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The effects of LIF and EGF on mouse oocyte maturation, fertilization and development in vitro

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Abstract

Background: Mammalian oocytes are exposed to a mixture of many different growth factors and cytokines which provides an optimized microenvironment for oocyte maturation. In the lack of this natural microenvironment in vitro, the quality of oocyte and embryos appears to be suboptimal.

Objective: This study was undertaken to investigate the effects of EGF and LIF on in vitro maturation, fertilization and cleavage rates in mouse oocytes.

Materials and Methods: The GV oocytes were collected from female NMRI mice and randomly divided into control and 3 treatment groups. Oocytes in treatment groups were cultured in the maturation medium supplemented with 50 ng/ml rhLIF (Treatment 1), 10ng/ml EGF (Treatment 2) and 50 ng/ml LIF+ 10ng/ml EGF (Treatment 3) for 24 hours at 37°C in humidified 5% CO_2 in air. The matured oocytes were fertilized in vitro and cultured for 96 hours. Finally, the developmental rates were assessed and embryos were stained using Hoechst 33258.

Results: There was a higher maturation rate in treatment groups compared to the control group. There was not any significant difference in the rate of fertilization among the groups. The rates of cleavage (79.1%) and blastocyst formation (62.2%) were significantly higher in LIF + EGF group comparing to the other groups. The rates of hatching in groups treatment 1 (35.2%) and 3(41%) was significantly higher comparing to the other groups. Also the mean of total cell number in treatment groups significantly was higher than control ($p< 0.05$).

Conclusion: The findings of this study suggest a beneficial effect of LIF and EGF on mouse oocyte maturation and cleavage rates.

Key words: *LIF, EGF, IVM, IVF, Embryo development, Mouse.*

Introduction

Recently, many attempts have been done to grow immature oocytes in vitro. Maturation of oocytes in vitro can be used not only to investigate the effects of endogenous and exogenous factors on folliculogenesis and oocyte quality but also in treatment of human infertility (1-3).

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In spite of many reports about successful maturation and development of mammalian immature oocytes in vitro (4-6), the quality of maturation appears to be suboptimal because embryos resulting from in-vitro matured oocytes show more frequent cleavage blocks and overall retarded cleavage rates as compared to oocytes matured in vivo (7, 8).

Oocyte maturation can be conceptually divided into nuclear and cytoplasmic procedures; the first refers to the resumption of meiosis and progression to metaphase II, the later refers to cytoplasmic events that prepare the oocyte for fertilization and preimplantation embryo development (9, 10). It is

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known that insufficient cytoplasmic maturation of the oocyte will fail to promote male pronuclear formation and will thus increase chromosomal abnormalities after fertilization (9).

Therefore, developing an optimal culture system is essential to improve quality of oocytes matured in vitro. There are several ways to improve oocyte quality during in vitro maturation. One way to improve the in vitro development of oocytes and embryos is co-culturing with somatic cells (11-14).

Improved developmental capacity following co-culture of embryos using oviduct epithelial cells, indirectly suggest that growth factors release from these cells are responsible for the positive effects of co-culture on embryos(14).

An alternative method to improve oocyte quality is supplementation of maturation media by growth factors and cytokines. The natural ovarian microenvironment provides the hormones and factors locally, for example, kit ligand, leukemia inhibitory factor (LIF), epidermal growth factor, bone morphogenetic proteins, keratinocyte growth factor, and basic fibroblast growth factor, that promote the ovarian follicle maturation (15, 16). LIF is a pleiotropic cytokine with a range of molecular weight 38–67 kDa and a remarkable range of biological actions in various tissues (17). LIF is present in follicular fluid, and its concentration rises around the time of ovulation (18). Expression of LIF and LIF receptor in human fetal and adult ovary and their roles in transition of primordial to primary follicle in rat ovary were reported (19, 20).

On the other hand, it has been shown that co-culture of preimplantation embryos with cells that express LIF enhance mouse blastocyst development in vitro (21). There are some other reports, in which LIF was added to culture medium and findings indicated that LIF is important in ovarian physiology (22-25). But the role of LIF in folliculogenesis has not been known clearly yet and also there is very limited research on its use in supplementing media for maturation of follicles in vitro.

Epidermal growth factor (EGF) is the other growth factor that its role in embryo development has been identified previously (22, 24, 25). EGF is a strong mitosis-promoting agent that stimulates the proliferation of different types of cells. The effects of EGF on embryo are still not clear well, but it has been shown that this factor improves the preimplantation embryo development by increasing the cell metabolism and proliferation and promotes nuclear and oocyte cytoplasmic maturation in a wide range of mammalian species (22, 24-26).

However, there are other reports that indicate EGF does not improve the cytoplasmic maturation of oocytes (27).

Due to these limited data and controversial reports about the effects of LIF and EGF on maturation and developmental capacity of embryo in vitro, this study was undertaken to investigate the effects of EGF and LIF alone and in combination on in vitro oocyte maturation, fertilization and cleavage rates in mouse embryo.

Materials and methods

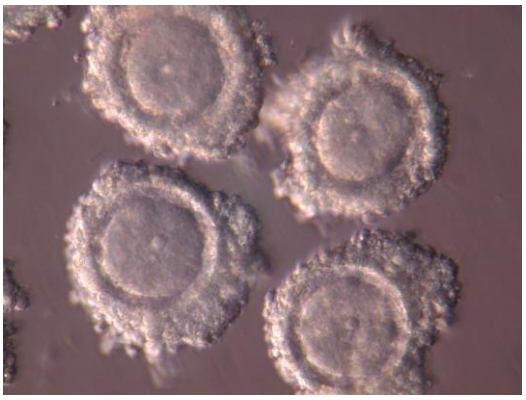
GV oocyte collection and in vitro maturation

Male and female NMRI mice were kept under controlled light and temperature conditions with free access to water and food. Overall 35 female mice (4 weeks old) were injected with 7.5 IU of pregnant mare serum gonadotropin (PMSG, Nasr, Iran) and were killed 44-46 hours later by cervical dislocation.

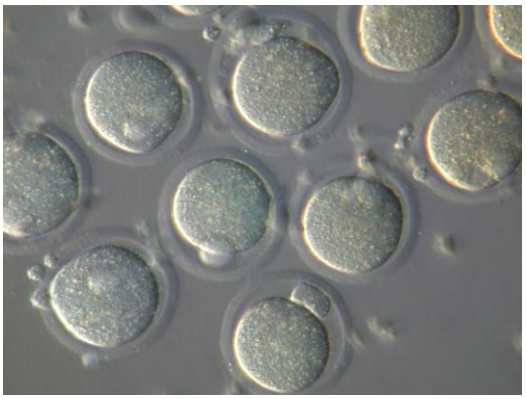
The ovaries were cut and collected in 1ml of HEPES-buffered Waymouth medium (Sigma) and the cumulus-enclosed oocytes were obtained by puncture of the antral follicles. Only oocytes with a uniform covering of cumulus cells (Figure1A) were used in this study.

After washing three times in HEPES-buffered Waymouth medium, the GV oocytes randomly divided into 4 groups that carried out in micro drops of maturation medium under mineral oil. Oocytes were cultured as the control group in Waymouth medium [containing 0.075 IU follicle stimulating hormone (FSH), 0.075 IU human chorionic gonadotropin (HCG)] + 15% fetal bovine serum (FBS; Gibco). Ova in treatment 1, 2 and 3 groups were cultured in the same medium supplemented with 50 ng/ml LIF (Sigma) (treatment 1), 10 ng/ml EGF (Sigma) (treatment 2) and 50 ng/ml LIF + 10 ng/ml EGF (treatment 3.). The experiments started with approximately 140 GV oocytes and replicated 5 times. In each experiment, the groups of up to 40 oocytes were placed in 50-µL microdrops of medium (5 oocytes in each droplet) under mineral oil (Sigma) within 35 × 10 mm Petri dishes (Falcon) and were stored at 37°C in humidified 5% CO_2 in air. 24 hour later, all oocytes were denuded using hyaluronidase (Sigma) and mechanical pipeting, and their maturation was assessed. Polar body extrusion observed under the inverted microscope (×200 magnification) was used as the maturation criterion (Figure1B).

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A



B

Figure 1. A) GV stage oocyte at the beginning of maturation. B) Matured (MII) stage oocytes after 24 hours culture in vitro (Magnification is 20X).

In vitro fertilization and cleavage

The epididymal spermatozoa were retrieved from the cauda epididymis of 10 to 12-week-old NMRI mice. The sperm suspension (1 $\times 10^6$ motile spermatozoa/ml) was capacitated for 1.5-2 hr in 400 µl of T6 medium supplemented with 5 mg/ml BSA. In vitro matured (MII stage) oocytes from each group were placed into 0.9 ml T6 then 0.1 ml capacitated spermatozoa were added. Inseminated oocytes were washed away from sperm by gentle pipetting 6-8 hours later, and fertilization was assessed by presence of two pronucleus (2PN). The oocytes were than cultured in droplets of T6 medium under mineral oil for 96 hour. The rates of 2-cell embryos, and blastocyst were assessed 24, and 96 hours later. Finally, the expanded and hatched blastocysts were collected and used to analysis total cell number (TCN) using Hoechst 33258 (Sigma) stain. Briefly, blastocysts were washed in PBS several times then treated with 0.5% (w/v) pronase (Sigma) in PBS for 3–5 min to dissolve the zona pellucida. Then zona-free and hatched blastocysts were placed in cold absolute ethanol (Merck, Germany) containing 10 μg/ml bisbenzimide (Hoechst 33258, Sigma) for 30 min at 4°C. This resulted in the staining of all cell nuclei. Finally, embryos were washed in absolute ethanol, then mounted in undiluted glycerol (Merck, Darmstadt, Germany) and squashed on a glass slide. The stained embryos were observed directly using a fluorescent microscope (Olympus, Japan) an ultraviolet excitation filter of 365 nm and a barrier filter of 420 nm. Bisbenzimide-stained cell nuclei appeared blue, the total cell number (TCN) for each blastocyst was counted.

Statistical analysis

Differences in the rates of maturation, fertilization, cleavage, blastocyst formation and hatching were analyzed with χ^2 test or Fisher exact test as appropriate. Differences in the mean of total cell number among the groups were analyzed by one way ANOVA and Tukey post-test. SPSS version 17 was used for all statistical analysis. Values were considered significant when $p < 0.05$.

Results

A total of 733 GV stage oocyte retrieved from 35 female mice were included in this investigation. The rates of matured, germinal vesicle break down (GVBD) and degenerated oocytes in the control and treatments groups are shown in table I. As shown in the table, in treatment groups a higher maturation rates were obtained compared with the control group ($p<0.01$). But, there were not any significantly different in maturation rate between the treatment groups. The rates of fertilization, cleavage, blastocyst formation and hatching are shown in table II. The fertilization rates in control and treatment groups were 79.5%, 83.6%, 79.7%, and 87.8%, respectively. In comparison to the control group, the rate of fertilization was not significantly higher in the treatment groups. However, there were a higher but not statistically different number of fertilized oocytes in groups which treated with LIF (treatment 1 and 3). The rates of cleavage (2 cell embryo) and blastocyst were significantly higher in all of treatment groups in compared with control. The percentage of two-cell embryos (79.1%) and blastocyst (62.2%) in group which treated with LIF + EGF were significantly higher than the groups which treated LIF or EGF alone ($p< 0.001$). The hatching rates in the groups treatment 1 and 3 were significantly higher comparing with the other groups ($p<0.05$). Also, there was a significant difference between these groups (treatment 1 and 3) in total cell number (Figure 2 & 3), comparing with the other groups ($p< 0.05$).

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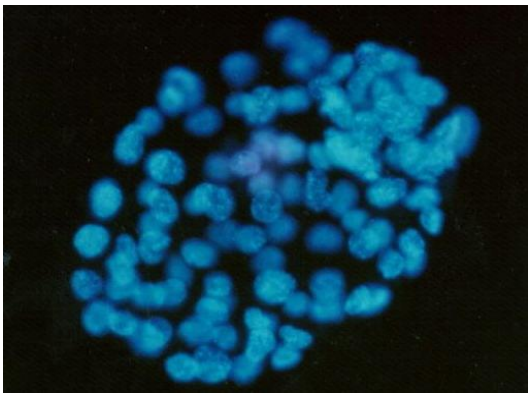


Figure 2. A blastocyst after staining with Bisbenzimide (Hoechst 33258). The stained cell nuclei under a fluorescent microscope (Olympus, Japan) using an ultraviolet excitation filter of 365 nm and a barrier filter of 420 nm appeared in blue (magnification is 40X).

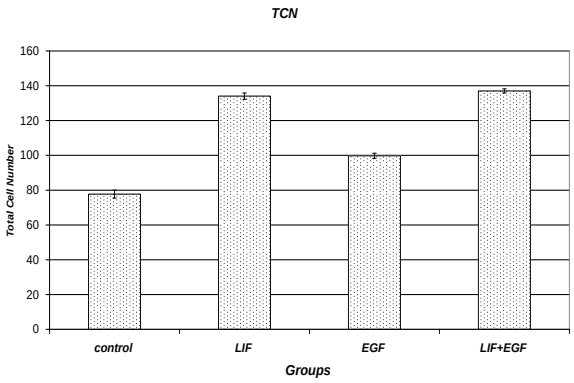


Figure 3. The mean (±SE) of total cell number (TCN) in control and treatment groups. As shown in the figure, there is a significant difference in LIF and LIF + EGF groups (treatment 1 and 3) comparing with the other groups ($p<0.05$).

Table I. Maturation rate of mouse oocytes after 24h culture. GV= germinal vesicle oocyte, GVBD=germinal vesicle breakdown oocyte.

Groups	No. of GV oocytes	No. (%) of GVBD oocytes	No.(%) of matured oocytes	No.(%) of undeveloped or degenerated oocytes
Control	189	62(32.8%)	112(59.3%)	15(7.9%)
Treatment 1(LIF)	181	43(23.7%)	128(70.7%)*	10(5.6%)
Treatment 2(EGF)	178	45(25.2%)	123(69.1%)*	10(5.7%)
Treatment 3 (LIF+EGF)	185	38(20.5%)	139(75.1%)*	8(3.4%)

* $p<0.01$

Table II. Fertilization, cleavage, blastocyst formation and hatching rates in control and treatments groups.

Groups	No.of oocyte	No.(%) of fertilized oocytes	No.(%) of cleaved oocytes	No.(%) of blastocyst	No.(%) of blastocysts hatched
Control	112	89(79.5%)	64(57.1%)	49(43.8%)	25(22.3%)
Treatment 1 (LIF)	128	107(83.6%)	89(69.5%)	67(52.3%)*	45(35.2%)*
Treatment 2 (EGF)	123	98(79.7%)	83(67.5%)	54(43.9%)	26(21.1%)
Treatment 3 (LIF+EGF)	139	122(87.8%)	110(79.1%)*	92(66.2%) **	57(41%) **

* $p<0.01$ ** $p<0.001$

Discussion

In this investigation, it was demonstrated that addition of LIF or LIF + EGF to the maturation medium, has significant stimulatory effect on the maturation of immature oocyte. Our data are comparable with previous reports. For example in agreement with our results, Das and his colleagues in 1992 showed the positive effect of EGF on human oocyte maturation (26). Also, De Matos *et al* in 2008 demonstrated that LIF induces cumulus expansion similarly in human and mouse cumulus-oocyte complexes, and recombinant follicle-stimulating hormone (FSH) plus LIF supplementation during mouse IVM significantly improved oocyte competence (23). Furthermore, Ptak *et al* in 2005 obtained significantly higher rates of pronuclear stage embryos from adult oocytes when medium was enriched with LIF (28).

In contrast to Ptak *et al* report, our results dose not show significant differences in fertilization rate. Also Haidari *et al* in 2008 showed that the developmental rates of immature oocytes treated with 50 ng/ml of LIF could increase during preantral to antral follicle transition but they did not find any significant difference between the maturation rate of MII oocytes and embryo development in the control and LIF-treated groups (29). In contrast to their report, our results show that LIF could increase the rate of maturation and embryo development. Similar to the previous reports (33, 34, 38), our results indicated the beneficial effects of EGF on oocyte maturation and embryo development. This study shows that the beneficial effects of LIF on oocyte maturation, cleavage rate and blastocyst formation are prominent in the presence of EGF. These synergistic effects may be is due to the biological

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properties of these factors. EGF is a strong mitosis-promoting agent that improves the preimplantation embryo development by increasing the cell metabolism and proliferation and promotes nuclear and oocyte cytoplasmic maturation (22, 24-26). Insufficient cytoplasmic maturation of the oocyte will fail to promote male pronuclear formation and increase chromosomal abnormalities after fertilization (9). Therefore addition of EGF to maturation culture media may promote nuclear and oocyte cytoplasmic maturation and affect positively on embryo development.

On the other hand, LIF may influence the follicular growth directly by its receptors on the follicular and theca cells and has an indirect effect on stimulating the other growth factors to improve oocyte and embryo development.

However the role of LIF in folliculogenesis has not been known clearly yet but our results shows positive effects of LIF and EGF on oocyte maturation in vitro and following embryo development. These findings confirm the theory which suggested that supplementation of maturation media by growth factors and cytokines provides an enviournment, like the natural ovarian microenvironment and improves oocyte and embryos quality.

In conclusion, our results suggest that LIF alone or in combination with EGF increases the maturation rate and embryo development. In spite of beneficial effects of EGF and LIF respectively on oocyte maturation and embryo development, it is strongly recommended that more animal studies should be undertaken to evaluate its safety, with particular attention to its potential teratogenic effects and the long-term outcome of the offspring, before it is applied to human-assisted reproductive programs.

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