFF-2nd were significantly higher than that with no addition or with bFF or bFF-3rd. However, there were no differences in developmental rate to each development stage between bFF-1st and bFF-2nd. In the oocytes with heterogeneous ooplasm, cleavage rates with bFF-1st and with bFF-2nd were significantly higher than that with no addition or with bFF-3rd, and the rates of development to the 8-cell stage or higher with bFF-1st and with bFF-2nd were significantly higher than that with no addition or with bFF or bFF-3rd. Furthermore, the rate of development to the blastocyst stage with bFF-2nd was significantly higher than that with no addition or with bFF, bFF-1st or bFF-3rd ($P < 0.05$).

Experiment 2: effect of fractions of FBS on IVM

The results of experiment 2 are presented in Table 2. In the oocytes with homogeneous ooplasm, the fractions of FBS had no effect on *in vitro* maturation. In addition, the rates of development to the 2-cell or higher, 8-cell or higher and blastocyst stages with FBS-1st were significantly lower than those with FBS, FBS-2nd and FBS-3rd. In contrast, in the oocytes with

Table 1. Development to the blastocyst stage of IVF bovine oocytes matured in medium supplemented with an ultracentrifuged bovine follicular fluid fraction

Type of oocytes	Supplement	No. of oocytes examined	No. (%) of oocytes developed to		
			≥2 cell (48 h) ^a	≥8 cell (96 h) ^a	Blastocyst (144–216 h) ^a
Homo ^b	– (no addition)	90	49 (54.4) ^{def}	29 (32.2) ^{df}	13 (14.4) ^d
	bFF	108	58 (53.7) ^{df}	36 (33.3) ^{df}	23 (21.3) ^d
	1st fraction (top)	125	79 (63.2) ^{de}	59 (47.2) ^c	44 (35.2) ^c
	2nd fraction	177	122 (68.9) ^e	79 (44.6) ^{de}	60 (33.9) ^c
	3rd fraction (bottom)	137	58 (42.3) ^d	35 (25.5) ^f	23 (16.8) ^d
Hetero ^c	– (no addition)	126	84 (66.7) ^{df}	67 (53.2) ^d	41 (32.5) ^d
	bFF	110	84 (76.4) ^{de}	62 (56.4) ^d	51 (46.4) ^c
	1st fraction (top)	150	115 (76.7) ^e	93 (62.0) ^{de}	66 (44.0) ^{de}
	2nd fraction	188	152 (80.9) ^e	130 (69.1) ^e	107 (56.9) ^f
	3rd fraction (bottom)	145	86 (59.3) ^f	71 (49.0) ^d	46 (31.7) ^d

^aHours after insemination, ^bHomo: Homogeneous ooplasm, ^cHetero: Heterogeneous ooplasm, ^{d, e, f}Values in the same column with different superscripts are significantly different for each type of oocyte ($P < 0.05$).

Table 2. Development to the blastocyst stage of IVF bovine oocytes matured in medium supplemented with an ultracentrifuged fetal bovine serum fraction

Type of oocytes	Supplement	No. of oocytes examined	No. (%) of oocytes developed to		
			≥2 cell (48 h) ^a	≥8 cell (96 h) ^a	Blastocyst (144–216 h) ^a
Homo ^b	FBS	119	81 (68.1) ^d	54 (45.4) ^d	42 (36.1) ^d
	1st fraction (top)	125	66 (51.6) ^c	34 (26.6) ^c	25 (19.5) ^c
	2nd fraction	99	70 (70.7) ^d	49 (49.5) ^d	38 (38.4) ^d
	3rd fraction	123	96 (78.0) ^d	55 (44.7) ^d	46 (37.4) ^d
Hetero ^c	FBS	189	122 (64.6) ^d	93 (49.2) ^{df}	79 (41.8) ^d
	1st fraction (top)	182	96 (52.7) ^d	80 (44.0) ^d	62 (34.1) ^d
	2nd fraction	194	152 (78.4) ^e	112 (57.7) ^{ef}	89 (45.9) ^{de}
	3rd fraction	229	179 (78.2) ^e	147 (64.2) ^e	119 (52.0) ^c

^aHours after insemination, ^bHomo: Homogeneous ooplasm, ^cHetero: Heterogeneous ooplasm, ^{d, e, f}Values in the same column with different superscripts are significantly different for each type of oocyte ($P < 0.05$).

heterogeneous ooplasm, the rates of development to the 2-cell or higher, 8-cell or higher and blastocyst stages with FBS-3rd were significantly higher than those with FBS and FBS-1st ($P < 0.05$). The rate of development to the blastocyst stage with FBS-3rd tended to be higher than that with FBS-2nd, but the difference was not significant.

Experiment 3: comparison of developmental competence to the blastocyst stage of oocytes, homogeneous and heterogeneous oocytes

The results of experiment 3 are shown in Table 3. The oocytes with heterogeneous ooplasm showed significantly higher rates of development to the 8- cell or higher and blastocyst stages than did oocytes with homogeneous ooplasm matured in maturation medium with bFF-2nd fraction ($P < 0.01$).

Discussion

In the present study, we examined the effects of different fractions of bovine follicular fluid (bFF) or fetal bovine serum (FBS) supplemented to maturation medium on development to the blastocyst stage of bovine oocytes matured and fertilized *in vitro*. The concentrations of the fractions of bFF obtained by ultracentrifugation were about 81% for bFF-1st, about 12% for bFF-2nd and about 7% for bFF-3rd. In experiment 1, the concentration of each fraction supplemented to the maturation medium (bFF-1st: 8.5%, bFF-2nd: 1.5%, bFF-3rd: 0.5%) corresponded to the concentration of 10% (v/v) bFF supplemented to the maturation medium. Although the concentration of each fraction of FBS obtained by ultracentrifugation was different from that of bFF, the concentration of FBS-3rd

Table 3. Comparison of developmental competence to blastocyst between oocytes with, homogeneous ooplasm and heterogeneous ooplasm^a

Supplement	Type of oocytes ^c	No. of oocytes examined	No. (%) of oocytes developed to		
			≥2 cell (48 h) ^b	≥8 cell (96 h) ^b	Blastocyst (144–216 h) ^b
bFF-2nd fraction	Homo	177	122 (68.9)	79 (44.6)	60 (33.9)
	Hetero	188	152 (80.9)*	130 (69.1)**	107 (56.9)**

Values in the same column with asterisks are significantly different for each supplement (* $P < 0.05$, ** $P < 0.01$). ^aThe same data in Table 1, ^bHours after insemination, ^cHomo: Homogeneous ooplasm; Hetero: Heterogeneous ooplasm.

supplemented to the maturation medium was the same as that of bFF-2nd in experiment 1. In the present study, a low concentration (1.5%) of bFF-2nd and FBS-3rd promoted developmental competence. The results indicate that the cytoplasmic maturation promoting factor(s) can be isolated from bFF or FBS by ultracentrifugation.

As shown in Table 1, supplementation of the maturation medium with bFF-2nd fraction improved the developmental competence compared to that with supplementation with the other fractions and bFF. In porcine oocytes, Daen *et al.* [17] found that when porcine FF (pFF) was separated into four fractions by ultracentrifugation, fraction 1 (top) promoted cumulus expansion and male pronucleus formation. They also reported that fraction 2 (2nd) did not promote cumulus expansion or the rate of male pronucleus formation. In contrast, cumulus expansion in oocytes matured in the maturation medium with bFF-2nd was observed in the present study (data not shown). This difference in results might be due to the difference in the efficacy of the bFF and pFF fractions obtained by ultracentrifugation.

As shown in Table 2, there were no differences in the proportions of oocytes with homogeneous ooplasm that reached the blastocyst stage with the FBS-3rd fraction and with FBS. However, the proportion of oocytes with heterogeneous ooplasm that reached the blastocyst stage was greater with the FBS-3rd fraction than with the FBS or the FBS-1st fraction. These results indicate that FBS-3rd had the ability to promote competence of bovine oocytes to develop to the blastocyst stage. In oocytes with homogeneous ooplasm, although the bFF-1st fraction had an effect on developmental competence, the FBS-1st fraction had no effect on developmental competence. Dean *et al.* [23] observed that when pig serum was separated into four fractions by ultracentrifugation, the 3rd fraction led to degeneration of all porcine oocytes. However, in experiment 2 in the present study, degeneration of

oocytes matured in the presence of each fraction obtained by FBS was not observed.

In the present study, the active substance(s) in the bFF-2nd fraction and in the FBS-3rd fraction was not identified. In cattle, it has been reported that addition of epidermal growth factor (EGF) [24–26], insulin-like growth factor (IGF-I) [27], EGF+IGF-I [28] or activin [29, 30] to the maturation medium enhances the developmental competence of oocytes. Two of these growth factors, EGF [31] and IGF-I [32], are found in human FF and bFF, and activin [33] is found in serum and bFF. Therefore, it is possible that these growth factors are contained in the bFF-2nd fraction and FBS-3rd fraction. Ikeda *et al.* [34] found that adding a heparin-binding fraction of bFF to the IVM medium was highly effective for enhancing the developmental competence of bovine oocytes *in vitro*. Ikeda *et al.* [35] examined the effect of recombinant bovine midkine (rbMK) on IVM of bovine oocytes and found that the presence of rbMK during IVM enhanced their developmental competence to the blastocyst stage after IVF. MK was also isolated from bFF [36]. Therefore, it is thought that MK is one of the cytoplasmic maturation factors of bovine oocytes.

In oocytes with homogeneous ooplasm, the fractions of FBS had no effect on *in vitro* maturation (experiment 2). The reason for this is not clear, but a possible reason is that because FBS itself is comparatively effective as a supplement to the maturation medium, the developmental competence of oocytes with homogeneous ooplasm was lower. As shown in Table 3, developmental competence to the blastocyst stage was significantly greater in oocytes with heterogeneous ooplasm than in oocytes with homogeneous ooplasm. This result is consistent with the results reported by Fukuda and Enani [21]. In our previous study [18], we found that the abilities of oocytes with heterogeneous ooplasm for *in vitro* maturation and *in vitro* fertilization were superior to those of oocytes with homogeneous ooplasm. Blondin and Sirard [37] concluded that bovine

oocytes acquire developmental competence late in the follicular phase, possibly when the first signs of atresia appear, and that oocytes with initial signs of degeneration (slight expansion in the outer layers of the cumulus and slightly granulated ooplasm) develop significantly more than all other classes. Recently, Nagano *et al.* observed [38] that a heterogeneous ooplasm indicated an accumulation of lipids and good developmental potential, whereas a homogeneous ooplasm indicated aging and low developmental potential. The results of the present study suggest that oocytes with heterogeneous ooplasm are developmentally more competent and that this developmental competence is induced by the presence of the bFF-2nd fraction or FBS-3rd fraction during IVM.

The embryo culture medium used in the present study was modified TCM199 (experiment 1) or modified KSOM/aa (experiment 2) as a serum-free medium. These culture media do not contain glucose. Modified KSOM/aa which is a comparatively simple culture medium would be especially useful for analyzing promoters or inhibitors in IVC of bovine embryos.

In conclusion, the bFF-2nd fraction and FBS-3rd fraction obtained by ultracentrifugation are superior as supplements for bovine oocyte maturation medium. The results of this study will contribute to improvement of the IVM system for bovine oocytes. Further study is required to determine which substance(s) in the bFF-2nd fraction and FBS-3rd fraction has an effect on IVM of bovine oocytes.

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