Effect of Human or Murine Leukemia Inhibitory Factor on In Vitro Development of Bovine Morulae Cultured Singly or in a Group

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Abstract: *This study was conducted to evaluate the effects of supplementation of human or murine leukemia inhibitory factor (hLIF or mLIF) to synthetic oviduct fluid medium (SOFM) containing BSA and to determine their effective dosage on in vitro development of bovine morulae cultured singly or in a group. Morulae were produced by culture of in vitro matured (IVM), fertilized (IVF) and cleaved (2- to 4-cell) embryos in SOFM+BSA for 124 h after IVF, and then cultured singly (1 embryo/30 μl drop) or in a group (4–5 embryos/30 μl drop) in SOFM+BSA supplemented with 0 (control), 500, 1,000, 2,000, 4,000 and 6,000 U/ml of each hLIF or mLIF at 39°C in an atmosphere of 5% CO_2, 7% O_2, 88% N_2 and ≥95% humidity. Culture of embryos in a group improved ($p<0.01$) embryo development to hatched blastocysts compared to a single culture. Both hLIF and mLIF significantly ($p<0.05$) increased development to hatched blastocysts when morulae were cultured singly, but not when cultured in a group. The most effective dosage of mLIF was 1,000 U/ml ($p<0.05$), but there was no significant difference in the addition of hLIF with different doses (500 to 6,000 U/ml) tested. This results indicate that a group culture may be more effective on embryo development than a single culture and supplementation of hLIF or mLIF to SOFM+BSA improve in vitro development into hatched blastocysts of bovine morulae derived from in vitro maturation and fertilization when cultured singly.*

Key words: *LIF, In vitro development, Oocyte, Cattle, Single culture.*

Leukemia inhibitory factor (LIF) is a cytokine with various activities in both the adult and the embryo. LIF induces differentiation and inhibits the proliferation of the M1 myeloid leukemia cell line [1]. The mRNA of LIF is expressed in human [2] and mouse [3] endometrium, peaks at the time of implantation in human, and LIF receptor mRNA is present in human blastocyst [2]. It has been reported that blastocyst implantation depends on maternal expression of leukemia inhibitory factor, and that LIF may play a crucial role in regulating the development of mice [4], ovine [5–7] and bovine [8–10] preimplantation embryos. These previous findings suggests that LIF may have a physiological role in the embryonic development. In previous studies, 1,000–5,000 U/ml [8, 9] of human LIF (hLIF) or 8,000 U/ml [6] of murine LIF (mLIF) were supplemented to synthetic oviduct fluid medium (SOFM) containing serum or BSA but, at present, there are few reports that have compared the effects of human or mouse LIF and their appropriate dosage added to culture medium on the development of IVM- and IVF-derived bovine embryos cultured singly or in a group.

It was reported that some growth factors genes were expressed in the mouse embryo [11] and that the stimulatory effect of growth factor on mouse embryonic development disappeared when embryos were cultured in a group (5 embryos/25 μl microdrop), which was attributed to autocrine effect by growth factors secreted by the embryos themselves [12]. Kato *et al.* [13] reported that embryonic development was affected by the number of cultured embryos and the volume of culture medium. And, therefore, it is considered that a comparative study is necessary to determine the precise role of specific substances, such as growth factor, supplemented to medium on embryonic development during a single or group culture.

Our aims in this study were to compare the effects of human and murine LIF, and to investigate the appropriate dosage of LIF added to SOFM supplemented with BSA on *in vitro* development of bovine morulae using a single or group culture.

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Materials and Methods

In vitro maturation: Ovaries were collected from Holstein cows and heifers slaughtered at a local abattoir and were transported in physiological saline (30–35°C) to the laboratory within 1 h. The ovaries were rinsed 3 times with fresh sterile saline. Follicular contents were aspirated from small antral follicles (2–5 mm) using an 18-gauge needle attached to a 5-ml disposable syringe, then allowed to settle in a petri dish and the supernatant was discarded and only the cumulus-oocyte complexes (COCs) with multilayered compact cumulus cells and evenly granulated cytoplasm [14] were selected for *in vitro* maturation. The COCs were washed three times in 2.5 ml of Tissue Culture Medium 199 (TCM199, Flow Laboratories Inc., Scotland) supplemented with 0.3% (w/v) BSA (fatty acid free, Fraction V; Sigma Chemical Co., St. Louis, MO, USA), 2 mM sodium bicarbonate and 10 mM HEPES (TCM199-HEPES). Thirty to 40 COCs were cultured in each well of a 4-well dish (Nunclon, Roskide, Denmark) containing 0.5 ml of TCM199 supplemented with 10% (v/v) fetal calf serum (FCS) and 25 mM $NaHCO_3$. The maturation medium was also supplemented with 2.5 μg FSH-B-1/ml (U.S. Department of Agriculture, USA), 2.5 μg LH-B-5/ml (National Hormone and Pituitary Program, USA), 1 μg estradiol/ml (Sigma Chemical Co., St. Louis, USA) and 2×10^6 granulosa cells/ml. Granulosa cells were collected from antral follicles of about 10 mm in diameter after dissection by the method of Moor and Trounson [15] and washed (500 × g, 10 min) twice with TCM199-HEPES. The final cell suspension was adjusted to the concentration of 50×10^6 cells/ml and a volume of 20 μl was added to each well. The cells were pre-incubated for 10 minutes before the initiation of oocyte maturation. The oocytes were statically cultured for 24 h at 39°C in an atmosphere of 5% CO_2 in air and ≥95% humidity.

In vitro fertilization: The media for *in vitro* fertilization used in this experiment were prepared as described by Fukui [16]. Each 0.5 ml frozen straw of semen from three Holstein bulls was thawed at 37°C water bath for 30 sec and prepared for swim-up treatment. An aliquot (about 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 [16, 17]. The top 0.8 ml of medium were then collected after incubation for 1 h at 39°C. The spermatozoa were washed twice (500 × g, 10 min) with capacitation medium. The final pellet of spermatozoa was resuspended in the capacitation medium to a concentration of 50×10^6 sperm/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 heparin concentrations 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.

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. Four to 6 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 (E.R. Squibb & Sons Inc., Princeton, NJ, USA). Then 4 μl of the heparin-treated sperm suspension was added to give a final concentration of 2.0×10^6 sperm/ml. Gametes were co-incubated for 30 h at 39°C and 5% CO_2 in air.

In vitro development: The basic medium for the *in vitro* culture of embryos was the synthetic oviduct fluid medium (SOFM) as described by Tervit *et al.* [18]. 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), 1% (v/v) MEM non-essential amino acids solution (Life Technologies, Inc., Grand Island, NY, USA) and 1 mM L-glutamine (Waco Pure Chemical Industries, Japan). All media were sterilized by passage through a 0.22 μm membrane filter (Gelman Sciences, Ann Arbor, USA) and equilibrated for one night in an incubator at 39°C with 5% CO_2 in air. The MEM essential and non-essential amino acids, glutamine were added to the medium immediately before use. LIF used in this study were recombinant hLIF (Batch No.; H1I3, AMRAD Corporation Ltd., Kew Victoria, Australia) and mLIF (Batch No.; M1F3, AMRAD Corporation Ltd., Kew Victoria, Australia). LIF were reconstituted according to manufacturer's recommendation and stored at 2–8°C.

After 30 h of fertilization, all cleaved embryos (2- to 4-cell) were cultured in SOFM+BSA. To obtain morulae, the 2- to 4-cell embryos were cultured in 24-well dishes (500 μl of medium/well, Becton Dickinson Labware, Lincoln Park, NJ, USA) at 39°C in an atmosphere of 5% CO_2, 7% O_2, 88% N_2 and ≥95% humidity. Morulae developed from the culture in SOFM+BSA at 124 h after IVF were selected and allocated randomly to each treatment group. Human or mLIF were supple-

mented to SOFM+BSA at the concentrations of 0 (control), 500, 1,000, 2,000, 4,000 and 6,000 U/ml, and morulae were cultured singly (1 embryo/30 μl drop) or in a group (4–5 embryos/30 μl drop) in microdrops containing each LIF-supplemented medium for additional 5 d at 39°C in an atmosphere of 5% CO_2, 7% O_2, 88% N_2 and ≥95% humidity. Embryonic development into expanded and hatched blastocysts were observed on Day 8 and 10 (Day 0; the day of IVF), respectively.

Statistical analysis: The percentages of embryonic development into expanded and hatched blastocyst stages among treatment groups were analyzed by a CATMOD procedure in the Statistical Analysis System (SAS).

Results

The supplementing effects of hLIF or mLIF to SOFM on embryonic development of bovine morulae cultured singly or in a group were shown in Table 1. A single culture with hLIF showed significantly ($p<0.01$) lower development to hatched blastocysts than the group culture in control and hLIF-supplemented groups. Supplementation of hLIF or mLIF to SOFM significantly ($p<0.05$) improved embryonic development to hatched blastocysts when cultured singly but not when cultured in a group. There was no significant difference in the proportions of expanded and hatched blastocysts with the two LIFs when embryos were cultured singly but supplementation with hLIF showed significantly ($p<0.05$) higher developmental rate to expanded blastocysts than that of mLIF, although hLIF was not significantly different from control, when embryos were cultured in a group. There was no significant difference in embryonic development with the concentrations of 500 to 6,000 U/ml of hLIF, but supplementation of 1,000 U/ml mLIF showed significantly ($p<0.05$) higher developmental rate to hatched blastocysts than 500, 2,000 and 6,000 U/ml in single culture, and also showed the highest embryonic development to expanded and hatched blastocysts in a group culture.

Discussion

Culture of embryos in a group (4–5 embryos/drop) improved the development to hatched blastocysts in control and hLIF groups compared to single culture. Supplementation of hLIF or mLIF had no stimulatory effect on embryonic development when the morulae were cultured in a group, but significantly increased develop-

Table 1. Effect of human or murine leukaemia inhibitory factor (LIF) on *in vitro* development of bovine morulae cultured singly or in a group

Dosage of LIF (U/ml)		Culture methods[a]					
		Single (1 embryo/drop)			Group (4–5 embryos/drop)		
		N	%ExBL	%HBL	N	%ExBL	%HBL
Control (0)		50	66	28^{b,A}	50	80^{d,e}	56^{B}
Human LIF	500	50	70	44	50	82	64
	1,000	50	68	36	50	78	68
	2,000	50	78	52	50	78	60
	4,000	50	70	40	50	82	62
	6,000	50	78	52	50	68	52
	Total	250	73	45^{c,A}	250	78^{d}	61^{B}
Murine LIF	500	50	58	40^{f}	50	70^{h,i}	46^{j}
	1,000	50	76	60^{g}	50	80^{h}	66^{k}
	2,000	50	68	38^{f}	50	72^{h,i}	56^{j,k}
	4,000	50	74	50^{f,g}	50	68^{h,i}	56^{j,k}
	6,000	50	70	38^{f}	50	58^{i}	44^{j}
	Total	250	69	45^{c}	250	70^{e}	54

[a]N; Number of morulae cultured (12 replicates). ExBL; expanded blastocyst, HBL; hatched blastocyst. $^{b-e}$ Different superscripts in the same column are significantly different ($p<0.05$). $^{f-k}$ Different superscripts in the same column are significantly different ($p<0.05$). $^{A, B}$ Different superscripts in the same row are significantly different ($p<0.01$).

mental rate to hatched blastocysts when cultured singly. Paria and Dey [12] reported similar observations that mouse embryos cultured in a group showed higher developmental capacity compared to embryos cultured singly, and that the beneficial effects of EGF on singly cultured embryos disappeared when embryos cultured in a group, which was due to autocrine actions among embryos. It is possible that autocrine action of growth factors secreted by embryos may have masked any growth promoting effect(s) of hLIF or mLIF supplemented to medium when embryos are cultured in a group.

Both hLIF and mLIF showed improvement of embryonic development to hatched blastocysts, but not to expanded blastocysts, which was consistent to the reports that hLIF increased the rate of hatching blastocysts in mouse [19], ovine [6] and bovine [8] embryos. Embryonic development to expanded blastocysts, in the present study, was not improved by supplementation of hLIF or mLIF, which is inconsistent to the previous report [9] that 5,000 U/ml of hLIF supplemented to SOFM containing BSA or PVA significantly increased embryonic development to expanded blastocysts, but not to hatched blastocysts of bovine morulae and early blastocysts. Recently, it has been reported that expression of LIF in uterine endometrium is necessary for implantation of mouse embryos [20], and that hLIF mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time [2]. Stewart [21] reported that mouse blastocysts with delayed implantation were recovered on Day 7 of pregnancy from LIF-deficient females that had been mated to a LIF-dificient male. Judging from these previous findings and the present results, it is considered that LIF plays roles at the expanding or hatching process, and affects implantation of mammalian embryos *in vivo*. There is no data available on implantation and pregnancy rate after trasnfer of bovine embryos treated with LIF and, therefore, further studies on the effects of LIF are required.

The homology of LIF is different among animal species that there is 74% of homology between mLIF and ovine LIF (oLIF) and 88% between hLIF and oLIF [22–24]. It is likely that there is difference in the effects of LIF on embryonic development according to animal species of LIF supplemented to culture medium. In the experiment utilizing mLIF and hLIF, Fry *et al.* [6] reported that 8,000 U/ml of mLIF supplemented to SOFM did not improve the development to hatching stage of ovine morulae and blastocysts. In the present study, there is no significant difference in effects of hLIF with various (500–6,000 U/ml) dosages, but supplementation of 1,000 U/ml of mLIF showed the highest stimulative effect on embryonic development to hatched blastocysts of bovine morulae when cultured singly, and 6,000 U/ml of mLIF showed lower development (58%) to expanded blastocysts than those of control and 1,000 U/ml group (80% and 80%, respectively) when embryos cultured in a group. It is unable to compare our results with other results directly, because there are few comparative data on dose-response of LIF on embryonic development in cattle, but it is considered that a higher concentration of mLIF in culture medium may inhibit embryonic development.

In conclusion, a group culture may be more effective on embryonic development than single culture, and the supplementation of a culture medium with hLIF or mLIF can improve *in vitro* development to hatched blastocysts of bovine morulae derived from IVM and IVF only when cultured singly *in vitro*.

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