Original Article

Relevance of LIF and EGF on Mouse Preimplantation Embryo Development

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Abstract

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Objective: Recent evidence suggests that Leukemia Inhibitory Factor (LIF), a member of interleukin-6 family, has biological actions on preimplantation embryo development. Also it is established that Epidermal Growth Factor (EGF), a strong mitosis-promoting agent, improves the preimplantation embryo development by increasing the cell metabolism and proliferation. The purpose of the present study is to investigate the effects of these factors, alone and in combination together, on preimplantation and development of the embryo.

Materials and Methods: Six to eight weeks old NMRI mice were super ovulated by injection of 10IU PMSG and 10IU hCG, then the mated mice were killed 46 hours later. Their oviducts were flushed, two-cell embryos collected and divided randomly to the four groups as following: Control, treatment 1 (LIF), treatment 2 (EGF), treatment 3 (LIF+EGF). In each group, the embryos were cultured in an incubator at 37°C with 5% CO_2 and 90% humidity for 72hrs. The state of embryo development was evaluated in 24,36,48,60 and 72hrs following the embryos cultures. By the end of the cultures, cell apoptosis was studied by the terminal deoxynucleotidyl transferas-mediated dUTP nick end-labeling (TUNEL) technique.

Results: Significant difference was detected in the rate of hatching in the LIF and LIF+EGF groups. This difference was also seen in the rate of blastocyst formation after 36hrs ($p<0.05$) and in the average of the total cell number ($p<0.05$) after 72hrs. In comparison to the apoptotic index, there was no significant difference between the control and treatment groups.

Conclusion: The findings in this study show a beneficial effect of LIF and EGF on the blastocyst formation, hatching and its total cell numbers in vitro.

Keywords: LIF, EGF, Mouse, Preimplantation Embryo

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Introduction

Despite of years of experience and many technical improvements in human in vitro fertilization and embryo transfer, a low implantation rate is still one of the major unsolved problems in this filed (1).

One of the factors associated with poor success rates in assisted reproductive technique (ART) is the suboptimal culture condition in which fertilization and early embryonic growth occur. The problems like: Embryo fragmentation, cell irregularity, reductions in total cell numbers, retarded cleavage rate ,and blastulation observed in blastocysts growth in vitro attribute to suboptimal in vitro conditions, which in turn contribute significantly to reduced embryo quality and implantation rate following embryo transfer (1, 2).

The results of recent studies about the optimal condition for growth and development of preimplantation embryo demonstrate the embryo, in vivo, is exposed to a number of embryotrophic factors and interactions between embryo and reproductive epithelial cells which are essential o embryo development (3-10). The secretion of human reproductive epithelial cells such as colony-stimulating factor-1, Interleukin-6 (IL-6), leukemia Inhibitory Factor (LIF), and Epidermal Growth Factor (EGF) as well as expression of their receptors on preimplantation embryos suggest a dynamic interaction between embryo and reproductive epithelial cells during the first week of embryonic period. These dynamic interactions are not present in the current static in vitro culture systems used in ART (1).

Improved development capacity following co-culture of embryos using oviduct epithelial cells indirectly suggests that the growth factors releases from these cells are responsible for the positive effects of co-culture on embryos (1, 11).

Supplementation of media by some of these factors such as EGF and LIF and their beneficial effects on embryo development are reported in recent years (11-14). For example, higher blastocyst formation rate and increased their total cell number, in media enriched with

EGF, were demonstrated in vitro (10). EGF is a strong mitosis-promoting agent that stimulates the proliferation of different types of cells. This factor improves the preimplantation embryo development by increasing the cell metabolism and proliferation (15, 16)
On the other hand, it is reported that LIF plays an important role in the embryo development (17). LIF enhances in vitro blastosyct development in mice (18, 19), in vitro blastocyst hatching in sheep and pregnancy rates for embryos cultured in vitro and transferred back into recipient ewes (20). It is reported that the expression of LIF in the endometrium is absolutely essential for mouse embryo implantation (12, 18).
In spite of positive effects in vitro, it is important to carefully study development of embryo before any supplementation is introduced into culture media. Supplementation of growth factors and cytokines to culture media require more caution because of their effects on cell proliferation and differentiation. Also, preliminary animal studies should be conducted to evaluate risks carefully and benefits of these factors before their applications to human embryo culture. The purpose of the present study is to investigate and compare the effects of EGF and LIF, alone and in combination together, on mouse preimplantation embryo development, cell proliferation and apoptosis in vitro.

Materials and Methods

Six to eight week-old NMRI female mice were kept under the controlled light and temperature conditions with free access to water and food. All animal experiments were approved by the institutional animal care and use committee. They were superovulated by intraperitoneal injections of 10IU of pregnant mare serum gonadotropin (PMSG), and then followed 48hrs later with 10IU of hCG. On the same day of hCG injection, designated as day 0, the female mice were paired with male mice of the same strain to mate. The mated females were then killed 46hrs after another hCG administration and two-cell stage embryos harvested from the oviducts in pre-warmed HEPES-buffered/HTF medium. After washing several times, the obtained 2-cell embryos with the normal morphology were divided randomly into the four groups of 15-20 embryos, cultured in droplets of human tubal fluid (HTF) medium supplemented with 4mg/ml bovin serum albumin (BSA, fraction V, Sigma) under mineral oil at 37ºC in 5% CO_2 for 72hrs. The experiment was repeated 7 times and totally 132-149 embryos were examined in each group.
In group I, the embryos were cultured in HTF+4mg/ml BSA as the control group. In group II (treatment1), the embryos were cultured in HTF+4mg/ml BSA contained 1000IU/ml of LIF. In group III (treatment 2), the embryos were cultured in HTF+4mg/ml BSA contained 10ng/ml of EGF. In group IV (treatment 3), the embryos were cultured in HTF+4mg/ml contained 10ng/ml of EGF and 1000IU/ml of LIF. The media in all groups were replaced every 24hrs. Embryonic development was evaluated 24,36,48,60 and 72hrs after culturing by morphological observation under an invert microscope. In all groups, the following embryos' developments were noticed: four cells, eight-cell, morula, blastocyst, expanded blastocyst and hatching blastocyst stages. At the end, the expanded and hatched blastocysts were collected and used to analysis of apoptotic cell and total cell number (TCN).

Apoptosis Detection

A combined technique for simultaneous nuclear staining and TUNEL by modification of the procedures of Brison and Scholtz (21) was used for apoptosis detection.
Briefly, the embryos were fixed overnight at 4ºC in 3.7% paraformaldehyde diluted in PBS. Following fixation, they were washed 4 times in PBS/PVP, permeabilized in PBS containing 0.1% Triton-X100 for 1hr and incubated in flurescein conjugated- dutp and TdT (TUNEL reagents), (Boehringer Mannheim, Germany) at 37ºC for 1hr. After TUNEL, the embryos were washed 3 times in PBS/PVP and incubated in RNAse (50 mg/ml incubated in PBS containing propidium iodide (PI, 50µg/ml)) for 1hr at the room temperature to label all nuclei.
Finally, they were washed 4 times in PBS/PVP, mounted on a glass slide and examined for 30 days under a fluorescence microscope with an excitation filter of 460-490 nm and a barrier filter of 514nm.
The number of total cells and nuclei labeled by TUNEL were counted and for each of the blastocyst, the incidence of TUNEL positive nuclei was calculated as percentage of the total cell number.

Statical analysis

The percent of embryos reaching each developmental stage, total cell number (TCN) and apoptotic cell indices were compared by one-way analysis of variance (ANOVA) using SPSS version 10. All post-hoc analyses corrected by the Bonferroni method for multiple group comparison $p<0.05$ was considered statistically significant.

Results

Table1 illustrates the effects of LIF and EGF on the development of 2 cell embryos (Fig.1A) after 24hrs of the culture. As shown in the table, there was not any significantly different about the mean of percentage of eight-cell, morula and fragmented embryos, between the control and treatment groups. In the case of blastocyst formation (Fig 1B), the rate of blastocyst formation in LIF+EGF group after 36hrs culture was significantly higher in comparison to the other groups ($p<0.05$).
But after 48h, 60h and 72h, there were no significant difference between the groups (Fig 2).
Also, the rate of hatching in groups of LIF and LIF+EGF was significantly higher than the other groups (Fig 3).
Total cell number (TCN) in LIF and LIF+EGF ($p<0.05$), in

comparing with the other groups were significantly higher (Fig 4).

Table 1: The number of 8-cell, morola and fragmented embryos in the study groups on 24hrs after culturing (the study was repeated 7 times). There is not any significant differences between the four groups (p<0.05)

Groups	Eight-cell embryo (٪mean±SE)	Morula (٪mean±SE)	fragmented embryo (٪mean±SE)
Control (HTF)	42/139 (30.28±8.1)	83/139 (60.45±8)	14/144 (9.25±3.8)
Treatment 1 (LIF)	49/144 (33.45±5.1)	85/144 (59/59±5.3)	10/144 (6.9±2)
Treatment 2 (EGF)	43/136 (30.38±6.91)	82/136 (61/30±7.9)	11/136 (8.31±2.2)
Treatment 3 (LIF+EGF)	18 /122 (15.07±3.5)	97/122m (73.49±12.73)	7/122 (5.59±4.87)

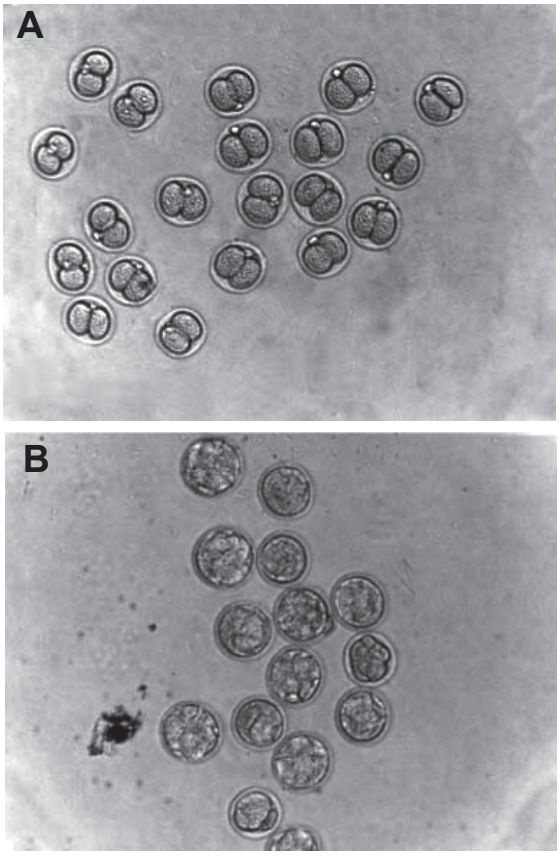

Fig 1: Mouse embryos at two-cell (A) and blastocyst stage (B)

In comparing the apoptotic index, there was no significant difference between the control and treatment groups (fig 5, 6).

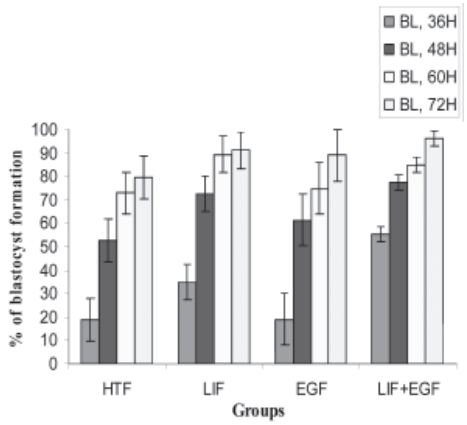

Fig 2: Effects of EGF and LIF on blastocyst formation rate in preimplantation embryos on 36, 48, 60 and 72hrs after culturing. Among all groups, the LIF and LIF+EGF groups showed significant differences with the control and EGF group on 36hrs after culturing (p<0.05). There is not any significant differences among the groups on 48, 60 and 72hrs after culturing (mean±SE).

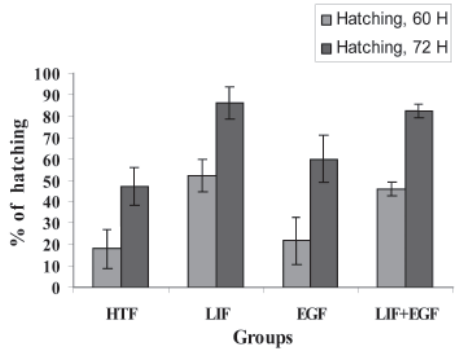

Fig 3: Effects of EGF and LIF on hatching rates in preimplantation embryos on 60 and 72hrs after culturing. Among all groups, the LIF and LIF+EGF groups showed significant differences with the control and EGF group (mean± SE) (p<0.05).

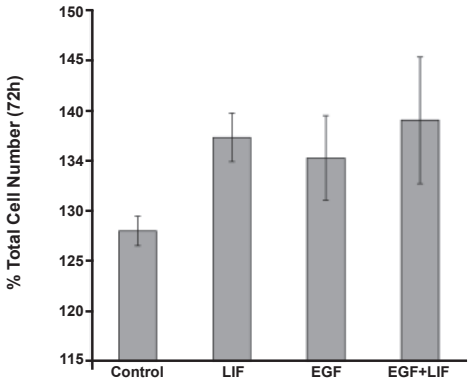

Fig 4: Effects of EGF and LIF on total cell number (TCN) in preimplantation embryos on 72hrs after culturing. Among all groups, the LIF and LIF+EGF groups showed significant differences with the control and EGF group (mean±SE) (p<0.05).

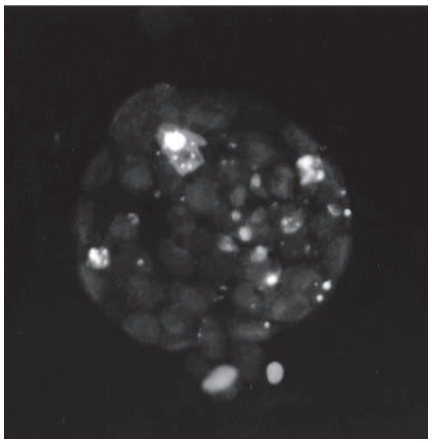

Fig 5: A blastocysts from the control group after TUNEL. The apoptotic nuclei that labelled by TUNEL are shown in green and the normal nuclei are shown in red. (This figure has also been printed in full-color at the end of the issue)

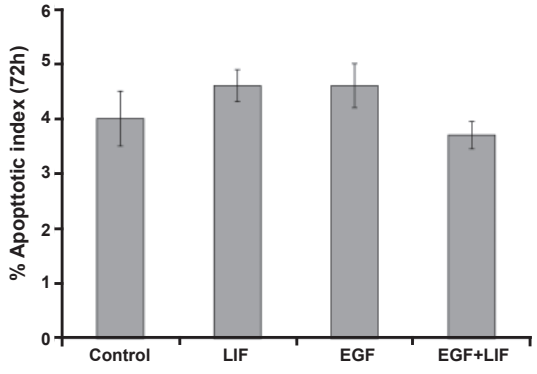

Fig 6: Effects of EGF and LIF on apoptosis index in preimplantation embryos on 72hrs after culturing. There were not any significant differences among the groups (mean±SE).

Discussion

In this investigation, it was demonstrated that the addition of LIF and EGF to the culture medium had no significant stimulatory and inhibitory effects on the development of early cleavage stage (2-cells, 4-cells, 8-cells and morula) and apoptotic cell death in mouse blastocyst. But LIF and EGF in combination increased the rate of the blastocysts formation on 36hrs after culturing as well as hatching rate and total cell number on 72hrs after culturing. These data are comparable with previous reports about the effects of theses factors on the development of the preimplantation embryo (12, 20, 22).

For example, Jurisicova in 1995 have demonstrated that the culture medium supplemented with rhLIF in the concentrations of 5-20 ng/ml had no effect on human blastocyst formation when used in very early cleavage stages in vitro (23). Lavranos you have to mention the year!!! explained the supplementing of the embryo culture media with 1000 IU/ml of rhLIF increased the number of eight-cell mouse embryos developing beyond the hatched blastocyst stage (24). In the present investigation, we used concentration of 1000 IU/ml rh-LIF and achieved similar results to Lavranous finding about the blastocysts formation and hatching. But we did not see any advantage for using LIF in the early stages of embryo development (2-cells, 4-cells,8-cells and morula) and it might be depends on the different requirements in the various stages of the embryo development.

However the previous studies showed the use of 10 ng/ml of EGF in the culture medium increasing the rate of blastocysts formation (10, 25), our study revealed a nonstimulatory and noninhibitory effects of LIF when added to the culture medium at the early cleavage stages. Furthermore, this study showed the use of EGF in the culture medium not promoting statistically significant improvement in the rate of blastocyst formation. Altogether, our results showed the use of LIF, alone or in combination with EGF, significantly improveed blastocysts formation, hatching rate and total cell number. Additional synergistic effects could not be observed by combining LIF and EGF.

Conclusion

The findings of this study show beneficial effect of LIF and EGF on the mouse blastocysts development in vitro. In spite of the indicated effects, it is strongly recommended that more animal studies should be conducted to evaluate its safety with particular attention to its potential teratogenic effects and long-term outcome of offspring before application to human-assisted reproductive program.

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References

1. Lai-Ping Cheung, Ho-Yin Leung. Ariff Bongso: Effect of supplementation of leukemia inhibitory factor and epidermal growth factor on murine embryonic development in vitro, implantation, and outcome of offspring. Fertil Steril 2003; 80, Suppl. 2: 727-753

2. Hardy K, Handyside AH, Winston RML. The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Develop 1989; 107: 597-604

3. Bhatt H, Brunet LJ, Stewart CL. Uterine expression of leukemia inhibitory coincides with the onset of blastocyst implantation. Proc Natl Acad Sci USA 1991; 88: 11408-11412

4. Harvey MB, Leco KJ, Arcellana-Panlilio MY, Zhang X, Edwards DR, Schultz GA. Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor. Develop 1995; 121: 1005-1014

5. Cullinan EB, Abbondanzo SJ, Anderson PS, Pollard JW, Lessey BA, Stewart CL. Leukemia inhibitory factor

(LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. Proc Natl Acad Sci USA 1996; 93: 3115-3120
6. Lass A, Weiser W, Munafo A, Loumaye E. Leukemia inhibitory factor in human reproduction, Fertil Steril 2001; 76: 1091-1096
7. Robb L, Dimitriadis E, Li R, Salamonsen LA. Leukemia inhibitory factor and interleukin-11: cytokines with key roles in implantation, J Reprod Immunol 2002; 57: 129-141
8. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F. Blastocyst implantation depends on marenanal expression of leukemia inhibitory factor. Nature 1992: 359: 76-77
9. Michell MH, Swanson RJ, Hodgen GD, Oehninger S. Enhancement of in vitro murine embryo development by recombinant leukemia inhibitory factor. J Soc Gynecol Investing 1994; 1: 215-219
10. Kane MT, Morgan PM, Coonan C. Peptide growth factors and preimplantation development. Hum Reprod Update 1997; 3: 137-157
11. Richter KS. The importance of growth factors for preimplantation embryo development and in-vitro culture. Curr Opin Obstet Gynecol 2008; 20(30): 292-304
12. Desai N, wason J, Goldfarb J. Assessment of growth factor effects on post thaw development of cryopreserved mouse morula to the blastocyst stage. Hum Reprod 2000; 15: 410-418
13. Grupen CG, Nagashima H, Nottle MB. Role of epidermal growth factor and insulin-like growth factor-I on porcine oocyte maturation and embryonic development in vitro, Reprod Fertil Develop 1997; 9: 571-575
14. Mitchell MH, Swanson RJ, Hodgen GD, Oehninger S. Enhancement of in vitro murine embryo development by recombinant leukemia inhibitory factor. J Soc Gynecol Investing 1994; 1: 215-219
15. Cheung Wong RW, Guillaud L. The role of epidermal growth factor and its receptors in mammalian CNS. Cytokine Growth Factor Rev 2004; 15: 147-156
16. Yamada M, Ikeuchi T, Hatanaka H. The neurotrophic action and signaling of epidermal growth factor, J. Prog Neurobio 1997; 51: 19-37
17. Tsai HD, Chang CC, Hsieh YY, Lo HY, Hsu LW, Chang SC. Recombinant human leukemia inhibitory enhances the development of Preimplantation mouse embryo in vitro. Fertil Steril 1999; 71: 722-725
18. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F. Blastocyst implantation depends on marenanal expression of leukemia inhibitory factor. Nature 1992; 359: 76-77
19. Michell MH, Swanson RJ, Hodgen GD, Oehninger S. Enhancement of in vitro murine embryo development by recombinant leukemia inhibitory factor. J Soc Gynecol Investing 1994; 1: 215-219
20. Fry RC, Batt PA, Fairclough RJ, Parr RA. Human LIF improves the viability of bovine embryos. Biol. Reprod 1992; 46: 470-474
21. Brison D, Schultz R. Apoptosis during mouse blastocyst formation; evidence for role of survival factors including transforming growth factor α1. Biol reprod 1997; 56:1088-1096
22. Marqunat-le Guinne B, Humbolt P, Guillon N, Thibier M. Murine LIF improves the development of IVF cultured bovine morula. Reprod Fertil 1993; 12: 61-67
23. Jurisicova A, Ben-Chetrit A, Varmuza SL, Casper RF. Recombinant human leukemia inhibitory factor does not enhance in vitro human blastocyst formation. Fertil Steril 1995; 64: 999-1002
24. Lavranos TC, Rathjen PD, Seamark RF. Trophic effects of myeloid leukaemia inhibitory factor (LIF) on mouse embryos. Reprod Fertil Dev 1995; 105: 331-338
25. Zhou P, Liu DJ, Cang M, Ma YZ, Yang DS, Li HJ, et al: TGF- and EGFR in ovine preimplantation embryos and effects on development, Anim Reprod Sci 2007; 10. 1016/j.anireprosci.02.024