Effect of Leukemia Inhibitory Factor Added to Maturation Medium on In Vitro Maturation, Fertilization and Development of Bovine Follicular Oocytes

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Abstract: *This study was conducted to evaluate the effect of supplementation of human or murine leukemia inhibitory factor (hLIF or mLIF) on* in vitro *maturation medium (TCM-199) containing FCS or BSA, and mLIF on semi-defined maturation medium containing BSA with or without hormones and in two different culture systems (single or group) on* in vitro *maturation, fertilization and development of bovine oocytes. Immature bovine oocytes were matured* in vitro *in TCM-199 supplemented with hLIF or mLIF (0 or 1,000 U/ml) containing FCS or BSA (Experiment 1), and mLIF as a supplement (0 or 1,000 U/ml) in TCM-199 containing BSA with or without hormones (Experiment 2) for 24 h. In Experiment 1, hLIF and mLIF added to a maturation medium have no positive effect on maturation, fertilization or development rates. In Experiment 2, the proportion of oocytes matured in TCM-199 with hormones showed significantly higher normal and total fertilization rates ($P<0.01$) and cleavage rates ($P<0.05$) than without hormones. The proportion of oocytes matured in a group showed significantly ($P<0.01$) lower normal and total fertilization rates, and higher polyspermy rates than those matured singly. There was no positive effect of mLIF added to the maturation medium on* in vitro *maturation, fertilization or development of bovine oocytes. These results indicate that there was no stimulating effect of hLIF or mLIF added to* in vitro *maturation medium on IVM, IVF or development of bovine oocytes.*

Key words: *LIF,* In vitro *maturation, Bovine oocyte, Single culture, Hormones.*

Growth factors (EGF [1–7], TGF-α [6], TGF-β [8] and IGF-I [7]) and cytokines (IL-1β [9]) play important roles on *in vitro* maturation (IVM) of mammalian oocytes, and their beneficial effects on IVM, *in vitro* fertilization (IVF) and culture (IVC) of mammalian oocytes have been reported in many species (mouse, rat, pig, cattle and man).

Leukemia inhibitory factor (LIF), a kind of cytokine, is a glycoprotein, first isolated in mice, that induces differentiation and inhibits the proliferation of the M1 myeloid leukemia cell line [10, 11]. Recently, it has been reported that LIF plays an important role in the development of mammalian oocytes of mouse [12], sheep [13–15] and cattle [16–18]. Lee *et al.* [18] suggested that the addition of human and murine LIF (hLIF and mLIF, respectively) to IVC medium improved development to hatched blastocysts when embryos derived from IVM and IVF were cultured singly. Furthermore, Marquant-Le Guienne *et al.* [19] showed that mLIF had positive effects on blastocyst growth and differentiation.

Although previous studies showed a positive effect of LIF on *in vitro* development of bovine embryos, the effect of LIF added to a maturation medium has not been investigated except in a recent study by Pugh *et al.* [20] showing that hLIF (1,000 or 2,000 U/ml) added to a maturation medium significantly increased both maturation and subsequent *in vitro* development of bovine oocytes, but the precise effects of LIF combined with hormones and culture systems (single and group) in IVM media have not been reported.

The present study was conducted to investigate the supplementing effects of hLIF and mLIF in maturation medium on IVM, IVF and *in vitro* development of bovine oocytes. In Experiment 1, the effects of hLIF and mLIF in maturation media containing FCS or BSA were compared. In Experiment 2, the supplementing effects of mLIF in combination with hormones were investigated in two culture systems (culturing singly or in a group).

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Materials and Methods

In vitro maturation

Ovaries from Holstein heifers and cows were collected at a local abattoir shortly after slaughter and were transported in physiological saline at 30–35°C to the laboratory within 2 h. Ovaries were then washed in fresh saline. Contents of small antral follicles (2–5 mm in diameter) were aspirated with an 18-gauge needle attached to a 5 ml disposable syringe, and pooled in a petri dish. Only oocytes with multilayered compact cumulus cells and evenly granulated cytoplasm were selected and washed three times in Tissue Culture Medium 199 (TCM199, Dainippon Pharmaceutical Co., Ltd. Australia) supplemented with 0.3% (w/v) bovine serum albumin (BSA, fatty acid free, Fraction V; Sigma Chemical Co., Louis, MO, USA), 2 mM sodium bicarbonate and 10 mM HEPES. Oocytes were cultured in microdrops (50 μl) of maturation medium covered with mineral oil for 24 h at 39°C in an atmosphere of 5% CO_2 in air and ≥95% humidity. LIF used in this study were recombinant hLIF (rh-LIF: Batch No.; H1I3, AMRAD Corporation Ltd., Kew, Victoria, Australia) and mLIF (rm-LIF: Batch No.; M1F3, AMRAD Corporation Ltd., Kew, Victoria, Australia). Molecular weights of the recombinant hLIF and mLIF are each 20,000. Biological activity of both hLIF and mLIF was assayed by the ability to induce differentiation in murine M1 colonies. The LIFs were reconstituted according to the manufacturer's recommendation and stored at 2–8°C for the present study.

Experiment 1

The media for *in vitro* maturation used in this experiment were TCM199 supplemented with 10% (v/v) fetal calf serum (FCS; heat-treated at 56°C for 30 min) or 0.8% bovine serum albumin (BSA, fatty acid free, Fraction V; Sigma Chemical Co., St. Louis, MO, USA) containing 25 mM $NaHCO_3$, 0.02 AU/ml Antrin (Denka Chemical Co., Ltd.) and 1 μg/ml estradiol (Sigma Chemical Co., St. Louis, Mo, USA). Both TCM199 + FCS and TCM199 + BSA media were supplemented with 0 (control), 1,000 U/ml of hLIF or mLIF. Oocytes were divided into 6 different treatment groups and cultured for 24 h at 39°C under 5% CO_2 in air.

Experiment 2

The medium for *in vitro* maturation used in this experiment was TCM199 + BSA containing 25 mM $NaHCO_3$ and supplemented with or without hormones (0.02 AU Antrin/ml and 1 μg estradiol/ml), and with or without mLIF (1,000 U/ml). Oocytes were allocated to each treatment group and cultured singly (1 oocyte/drop) or in a group (10 oocytes/drop).

Some oocytes in Exps. 1 and 2 were fixed to evaluate nuclear maturation *in vitro*. After 24 h of maturation, cumulus cells surrounding the oocytes were removed by repeated pipetting and the oocytes were mounted on slides. They were then fixed in acetic acid : ethanol (1:3) for at least 24 h, stained with aceto-orcein (1% orcein in 45% acetic acid solution) and examined for the stage of meiotic maturation under a phase-contrast microscope. Oocytes were considered matured when they reached metaphase II of meiosis.

In vitro fertilization

The media for *in vitro* fertilization used in this experiment were prepared as described by Fukui [21]. Each 0.5 ml frozen straw of semen from three Holstein bulls was thawed at 37°C in a water bath for 30 sec. An aliquot (0.2 ml) of thawed semen was placed under 1 ml of modified Tyrode's calcium-free medium (capacitation medium, pH 7.4) in conical tubes (Becton Dickinson Labware, Lincoln Park, NJ, USA) for a swim-up procedure [21, 22]. The top 0.8 ml of the medium was then collected after incubation for 1 h at 39°C. The pooled medium containing spermatozoa was washed twice (500 × g, 5 min) with capacitation medium. The final pellet of spermatozoa was resuspended in the capacitation medium to a concentration of 50 × 10^6 sperms/ml. An equal volume of a 200 μg/ml heparin (Sigma Chemical Co., St. Louis, USA) solution was added to the sperm suspension to yield spermatozoa and a heparin concentration of 25 × 10^6 sperm/ml and 100 μg/ml, respectively. The heparin-treated spermatozoa were incubated for 15 min at 39°C in 5% CO_2 in air and ≥95% humidity.

After *in vitro* maturation the oocytes were washed three times with a modified Tyrode's medium (washing medium, pH 7.4) containing 2 mM $CaCl_2$, 2 mM $NaHCO_3$ and 10 mM HEPES, and the expanded cumulus cells were partly removed by gentle pipetting. One (Exp. 2) or 5 (Exps. 1 and 2) oocytes together with 3 μl of washing medium were introduced into a fertilization drop (43 μl, pH 7.8) of a modified Tyrode's medium under mineral oil. Four μl of the heparin-treated sperm suspension was then added to give a final concentration of 2 × 10^6 sperms/ml. Gametes were co-incubated for 18 h at 39°C in 5% CO_2 in air.

The oocytes were fixed to ascertain sperm penetration and formation of pronuclei. After 18 h of fertilization, oocytes were mounted, fixed and stained as described

before. Oocytes were considered normally fertilized when female and male pronuclei with a residual spermtail were visible in the ooplasm, and polyspermic fertilization when there were more than two male pronuclei and spermtails in an oocyte.

In vitro development

The medium for *in vitro* culture of oocytes was the synthetic oviduct fluid medium (SOFM) as described by Tervit *et al.* [23]. It was modified by lowering the concentration of BSA to 8 mg/ml. SOFM was supplemented with 2% (v/v) MEM essential amino acids (Life Technologies, Inc., Grand Island, NY, USA) and 1% (v/v) MEM non-essential amino acids (Life Technologies, Inc., Grand Island, NY, USA). All media were sterilized by passage through a 0.2 μm membrane filter and equilibrated for one night in an incubator at 39°C in 5% CO_2 in air and ≥95% humidity.

After 18 h of fertilization, cumulus cells and residual spermatozoa were removed by repeated pipetting. Then the oocytes from each treatment group were cultured singly (1 oocyte/drop, Exp. 2) or in a group (4–6 oocytes/drop, Exps. 1 and 2) in microdrops (30 μl) of SOFM at 39°C in an atmosphere of 5% CO_2, 7% O_2, 88% N_2 and ≥95% humidity. Cleavage and embryonic development into blastocysts (BL) and hatched blastocysts (HBL) were observed on Days 3, 8 and 10 (Day 0; the day of IVF), respectively.

Statistical analysis: In both Experiments 1 and 2, mean proportions of the replicates for IVM, IVF and IVC were analyzed for the analysis of variance by a general linear models (GLM) procedure using a Statistical Analysis System (SAS). Furthermore, significant difference was tested for statistical difference among means, with $P<0.05$ accepted as being significant.

Table 1. Effect of human or murine LIF on *in vitro* maturation of bovine oocytes

Medium	LIF	No. examined*	Percent matured
TCM199 + FCS	Control	120	83.7
	Human	126	82.1
	Murine	129	84.6
	Total	375	83.6
TCM199 + BSA	Control	134	88.9
	Human	127	83.4
	Murine	128	82.4
	Total	389	84.9

*11 replicates.

Results

Experiment 1

The effects of hLIF and mLIF on *in vitro* maturation of bovine oocytes are shown in Table 1. There was no significant difference in the mean of maturation rates between FCS and BSA groups, or between control and hLIF or mLIF groups.

The supplementing effects of hLIF or mLIF during *in vitro* maturation on the *in vitro* fertilization rate are shown in Table 2. There was no significant difference between control, hLIF and mLIF groups in normal, polyspermy and total fertilization rates. Although there were no significant effects on normal and total fertilization rates, the polyspermy rate in the mean of the FCS group (45.7%) was significantly higher ($P<0.05$) than that of the BSA group (37.7%). In this experiment, polyspermy

Table 2. Effect of human or murine LIF on *in vitro* fertilization of *in vitro* matured bovine oocytes

			Fertilization rate (%)		
Medium	LIF	No. examined*	Normal	Polyspermy	Total
TCM199 + FCS	Control	117	50.9	47.9	98.8
	Human	115	56.5	42.3	98.8
	Murine	115	49.5	46.8	96.3
	Total	347	52.2	45.7[a]	97.9
TCM199 + BSA	Control	108	59.4	35.6	95.0
	Human	108	58.5	37.9	96.4
	Murine	113	57.7	39.6	97.3
	Total	329	58.5	37.7[b]	96.2

[a, b] Different superscripts in the same column indicate significantly different ($P<0.05$). *10 replicates.

rates were high in both FCS and BSA groups.

The supplementing effects of hLIF or mLIF during *in vitro* maturation on the *in vitro* development rate are shown in Table 3. There was no significant difference between FCS (81.6, 23.7 and 17.3%, respectively) and BSA (82.5, 27.4 and 17.9%, respectively) groups in the rates of cleaved, developed to blastocyst and hatched blastocyst. The proportions of oocytes that cleaved and developed to blastocysts and hatched blastocysts were not affected by the addition of either hLIF or mLIF during *in vitro* maturation. There was no significant interaction of two factors (culture media and addition of LIF) on the proportions of maturation, fertilization and development in the oocytes examined.

Experiment 2

The effect of mLIF and hormones on *in vitro* maturation of bovine oocytes cultured singly or in a group are shown in Table 4. There were no effects of mLIF and hormones on the proportion of oocytes reached Metaphase II. There was also no significant difference between oocytes cultured singly and in a group in maturation rates. There was no significant interaction in maturation rates among the three factors examined.

The supplementing effects of mLIF and hormones during *in vitro* maturation on *in vitro* fertilization rate are shown in Table 5. There was no significant difference in the fertilization rate with or without mLIF. Regardless of the culture method (group or single), the normal and total fertilization rates of oocytes cultured with hormones

Table 3. Effect of human or murine LIF on *in vitro* development of *in vitro* matured bovine oocytes

Medium	LIF	No. cultured*	% cleaved	% BL	% HBL
TCM199 + FCS	Control	224	81.7	24.6	16.2
	Human	226	77.3	21.5	14.1
	Murine	220	85.7	25.2	21.5
	Total	670	81.6	23.7	17.3
TCM199 + BSA	Control	233	77.8	26.5	15.4
	Human	232	85.6	27.6	20.0
	Murine	227	84.1	28.1	18.2
	Total	692	82.5	27.4	17.9

BL; blastocyst, HBL; hatched blastocyst. *11 replicates.

Table 4. *In vitro* maturation (mean ± SEM) of bovine oocytes matured in the presence or absence of murine LIF and hormones, and cultured singly or in a group

LIF	Hormones	Culture method	N	Percentage of oocytes matured to metaphase-II
−	−	group	98	74 ± 5.2
		single	100	86 ± 2.6
	+	group	107	88 ± 1.5
		single	101	87 ± 2.5
+	−	group	98	81 ± 5.1
		single	97	84 ± 3.5
	+	group	104	80 ± 4.7
		single	100	88 ± 2.7
Means, no Hormones			393	81 ± 2.1
Means for Hormones			412	86 ± 1.5
Means for group			407	81 ± 2.3
Means for single			398	86 ± 1.4

N; Number of oocyte fixed (11 replicates).

(66 ± 2.6 and 96 ± 1.0%, respectively) were significantly higher (P<0.01) than without hormones (53 ± 2.8 and 89 ± 1.7%, respectively). The normal fertilization rates for oocytes cultured singly (67 ± 2.2%) were significantly higher (P<0.01) than in a group (51 ± 3.0%), and the proportion of oocytes cultured singly with mLIF and hormones had the highest normal fertilization rate (75 ± 3.6%). The polyspermic fertilization rates for oocytes cultured in a group (40 ± 2.8%) were significantly higher (P<0.01) than those cultured singly (26 ± 2.3%), and the proportion (49 ± 5.1%) of oocytes cultured in a group with mLIF and without hormones had a significantly higher (P<0.05) polyspermic fertilization rate than that of those singly cultured in each treatment group. There was a significant interaction (P<0.05) in total fertilization rates between hormonal addition and culture systems (single or group culture).

The effects of mLIF and hormones during *in vitro* maturation on the *in vitro* development rate are shown in Table 6. The proportions of oocytes that cleaved and developed to BL and HBL were not affected by the addition of mLIF during *in vitro* maturation. The cleavage (≥2-cell) rates of oocytes cultured with hormones (77 ± 3.0%) were higher (P<0.05) than without hormones (70 ± 2.5%). Significantly more oocytes cultured in a group (29 ± 2.8, 24 ± 2.6, and 17 ± 2.5%, respectively) (P<0.05) developed to 8-cell, BL and HBL than singly cultured oocytes (15 ± 2.1, 13 ± 1.8, and 6 ± 1.6%, respectively). The percentages of oocytes cultured in a group with mLIF and hormones showed the highest rates of developing to 8-cell, BL and HBL (35 ± 4.8, 34 ± 4.3, and 26 ± 4.4%, respectively). There was a significant interaction (P<0.05) in all categories (development to ≥2-cell, 8-cell, BL and HBL) for the developmental capacity of oocytes cultured in the hormonal addition and culture systems (single or group culture).

Discussion

One of purposes of the present study was to investigate the effect of hLIF or mLIF added to semi-defined maturation medium with BSA on the IVM, IVF and *in vitro* developmental rates of bovine oocytes. As serum contains many unknown factors such as steroid hormones, proteins and growth factors, it is difficult to interpret the precise role of a specific substance in oocyte maturation, fertilization and development, when added to a medium containing serum.

In Experiment 1, there was no significant difference between oocytes cultured in TCM-199 containing FCS or BSA during IVM in IVM, IVF and *in vitro* developmental rates. The results suggest that BSA added to TCM-199 is as effective as FCS.

It was reported that the addition of growth factors such as EGF, TGF-α, TGF-β and IGF-I to a chemically defined medium improved oocyte maturation and subsequent development *in vitro*, but no supplementing effect of LIF on oocytes maturation has been reported.

Table 5. *In vitro* fertilization (mean ± SEM) of bovine oocytes matured in the presence or absence of murine LIF and hormones, and cultured singly or in a group

LIF	Hormones	Culture method	N	Percentage of fertilization		
				Normal	Polyspermy	Total
−	−	group	95	47 ± 4.6^{cd}	39 ± 5.6	86 ± 4.4^{c}
		single	97	58 ± 2.0^{bc}	29 ± 4.5^{b}	89 ± 3.2^{bc}
	+	group	105	62 ± 5.9^{ab}	36 ± 5.8	98 ± 1.8^{a}
		single	104	69 ± 4.9^{ab}	24 ± 4.7^{b}	92 ± 2.6
+	−	group	102	38 ± 5.5^{d}	49 ± 5.1^{a}	86 ± 3.2^{bc}
		single	97	67 ± 5.4^{ab}	28 ± 5.8^{b}	95 ± 2.5^{ab}
	+	group	106	59 ± 5.2^{abc}	37 ± 5.4	97 ± 1.5^{a}
		single	103	75 ± 3.6^{a}	23 ± 3.5^{b}	98 ± 1.4^{a}
Means, no Hormones			391	53 ± 2.8^{A}	36 ± 2.9	89 ± 1.7^{A}
Means for Hormones			418	66 ± 2.6^{B}	30 ± 2.6	96 ± 1.0^{B}
Means for group			408	51 ± 3.0^{A}	40 ± 2.8^{A}	92 ± 1.7
Means for single			401	67 ± 2.2^{B}	26 ± 2.3^{B}	93 ± 1.3

N; Number of oocyte fixed (10 replicates). $^{a-d}$Different superscripts in the same column indicate significantly different (P<0.05). $^{A-B}$Different superscripts in the same column indicate significantly different (P<0.01).

Table 6. *In vitro* development (mean ± SEM) of bovine oocytes matured in the presence or absence of murine LIF and hormones, and cultured singly or in a group

LIF	Hormones	Culture method	N	Percent of embryos developed to			
				≥2-cell	8-cell	BL	HBL
−	−	group	100	67 ± 6.9^{b}	22 ± 6.8^{bc}	20 ± 6.5^{bc}	13 ± 5.2^{bc}
		single	100	71 ± 3.3	19 ± 5.3^{c}	17 ± 3.8^{bc}	11 ± 4.4^{bc}
	+	group	100	84 ± 4.9^{a}	32 ± 6.9^{ab}	27 ± 4.4^{ab}	20 ± 4.7^{ab}
		single	100	64 ± 7.6^{b}	9 ± 2.9^{c}	9 ± 2.4^{c}	3 ± 1.6^{c}
+	−	group	100	66 ± 4.2^{b}	26 ± 3.3^{ab}	15 ± 3.4^{bc}	9 ± 3.9^{bc}
		single	100	74 ± 5.2	16 ± 3.4^{bc}	12 ± 3.7^{c}	4 ± 1.6^{c}
	+	group	100	81 ± 2.7	35 ± 4.8^{a}	34 ± 4.3^{a}	26 ± 4.4^{a}
		single	100	79 ± 6.2	17 ± 4.8^{bc}	13 ± 4.6^{c}	7 ± 3.6^{bc}
Means, no Hormones			400	70 ± 2.5^{A}	21 ± 2.4	16 ± 2.2	9 ± 2.0
Means for Hormones			400	77 ± 3.0^{B}	23 ± 3.1	21 ± 2.6	14 ± 2.5
Means for group			400	75 ± 2.7	29 ± 2.8^{A}	24 ± 2.6^{A}	17 ± 2.5^{A}
Means for single			400	72 ± 2.9	15 ± 2.1^{B}	13 ± 1.8^{B}	6 ± 1.6^{B}

N; Number of oocyte cultured (8 replicates). BL; blastocyst, HBL; hatched blastocyst. $^{a-c}$Different superscripts in the same column indicate significantly different ($P<0.05$). $^{A-B}$Different superscripts in the same column indicate significantly different ($P<0.05$).

Pugh *et al.* [20] reported that the addition of hLIF to maturation medium significantly improved the rate of maturation and development of bovine oocytes. We previously observed an improvement in the rate of development of *in vitro* matured and fertilized bovine oocytes cultured in SOFM containing hLIF and mLIF (1,000–5,000 U/ml) [16–18]. In the present study, however, neither hLIF nor mLIF in a maturation medium improved *in vitro* maturation, fertilization or the rate of development of bovine oocytes.

Overall polyspermy rates in the experiments were high (23–49 %), especially when FCS was used for IVM. Some reasons for the high polyspermy rates in this study would be a high sperm concentration in a fertilization drop (2×10^6 sperms/ml) and deletion of glucose from the fertilization medium as described by Parrish *et al.* [24] in comparison with our previous results of IVF [25–27]. In the present study, oocytes cultured singly had a higher ($P<0.01$) normal fertilization rate and a lower polyspermy rate than those cultured in a group. Although the fact that the volume of medium per oocyte in group culture was less than that in single culture might have resulted in the higher polyspermy rates in a group culture during IVF, the exact reason is unknown.

The other purpose of the present study was to investigate the effect of mLIF in combination with or without hormonal addition in two different culture systems (single or group). The addition of hormones to the maturation medium significantly improved normal and total fertilization rates ($P<0.01$) and cleavage rates ($P<0.05$), but did not stimulate oocyte maturation or embryonic development *in vitro*. Eyestone and Boer [28] reported that the addition of FSH to a maturation medium significantly improved cleavage and embryonic development to 8-cells, compacted morulae or blastocysts, but had no beneficial effect on the maturation and fertilization of bovine oocytes. On the other hand, Sanbuissho and Threlfall [29] reported that oocytes maturation to metaphase II was improved when FSH was added to maturation media containing estrous cow serum, FCS or BSA.

Paria and Dey [30] reported that mouse embryos cultured in a group showed higher developmental capacity and had a lager number of blastocyst cells than embryos cultured singly, and that the beneficial effect of EGF was lost when the volume of media was doubled, which was due to autocrine actions among embryos. In the present study, embryonic development to 8-cells, BL and HBL were much better ($P<0.05$) in group culture than in single culture, which is similar to the finding of Paria and Dey [30].

In conclusion, the present results suggest that hLIF and mLIF added to a maturation medium have no stimulating effect on IVM, IVF or development of bovine oocytes regardless of the hormonal addition and culture systems.

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