Effect of Leukemia Inhibitory Factor (LIF) on the Quality of In Vitro Produced Mouse Blastocysts and Subsequent Derivation of Embryonic Stem (ES) Cells

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Objective: *To determine the effect of leukemia inhibitory factor (LIF) on the quality of in vitro produced mouse blastocyst and the efficiency of embryonic stem (ES) cell derivation.*
Design: *Experimental study*
Setting: *Reproductive Medicine Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University*
Material and Method: *In vivo fertilized zygotes were collected and subjected to in vitro culture in potassium simplex optimized medium (KSOM) containing 1,000 unit/ml LIF. The developmental ability of the zygote to blastocyst-stage and the cell numbers in blastocysts were evaluated. Expanded blastocysts developed in different culture media were subsequently subjected to ES cell derivation.*
Main Outcome Measure (s): *The influence of LIF on the quality of and the total cell numbers of blastocyst developed in vitro.*
Results: *Supplementation of LIF in KSOM increased the rate of hatching blastocysts (63.8% vs. 53.7%; $p < 0.05$) and total cell numbers (91.4 ± 15.0 vs. 85.1 ± 7.7; $p < 0.05$) compared to KSOM alone. ES cells were obtained 66.7% from blastocysts developed in KSOM-LIF versus 41.7% in KSOM ($p > 0.05$). Established ES cell lines showed typical colony and characteristics of pluripotent murine ES cells.*
Conclusion: *LIF improved the quality of in vitro produced blastocysts but not enhanced ES cell derivation.*

Keywords: *LIF, in vitro, Blastocyst, Embryonic stem cells*

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The pluripotent embryonic stem (ES) cells have unique characteristics, for instance, self-renewal ability and differentiation potential[1,2]. ES cells have been extensively used as a tool for the study of developmental biology, gene function as well as the animal model for human diseases. It has been reported previously that mouse ES cells can be established from both in vivo and in vitro produced blastocyst[3,4]. Culturing embryos in vitro can potentially cause the fragmentation of blastomere, reducing rates of embryo cleavage to blastocyst formation as well as the total cell numbers. Improving the embryo development and enhance the viability by means of co-cultured system due to some embryo-trophic factors released by the co-cultured cells such as growth factor, cytokines, and low molecule weight glycoprotein. Furthermore, supplement of growth factor(s) or cytokine(s) into the culture medium has been proved beneficial in yielding a high rate of blastocyst development[5]. The cytokine, a leu-

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kemia inhibitory factor (LIF), was identified as a factor inhibiting the growth of myeloid leukemia cells[6,7] and spontaneous differentiation of embryonic stem cells[8]. LIF is a multifunctional cytokine and has diversity of target cells as determined by its capacity for intracellular signaling via binding to LIF receptor and transmembrane transmitter gp130[9,10]. LIF plays an important role in the embryo development, implantation, and pregnancy[11-13]. However, results obtained so far from in vitro cultured embryos are inconclusive and LIF has been demonstrated either to be stimulatory[14-16] or to have no effect[17] on the in vitro development of embryos.

Although mouse ES cells can be derived from the in vitro produced blastocysts, the efficiency is less than that of those produced in vivo[3]. Establishing ES cells from the in vitro produced blastocysts, the culture condition that helps to increase the quality of the blastocysts may improve the efficiency of ES cell establishment.

In the present study, the authors evaluated the supplementation of LIF in a single culture medium on the formation and quality of blastocysts developed in vitro and subsequent derivation of ES cell lines.

Material and Method

Collection and in vitro culture of zygote-stage embryos

Hybrid B6D2F1 mice (C57BL/6 x DBA) were obtained from the National Animal Laboratory Center, Nakhonpathom, Thailand. Mice were maintained under 14 h: 10 h light-dark cycle. Female mice at 6-8 weeks of age were superovulated with 7.5 IU pregnant mare serum gonadotropin (PMSG; Folligon□, Intervet) followed by 7.5 IU human chorionic gonadotropin (hCG; Chorulon□, Intervet) given 48 h later. After mating by caging with males of the same strain subsequent to the injection of hCG, the vaginal plug was checked the following day. Females exhibiting a vaginal plug were sacrificed by cervical dislocation at 20 h post hCG injection. Zygote-stage embryos were collected from oviductal ampulae, freed from cumulus cells by brief exposure to 0.1% hyaluronidase in M2 medium (Specialty Media) followed by three times of washing before culture. Zygotes were randomly assigned to control and treatment groups with different in vitro culture conditions.

Staining of total cell number in blastocyst-stage embryos

Blastocyst-stage embryos were stained with 10 □g/ml Hoecsht 33258 in M2 for 10 min at room temperature (RT). After rinsing with M2, embryos were fixed and mounted onto a glass slide, gently flattened with a cover slip and visualized for cell counting on an inverted microscope fitted with an ultraviolet lamp and excitation filters.

Derivation, culture and characterization of mouse ES cell lines

Blastocyst-stage embryos were plated individually on a 24-well dish covered with mitomycin C treated mouse embryonic fibroblast (MEF) feeder monolayer. MEFs were obtained from 13.5 dpc mouse embryos[8]. Blastocysts were allowed to hatch from the zona pellucida, attached to the feeder, and left without disturbance for the first 48 h. The cultured blastocysts were re-fed daily with Knock out DMEM (Invitrogen) supplemented with 20% Knock out serum replacement (Invitrogen), 0.1 mM non-essential amino acid (Sigma-Aldrich), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 50 IU penicillin/ml, 50 mg streptomycin/ml (Invitrogen) and 1,000 IU/ml ESGRO-LIF (Chemicon). After 5 days in culture, the inner cell mass (ICM) outgrowths were mechanically removed from the trophectoderm and dissociated with 0.25% trypsin-EDTA (Gibco) solution. The small clumps of ICM were re-plated on fresh feeder. The ES cells were grown to subconfluency and were gradually replated on a larger culture dish until freezing or characterization. The culture dishes were kept at 37□C, 5% CO_2 in a humidified atmosphere. The ES cells were fed daily with ES medium and passaged every 2-3 days onto fresh feeder.

The ES cells were characterized enzymatically and immunocytochemically using antibodies against markers of undifferentiated mouse ES (mES) cells. ES cells were fixed in 4% paraformaldehyde for 15 min at RT and washed three times with phosphate buffered saline (PBS). ES cells were blocked in PBS supplemented with 1% BSA, 5% FBS and 0.1% Triton-X (Sigma) for 1 hour at RT. Primary antibodies [mouse monoclonal SSEA-1 (MC-480, Abcam; 1:200), goat polyclonal Oct-4 (Abcam; 1:300) were incubated overnight at 4□C. Fluorescent secondary antibodies [anti-mouse IgM-Cy3 (1:200); anti-mouse IgG-FITC (1:200) respectively from Abcam] were incubated with the ES cells for 1 h at RT. The nuclei of ES cells were counter-stained with 4'-6-Diamidino-2-phenylindole (DAPI) and visualized by fluorescent microscopy. Alkaline phosphatase expression was determined using standard methodology[18].

Chimera analysis

Blastocyst injection was performed as described previously[8]. In brief, 10-15 ES cells were

injected with a beveled pipette into host blastocysts of ICR mouse strain, in hanging drops of M2 on a cooled stage. Embryos were allowed to recover for 45-90 min in potassium simplex optimized medium (KSOM) at 37□C and then transferred to E2.5 pseudopregnant ICR females.

Experiment 1

The in vivo fertilized zygotes were randomly assigned into control and treatment groups. There were 177 zygotes in the control group and 177 zygotes in the treatment group. For the control group, the zygotes were cultured in KSOM medium. Recombinant murine leukemia inhibitory factor (r-mLIF; chemicon) at the concentration of 1,000 unit/ml was supplemented into KSOM (KSOM-LIF) in the treatment group. Zygotes were cultured continuously without renewing the medium at 37°C, 5% CO_2 in a humidified atmosphere. The developmental stages of the various cultured embryos were evaluated daily until day 6 (144 h post-hCG injection). The data from four replications were pulled and analyzed statistically.

Experiment 2

The cell numbers of blastocysts cultured in KSOM with or without LIF were evaluated after Hoescht staining. The staining was performed on 58 expanded blastocyst-stage embryos from different culture medium at 120 h post- hCG injection. The cell numbers of blastocysts were then enumerated under a fluorescence microscope.

Experiment 3

The level of derivation efficiency and culture of ES cells from different groups of cultured blastocysts were compared among three replications. Twelve blastocyst-stage embryos from each group were subjected to ES cell derivation, subsequent characterization, and germ-line chimera production.

Statistical analysis

The proportions of embryos reaching expanded, hatching or hatched blastocyst stage, as well as the number of ES cell lines, were analyzed using Chi-square analysis. The cell numbers of blastocysts were compared by one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered statistically significant.

Results

Experiment 1: In vitro development of mouse embryos cultured in KSOM with or without LIF

A total of 354 zygotes were collected from 16 vaginal plug positive mice; 146 of 177 (82.5%) developed to blastocyst stage in KSOM and 151 of 177 (85.3%) in KSOM-LIF (Fig. 1). After 6 days of in vitro culture, the proportion of hatching blastocysts cultured in KSOM-LIF was significantly higher ($p < 0.05$) than those cultured in KSOM (63.8 vs. 53.6% respectively) as shown in Table 1.

Experiment 2: Effect of LIF supplementation on the cell numbers of blastocysts

The results of cell number were pulled from three replications of blastocysts staining experiments. The cell numbers (mean $\pm$ SD) of expanded blastocysts after cultured for 5 days in KSOM-LIF (91.4 ± 15.0; n = 29) was significantly different ($p < 0.05$) to those in KSOM (85.1 ± 7.7; n = 29).

Experiment 3: Effect of blastocysts cultured in the media with or without LIF on the efficiency of ES cell derivation

The efficiency of ES cell lines derived from blastocysts developed in vitro in KSOM and KSOM-LIF is shown in Table 2. Of KSOM produced blastocysts 41.7% (n = 5) resulted in ES cell lines versus 66.7% (n = 8) of KSOM-LIF ($p > 0.05$). All established cell lines have been enzymetically dissociated every

Table 1. Effect of LIF supplementation in KSOM on the in vitro development

Culture media	Zygote n	Arrested embryos n (%)	Blastocyst n (%)	Blastocyst quality at Day 6		
				Expanded n (%)	Hatching n (%)	Hatched n (%)
KSOM	177	31 (17.5)	146 (82.5)	12 (6.8)	95 (53.7)[a]	39 (22.0)
KSOM-LIF	177	26 (14.7)	151 (85.3)	13 (7.3)	113 (63.8)[b]	25 (14.1)

[a,b] Different superscripts denote statistically significant difference within a column ($p < 0.05$)

Table 2. Efficiency of ES cell establishment from in vitro produced blastocysts cultured in KSOM with or without LIF supplementation

Culture media	Cultured blastocysts n	ES-like colonies n	ES cell lines n (%)
KSOM	12	6	5 (41.7)
KSOM-LIF	12	8	8 (66.7)

48-72 h and frozen at passage number 2 for further studies. The ES cell lines established from two types of in vitro produced blastocysts showed similar cell and colony morphology. ES cells showed a high ratio of nucleus to cytoplasm and colony structure was compact and identifiable edge (Fig. 2A). ES cells from a different type of culture system were strongly positive for molecular markers of undifferentiated pluripotent mouse ES cells, including ALP, SSEA-1 and Oct-4. ES cell lines derived from blastocyst cultured in KSOM or KSOM-LIF medium were able to form cystic embryoid bodies (EBs) in suspension culture (Fig. 2B) and one cell line from KSOM-LIF showed germ-line transmission (Fig. 2C).

Discussion

Mouse ES cell is a beneficial tool for studying gene function, cell biology, as well as regenerative medicine. Although ES cells are now commercially available, they can be used during a limited period of time due to their aging and karyotypic instability after long-term culture. However, surviving ES cells may be damaged or lost during transportation, and therefore establishing ES cells in the laboratory is still necessary. Unlike in vivo produced blastocysts, establishment of ES cells from blastocysts developed in vitro displayed lower efficiency[3]. Improving the culture condition by, for example, adding growth factor(s) in the culture medium or using sequential culture system showed the improvement of blastocyst quality[4,19,20].

The effect of LIF on the in vitro development of embryos has been widely studied but results are often contradictory. Recombinant human (rh) LIF has

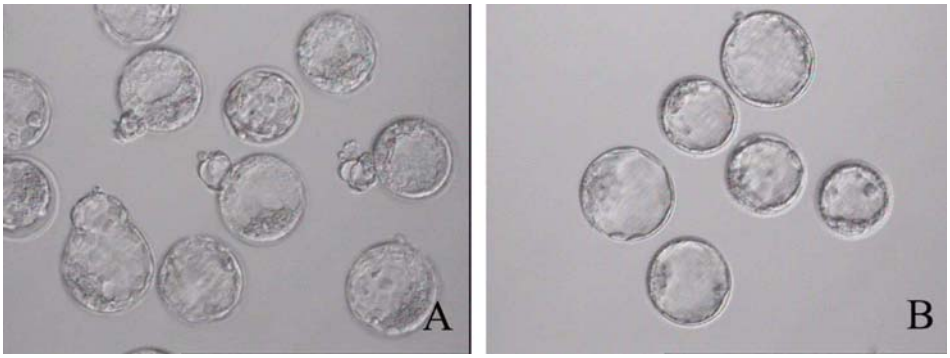

Fig. 1 The in vitro produced blastocyst embryos developed in KSOM supplemented with 1,000 U/ml LIF (A), and KSOM (B) (magnification: 200x)

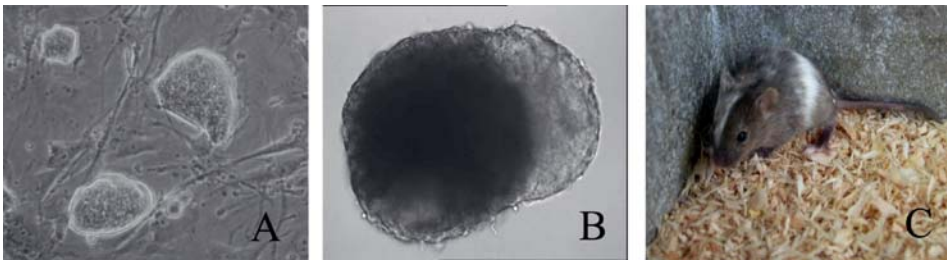

Fig. 2 Mouse ES cell colonies derived from blastocyst embryo cultured in KSOM supplemented with 1,000 U/ml LIF (A) (magnification: 100x), this ES cell line was able to form cystic EBs after culture in suspension for 7 days (B) (magnification: 100x) and contribute to germ-line transmission (C)

been demonstrated to enhance blastocyst formation and decrease embryo fragmentation in the embryos in some studies[15,21]. While other studies have shown the opposite results that rhLIF in standard medium does not enhance the development of early embryos[17]. However, these contradictory results may be attributable to the different species or strains of embryos and culture systems used for different experiments. Thus, it is interesting to investigate whether or not the embryos cultured and developed in vitro, in the media supplemented with LIF are suitable for ES cell derivation.

In this study, the concentration of 1,000 unit/ml of LIF was chosen for supplementation due to its stimulatory effect demonstrated in the previous reports[15] and the fact that this concentration has been adopted as the standard concentration for culture media supplement[8,22]. The present results showed that the blastocyst formation rates were not significantly different in KSOM and KSOM-LIF media. However, the blastocyst rate (82.5%) for the control group in the present study (Experiment 1) was similar to that (80%) reported by Tielens et al[3] using the same strain of mouse and 81.9% reported by Lin et al[4] using C57BL/6xC3H strain.

LIF supplementation in KSOM does not enhance the blastocyst formation, which supported the previous report[17]. However, the results show the beneficial effect of LIF supplementation in KSOM, not by promoting the blastocyst formation, but by improving of blastocyst quality with the evidences of increasing the proportion of hatching and total cell numbers. The authors found that total cell numbers of blastocysts grown in KSOM-LIF were higher than blastocysts grown in KSOM and embryos grown in KSOM-LIF reached the stage of hatching in a higher proportion than KSOM after having been cultured for 6 days. The increasing of total cell numbers in blastocyst in the present study related to the increasing of hatching embryos as the in vitro hatching is initiated as soon as a sufficient number of embryonic cells are available to overcome the resistance of the zona pellucida[23]. The increase in total cell numbers may be enhanced by LIF supplementation in KSOM as LIF can increase the proliferative activity of cell in blastocyst embryos especially the ICM[21].

Thirteen independent ES cell lines described in the present study were established from in vitro produced blastocysts, cultured in KSOM or KSOM-LIF. The authors found that the culture media in which blastocysts were produced does not influence the success rates of ES cell establishment. The efficiency in establishing ES cell lines was not enhanced when using embryos cultured in KSOM-LIF even when the total cell numbers were increased. The establishment of ES cell lines derived from in vitro produced blastocysts in the present study is comparable to that in other studies using the same strain of mouse[3,4].

In the standard ES cell derivation method reported by Robertson (1987)[8], mouse ES cell lines can be established by using the culture medium containing fetal bovine serum (FBS). In the present study knock-out serum replacement (K-SR) was used instead of FBS. The authors found that expanded blastocysts were able to hatch from zona pellucida, form ICM outgrowth and eventually ES cells can be established. It is generally accepted that K-SR promoted the proliferation of undifferentiated cells and enhanced the derivation of ES cells[24]. ES cells derived in the present study exhibited pluripotent markers of murine ES cells as seen by the positive characterization of AP, SSEA-1 and Oct-4. ES cell lines are able to maintain the undifferentiated status during in vitro cultivation and able to form EBs after induced differentiation by the hanging drop method. In addition, one ES cell line can contribute to germ-line chimera and it will be used for generating a mouse model for human diseases and the study of regenerative medicine in a further study.

Conclusion

The present study demonstrated that the quality of in vitro produced blastocysts, with the evidence of hatching blastocyst and the total cell numbers in blastocysts was improved by LIF supplementation in the culture medium. ES cell lines can be derived from both types of in vitro produced embryos with similar efficiency.

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ผลของ Leukemia inhibitory factor (LIF) ต่อคุณภาพของตัวอ่อน จำนวนเซลล์ระยะบลาสโตซิสของหนูที่เจริญภายนอกร่างกาย และการสร้างเซลล์ต้นกำเนิดจากตัวอ่อน

รัฐจักร รังสิวิวัฒน์, ศศิธร รุ่งอรุณเลิศ, ปราณี นำชัยศรีค้า, กำธร พฤกษานานนท์, มงคล เตชะกำพุ, ประมวล วีรุตมเสน

วัตถุประสงค์: *เพื่อศึกษาผลกระทบของ LIF ต่อคุณภาพของตัวอ่อนระยะบลาสโตซิสของหนูที่เลี้ยงภายนอกร่างกาย และประสิทธิภาพในการสร้างเซลล์ต้นกำเนิดตัวอ่อน*

ชนิดของการวิจัย: *การวิจัยแบบทดลอง*

สถานที่ทำการวิจัย: *หน่วยชีววิทยาการเจริญพันธุ์ ภาควิชาสูติศาสตร์นรีเวชวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย*

วัสดุและวิธีการ: *เก็บตัวอ่อนระยะไซโกตที่ปฏิสนธิในร่างกาย เลี้ยงในน้ำยาเลี้ยงตัวอ่อนชนิด KSOM ที่เติม LIF ในระดับ 1000 ยูนิต ต่อ มล. ประเมินคุณภาพของตัวอ่อน จากอัตราการเจริญถึงระยะบลาสโตซิส, จำนวนเซลล์ในตัวอ่อนระยะบลาสโตซิส และผลของการสร้างเซลล์ต้นกำเนิดตัวอ่อนจากตัวอ่อนระยะบลาสโตซิสที่เจริญในน้ำยาเลี้ยงทั้งที่เติมและไม่เติม LIF*

การประเมินผล: *ประเมินคุณภาพและจำนวนเซลล์ของตัวอ่อนระยะบลาสโตซิสและประสิทธิภาพการสร้างเซลล์ต้นกำเนิด*

ผลการศึกษา: *เปรียบเทียบการเจริญของตัวอ่อนที่เลี้ยงในน้ำยาที่เติม LIF และไม่เติม LIF พบว่า การเติม LIF ในน้ำยาเลี้ยงตัวอ่อน KSOM ช่วยเพิ่มอัตราการหลุดจากเปลือกหุ้มของตัวอ่อนอย่างมีนัยสำคัญทางสถิติ (63.8 % และ 53.7%, $p < 0.05$) และเพิ่มจำนวนเซลล์ในตัวอ่อนระยะบลาสโตซิสอย่างมีนัยสำคัยทางสถิติ (91.4 ± 15.0 และ 85.1 ± 7.7, $p < 0.05$) แต่ไม่เพิ่มประสิทธิภาพการสร้างเซลล์ต้นกำเนิดจากตัวอ่อน (66.7% และ 41.7%) เซลล์ต้นกำเนิดจากตัวอ่อนที่สร้างได้จากการศึกษานี้ สามารถแสดงคุณลักษณะที่สำคัญที่บ่งชี้ถึงการเป็นเซลล์ต้นกำเนิดตัวอ่อน*

สรุป: *LIF เพิ่มคุณภาพของตัวอ่อนระยะบลาสโตซิสที่เจริญภายนอกร่างกาย โดยเพิ่มอัตราการหลุดจากเปลือกหุ้มตัวอ่อน และเพิ่มจำนวนเซลล์ภายในตัวอ่อน แต่ไม่เพิ่มประสิทธิภาพของการสร้างเซลล์ต้นกำเนิดจากตัวอ่อน*