The Effects of Different Concentrations of Leukemia Inhibitory Factor on the Development of Isolated Preantral Follicles from Fresh and Vitrified Mouse Ovaries

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ABSTRACT

Background: Leukemia inhibitory factor (LIF) is a pleiotropic cytokine of interleukin-6 family with a remarkable range of biological actions such as proliferative effects on the granulosa and theca cells. The aim of this study was to evaluate the effects of LIF on the growth and maturation of mouse fresh and vitrified preantral follicles, an *in vitro* model was developed. **Methods:** The ovaries of 14-day-old mice were vitrified in a mixture of ethylene glycol, ficoll 70 and sucrose in PB1 for 5 min. The preantral follicles were mechanically isolated from vitrified-warmed and non-vitrified ovaries. They were cultured in α-minimum essential medium supplemented with 5% fetal bovine serum, 100 mIU/ml recombinant follicle stimulating hormone, 1% insulin, transferrin and selenium, 20 ng/ml murine recombinant epidermal growth factor and different concentrations of LIF (25, 50, 100 ng/ml) for 12 days. On day 12, ovulation was induced using 1.5 mIU/ml human chorionic gonadotropin. In this study, the follicle diameter, survival rate and maturation rate were assessed. **Results:** The mean diameter of fresh and vitrified preantral follicles cultured in 50 ng/ml concentration of murine recombinant LIF was significantly higher than that of other concentrations in each group on day 2 (229.42 ± 30.40, 222.55 ± 33.4) (*P*<0.001) and on day 4 (340.45 ± 61.05, 299.50 ± 65.55), respectively (*P*<0.01). The survival rates of follicle in fresh and vitrified groups were 80.56% and 77.78, respectively. There was no significant difference between control and treated groups. The percentage of follicles which released metaphase II (MII) oocyte in fresh groups in the presence of 0, 25, 50, 100 ng/ml of LIF was 16%, 14.28%, 40% and 21.05% (*P*<0.01) and so in vitrified groups were 11.76%, 14.28%, 28.57% and 13.38%, respectively (*P*<0.05). There were significant differences between 50 ng/ml LIF-treated groups with other concentrations in each group. **Conclusion:** Therefore, *in vitro* growth and maturation of mouse preantral follicles were improved in the presence of 50 ng/ml LIF. *Iran. Biomed. J. 10 (4): 185-190, 2006*

INTRODUCTION

Many systems have been developed for *in vitro* culture and maturation of preantral follicles for studying oogenesis, folliculo genesis and oocytes-somatic cell interactions [1- 3]. Isolated follicles have been cultured in the media supplemented by serum, growth factors and hormones [2, 4] and also supported using collagen gels and collagen-impregnated membranes [4].

Ovarian follicles are known to produce peptides, proteins and growth factors which can either interact with the same cell type via autocrine action or with other cell types within the developing follicle as paracrine action, therefore involved in follicular development [4, 5]. The granulosa and theca cells can control their behavior via juxtacrine by activation of receptors on adjacent cells by membrane bound growth factors [5]. Leukemia inhibitory factor (LIF) is a pleiotropic cytokine of interleukin-6 family with a remarkable range of biological actions in various tissues [6, 7]. Although named for its ability to inhibit proliferation of a myeloid leukemic cell line by inducing differentiation [7- 10]. It also regulates the growth and differentiation of embryonic stem cells [11, 12], primordial germ cells [13], peripheral neurons [14], osteoblast [15], adipocytes [16] and endothelial cells [17] and also improves mouse bastocyst development *in vitro* [18]. LIF is presented in

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human follicular fluid and its level is regulated according to the stage of follicle development [6-8]. Its concentration in the follicular fluid rises around the time of ovulation and its levels correlate with that of estradiol in follicular fluid [6, 8] in responsive to human chorionic gonadotropin (hCG) [8]. It was shown that LIF may play a role in ovulatory events, embryonic development and implantation [6]. LIF also promotes the transition of primordial to primary follicle in cultured ovaries and may be an important element of regulatory pathways controlling recruitment of follicles from the primordial follicle pool [8]. It was demonstrated that both granulosa- lutein cells and ovarian stromal cells express LIF mRNA and produce this protein [6]. Fully grown mouse follicles *in vivo* reach diameters of approximately 500 µm [19] whereas *in vitro* grown follicles generally reach 400 µm [20] and there is a number of evidence which strongly suggest that *in vitro* follicle culture condition is not optimal yet [21]. Also, after cryopreservation of ovarian tissue, it needs to improve the development and maturation of follicles.

In this study, we try to improve the rates of growth and maturation of isolated preantral follicles from fresh and vitrified ovaries using different concentration of LIF in the base α-MEM media.

MATERIALS AND METHODS

Animal. Five female NMRI mice (14-day-old) used in this study were obtained from Razi Vaccine and Serum Research Institute and provided with food and water ad libitum and housed in 12 h light: 12 h dark. Their ovaries were removed and stored in α- minimal essential medium (α-MEM; Gibco, UK) supplemented with 5% fetal bovine serum (FBS; Gibco, UK) (replicated 3-5 times). They were divided randomly in fresh (non-vitrified) and vitrified groups.

Vitrification and warming of ovaries. The Vitrification procedure was based on the previous method [22]. Briefly, the ovaries were immersed in vitrification medium containing 40% (V/V) ethylene glycol, 30% (W/V) ficoll 70, 1 mol sucrose for 5 min at room temperature. The ovaries were placed in the straw with a minimum volume of the vitrification medium at room temperature, transferred to nitrogen vapor for 30 s and then immersed and stored in liquid nitrogen for one week.

For warming, vitrified samples were warmed at room temperature and then placed in 25°C water bath for 20 s. The contents of straw transferred into 1, 0.5 and 0.25 mol sucrose for 5 min at room temperature. The recovered ovaries were transferred to α- MEM supplemented with 5% FBS for washing and equilibration and then the preantral follicles were isolated.

Preantral follicle isolation. The preantral follicles from fresh and vitrified-warmed ovaries with a diameter of 140-170 µm were mechanically isolated using 29-guage needle under stereomicroscope. Isolated follicles containing several layers of granulosa cells with a centrally located healthy oocyte and a thin layer of theca cells were randomly selected and cultured in medium supplemented with different concentrations of murine recombinant LIF (control without LIF, 25, 50 and 100 ng/ml of LIF; Sigma, Germany) for 12 days.

The culture medium. The selected preantral follicles were cultured individually in 20 µl droplets of α- MEM (Gibco; UK) supplemented with 5% FBS, 100 mIU/ml recombinant follicle stimulating hormone (rFSH or Gonal-f; Serono, Switzerland), 1% insulin, transferrin, and selenium (ITS; Gibco, UK), 20 ng/ml murine recombinant epidermal growth factor (Sigma, Germany), 100 µg/ml penicillin and 50 µg/ml streptomycin under mineral oil in a humidified atmosphere of 5% CO_2 in air at 37°C for 12 days [23, 24]. The medium was refreshed by changing half of the droplet every other day. For each group, different concentrations of LIF (25, 50, 100 ng/ml) was added except for the control groups. Mean follicle diameter excluding the theca cell layer was estimated by measuring two perpendicular diameters at × 100 magnification with precalibrated ocular micrometer under inverted microscope on day 2 and 4 [23]. In addition, the survival rate of the follicles was checked by evaluation of follicle degeneration and loose oocyte every other day under inverted microscope during culturing period and they were compared at the end of the study [25]. At day 12 of culture, final oocyte maturation and ovulation was induced by the addition of 1.5 IU/ml hCG (Organon) to the media. Released oocytes were classified as germinal vesicle (GV), GV breakdown metaphase I (MI) and metaphase II (MII) after 16 to 48 h.

Statistical analysis. The survival, degeneration, antral formation and developmental rates of follicles

were assessed by chi-square and follicular diameter analyzed by ANOVA. $P<0.05$ was considered to be statistically significant.

RESULTS

In order to determine if LIF improves the *in vitro* development of mouse isolated preantral follicles, three concentrations of LIF were used to get the optimal condition. The isolated follicles were cultured in the absence (control) or presence of LIF (25, 50, 100 ng/ml).

By day 2, the follicles were immobilized by growing and adhesion of theca cells (Fig. 1A and B). After that granulosa cells were proliferated and grown through the basal membrane. The outgrowth of granulosa cells was irregular and also they had diffuse appearance (Fig. 2A and B). During *in vitro* culture period, follicle degeneration occurred by showing either spontaneous release of the oocytes or failure of further proliferation of granulosa cells.
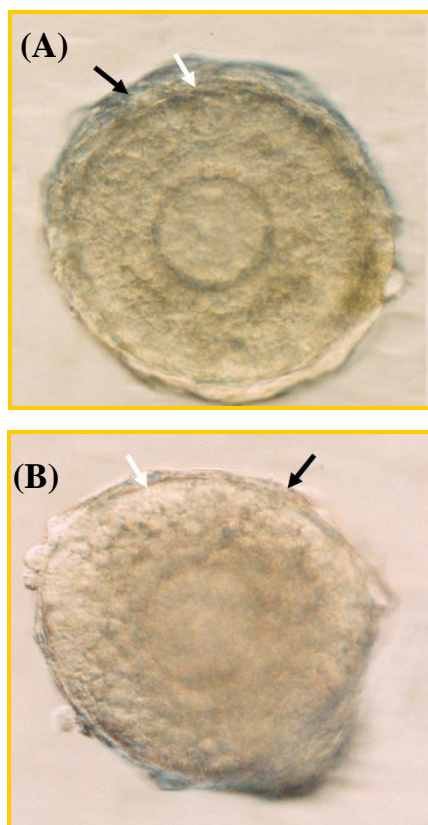

Fig. 1. **(A)**, Fresh isolated follicle after 48 h culturing with membrana granulosa (white arrow) and thin theca layer (black arrow); **(B)**, Vitrified isolated follicle × 200.
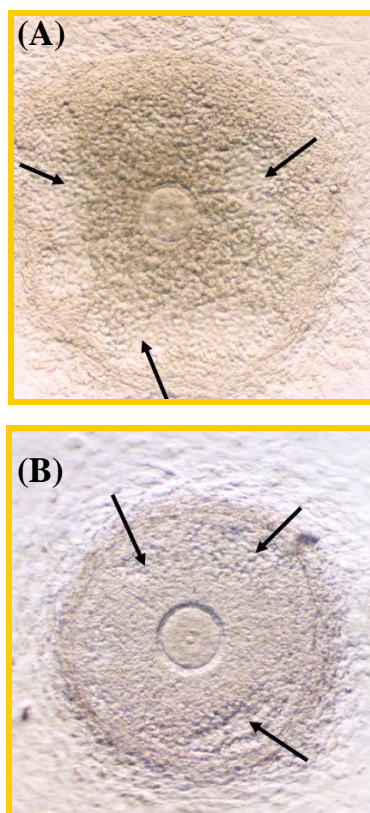

Fig. 2. **(A)**, Fresh isolated follicles after 8 days culturing with antral cavity (black arrows); **(B)**, Vitrified isolated follicles × 100.

The follicle diameter of fresh and vitrified preantral follicles was increased during *in vitro* culturing and their data were summarized in Table 1. As results show, the mean diameter of the fresh and vitrified follicles, which was cultured in medium containing 50 ng/ml LIF, were significantly higher on day 2 (229.42 ± 30.40 and 222.55 ± 33.4, respectively) and day 4 (340.45 ± 61.05 and 299.50 ± 65.55, respectively) in comparison with other treatments in the same groups ($P<0.001$).

By day 12 of culture, the survival rates of fresh isolated follicles cultured in α- MEM containing 0, 25, 50, 100 ng/ml LIF were 71.43%, 87.5%, 80.56%, 73.08% and that of vitrified follicles were 68%, 58.34%, 77.78%, 68.19%, respectively. There was no significant difference between control and LIF treatments in each group. Antrum formation was observed in some follicles from day 8 on (Table 2) however, there was no significant difference between groups. The percentage of follicles which released MII oocyte (Fig. 3) in fresh groups in the presence of 0, 25, 50, 100 ng/ml of LIF were 16%, 14.28%, 40% and 21.05% and so in vitrified groups

Table 1. Follicular diameter of fresh and vitrified isolated preantral follicles cultured in the absence and presence of different concentrations of LIF.

Group	LIF Concentration (ng/ml)	Follicular diameter (Mean ± SD; μm)		
		Day 0	Day 2	Day 4
Fresh	Control (n = 35)	163.68 ± 12.68	194.20 ± 28.27	246.08 ± 58.36
	25 (n = 24)	166.45 ± 15.63	212.54 ± 24.13	288.87 ± 56.58
	50 (n = 35)	166.00 ± 12.53	229.42 ± 30.40	340.45 ± 61.05[a]
	100 (n = 26)	170.57 ± 28.01	212.15 ± 30.54	297.80 ± 59.05
Vitrified	Control (n = 25)	162.40 ± 13.65	189.16 ± 20.06	239.00 ± 31.45
	25 (n = 24)	167.29 ± 14.51	197.91 ± 23.06	269.25 ± 58.83
	50 (n = 20)	166.50 ± 14.40	222.55 ± 33.40	299.50 ± 65.55[b]
	100 (n = 22)	165.45 ± 15.72	205.04 ± 24.95	275.77 ± 45.73

There was significant difference with other treatment and control. [a]$P<0.001$, [b]$P<0.002$.

were 11.76%, 14.28%, 28.57% and 13.38% respectively (Table 2). There were significant differences in developmental rates between 50 ng/ml concentration of LIF treated follicles with others in each group ($P<0.01$).

DISCUSSION

Several studies have shown that a variety of cytokines have potential to affecting on ovarian functions especially follicular development [26, 27].

In this study, we have shown that mouse isolated preantral follicles could be *in vitro* matured and released MII oocyte (16%). The basal media were supplemented with some factors especially gonadotropin hormones (rFSH and hCG) and growth factors such as EGF and ITS (contains: insulin, transferrin and selenium).

Gonadotropins are necessary for follicular cell proliferation and ovulation [2, 28]. Also, insulin that has pleiotropic anabolic effects on ovarian cells. It promotes glucose and amino acid uptake, lipogenesis, phosphate transport, protein and nucleic acid synthesis [29, 30]. Transferrin serves as a carrier for iron. It may also help to reduce toxic levels of oxygen radicals and peroxide [31]. Selenium is a co-factor for glutathione peroxidase and other proteins and is used as an anti-oxidant in media [32]. In similar results, Demeestre *et al.* [30] demonstrated that isolated mouse preantral follicles cultured in a medium with FCS, FSH, selenium, transferrin and IGF-I were able to support follicular growth and maturation. Our results showed that during *in vitro* maturation of isolated follicles, the size and diameter of follicles were increased in all groups. However, in medium containing 50 ng/mL of LIF it was higher than that of other concentrations of LIF in both fresh and vitrified groups. This

increase in size of follicles may be due to some mechanisms including direct and indirect effects of LIF on the granulosa cells and oocyte. It is shown that LIF is presented in follicular fluid and the granulosa-lutein and ovarian stromal cells are expressed the LIF mRNA and produced this protein [6]. Also, we postulated that LIF maybe affect directly on the oocyte via its receptors, as van Eijk *et al.* [33] show that the LIF receptors were presented in human oocyte and preimplantation embryos.

The indirect effect of LIF on the proliferation of granulosa and theca cells by increasing kit ligand mRNA expression that in turn promotes granulosa cell proliferation. Another possibility is that LIF may act to increase expression of other growth factor that affect on granulosa and theca cell proliferation [8]. Therefore LIF may promote the increase of follicles diameter after a four-day culture especially in concentration of 50 ng/mL.

It seems that, LIF have no effect on survival and antrum formation rates of isolated preantral follicles in fresh and vitrified groups. Also, the results of our

Fig. 3. MII oocyte Ovulated from the cultured fresh preantral follicle (16 h after hCG administration) × 400.

Table 2. The developmental rates of fresh and vitrified isolated preantral follicles after 12 days culturing in the presence and absence of different concentrations of LIF.

Groups	LIF Concentration (ng/ml)	Survived follicles N (%)	Degenerated follicles 120 N (%)	Antrum formation N (%)	GV N (%)	MI N (%)	MII N (%)
Fresh	Control (n = 35)	25 (71.43)	10 (28.57)	13 (37.14)	14 (40.00)	7 (32.00)	4 (16.00)
	25 (n = 24)	21 (87.50)	3 (12.50)	10 (41.66)	7 (33.33)	10 (47.61)	3 (14.28)
	50 (n = 31)	25 (80.56)	6 (19.35)	13 (41.93)	6 (24.00)	9 (36.00)	10 (40.00)[a]
	100 (n = 26)	19 (73.08)	7 (26.92)	11 (42.30)	6 (31.57)	9 (47.36)	4 (21.05)
Vitrified	Control (n = 25)	17 (68.00)	8 (32.00)	7 (28.00)	7 (41.17)	7 (41.17)	2 (11.76)
	25 (n = 24)	14 (58.34)	10 (41.66)	8 (33.33)	6 (42.58)	6 (42.58)	2 (14.28)
	50 (n = 18)	14 (77.78)	4 (22.22)	7 (38.88)	5 (35.71)	5 (35.71)	4 (28.57)[b]
	100 (n = 22)	15 (68.19)	7 (31.81)	9 (40.90)	5 (33.33)	7 (46.66)	2 (13.33)

There was significant difference with other treatment and control. [a]$P< 0.01$, [b]$P< 0.013$.

study showed that the percentage of ovulation was higher in treated with 50 ng/ml LIF than control and other treated follicles. On the other hand, the developmental rates of different groups treated with LIF showed that 50 ng/ml of LIF could act to increase oocytes maturation of the preantral follicles during preantral to antral follicle transition. In similar results, Bishonga *et al.* [23] and Cortvrindt *et al*. [34] showed that near 50% of MII oocytes matured from *in vitro* culture of isolated preantarl follicles.

In conclusion, our results demonstrated that LIF could improve optimal follicular growth in the fresh and vitrified preantral follicles. We have shown that optimal concentration of LIF was 50 ng/ml.

ACKNOWLEDGMENTS

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