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# LIF in embryo culture medium is a predictive marker for clinical pregnancy following IVF-ET of patients with fallopian tube obstruction

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## Highlights

- LIF levels could predict the pregnancy outcome of IVF-ET.
- LIF levels may help to seek new biomarkers for pregnancy outcome.
- Blastocyst-derived LIF may be used an indicator for embryo quality.

**Abstract**

Leukemia inhibitory factor (LIF) has played a vital role in a series of reproductive events, including follicle growth, embryo growth and differentiation. However, it is unclear whether the level of LIF in embryo culture medium can be used as a marker for clinical pregnancy. In this study, we aimed to investigate whether LIF level in embryo culture medium can act as a predictive marker for pregnancy outcome of in vitro fertilization-embryo transfer (IVF-ET) in infertile women due to tubal problems. A total of 208 infertile women due to tubal problems underwent IVF-ET treatment. The women were divided into two groups according to whether they were clinically pregnant. The level of LIF in the embryo culture medium was measured, and the correlation between LIF level and embryo quality and clinical pregnancy outcome was analyzed. The embryo culture medium was collected on the day of blastocyst transplantation. Compared to non-pregnant group, LIF level in the embryo culture medium on the day of blastocyst transplantation was significantly higher in the pregnant group. LIF level in the embryo culture medium may be used as a non-invasive auxiliary biomarker for predictive clinical pregnancy in infertile women with tubal problems that using single blastocyst transfer method.

**Keywords:** Embryo culture medium; LIF; Pregnancy outcome; IVT-ET

## Introduction

The selection of the most competent embryos for transfer is a crucial step for successful pregnancy using assisted reproductive technologies (ART). For decades, *in vitro* development of mammalian embryos has been performed extensively under laboratory conditions. Detailed morphological assessment is routinely used to select the embryos with the highest implantation potential (Ebner *et al*., 2003; Liu *et al*., 2016). Although this method is relatively successful and has led to significant improvement in the pregnancy rate, ART has its own limitations, with >70% of failure rate during ET (Holm *et al*., 2002). In recent decade, further refinements of morphological features for monitoring embryo development have been introduced and semi-automated in a number of different scenarios (Pribenszky *et al*., 2010; Rubio *et al*., 2014). However, live birth rate using ART has not increased much over recent years (Ferraretti *et al*., 2013; Kupka *et al*., 2014). It is imperative to develop appropriate approaches and screen ideal biomarkers to evaluate embryo quality and clinical pregnancy outcome.

The process of embryo implantation is an extremely complex physiological event that depends on the development of the embryo and the receptivity of endometrium. Leukemia inhibitory factor (LIF) is a class of cytokines with a wide range of biological functions. Studies have shown that LIF is involved in a series of reproductive events, including follicle growth, embryo growth and differentiation (Aghajanova, 2004). LIF level in the follicular fluid is abruptly increased before ovulation and its levels in the follicular fluid are positively correlated with egg cell mass. Our previous study has proved that concentrations of LIF in embryo culture medium were higher in pregnancy group following IVF compared non-pregnancy group in PCOS patients. On the basis of this study (Li *et al*., 2018), we tested LIF levels in the culture medium of single

blastocyst and investigated the correlation between LIF level and pregnancy outcome in infertile women due to tubal problems, which may evaluate whether LIF level in the embryo culture medium can predict the developmental potential of embryos and clinical pregnancy outcomes in women who undergo IVF-ET treatment.

## Materials and Methods

### Study patients

A total of 236 patients who underwent IVF–ET between February 2018 and March 2019 in the Center for Reproductive Medicine, Affiliated Hospital of Jining Medical University, were recruited for this study. Two hundred and eight women participated in the study because 28 patients were lost to follow-up. All of participated women had regular menstrual cycles (28 ± 2 days, blood progesterone concentrations measured between the 18th and 21st days of the menstrual cycle, >10 ng/ml in two consecutive cycles) without clinical or bio-chemical hyperandrogenism or polycystic ovary and with no history of any drug intake for at least 3 months. We judge the degree of tubal obstruction by performing hysterosalpingography on the patient. All of selected patients had fallopian tube obstruction. Sex hormone concentrations and routine biochemical examinations of subjects were normal, and patients had no history of genitourinary diseases, or severe cardiovascular, liver or kidney disease. Additional exclusion criteria were smoking and alcohol consumption. The data for all subjects were obtained from clinical and pathologic records including age and history of menstruation. All subjects provided written informed consent in accordance with Institutional Review Board guidelines for the protection of human subjects. The study received ethical approval from the Institutional Review Board on 16 March 2018.

**Ovarian stimulation and supernatant collection**

All patients were stimulated by GnRH agonist long protocol. Ovarian follicular development was stimulated with recombinant human FSH (Merck Serono, Switzerland) at doses of 150–225IU/day. Ovulation was triggered by human chorionic gonadotrophin (HCG 4000–10000 IU) (Livzon Pharmaceutical, China) when at least two follicles were 18 mm and half of the remainder were > 15 mm. Oocytes were recovered transvaginally under ultrasound guidance approximately 34.5 h later. All monitoring of controlled ovarian hyperstimulation (COH) as well as egg retrievals and embryo transfers were performed by one of five physicians. Specific methods refer to previous study (Li *et al*., 2018). Fertilized eggs were cultured to blastocysts. All embryos were graded microscopically according to Gardner grading standard (Gardner *et al*., 1998). All women chose to transplant the embryo at the blastocyst stage on the fifth day. Each patient selected the embryo with the highest morphological score for single-embryo transfer. All embryo transfers were performed using a Wallace catheter under direct ultrasound guidance 120 h after egg retrieval.

**LIF and β-HCG Assay**

LIF levels were assayed using a specific sandwich enzyme ELISA kit (RD) using a specific monoclonal antibody (mAb). When embryos were transferred to 50 μL droplets, they were transferred together with a small amount of medium. To ensure that equal volume of embryo supernatant was tested, 100 μL of dilution buffer were then added to the microplate testing wells. Forty microliters of embryo supernatant from each droplet were loaded manually into the wells. The standard substance in LIF kit was diluted to give a calibration curve within the range value of the embryos. Embryo culture supernatant LIF concentrations were measured by enzyme-linked immunosorbent assay

(R and D Systems, USA). Both inter-assay and intra-assay coefficients of variation for LIF assays were less than 10%. The lower limit of detection for LIF was 8 pg/ml. The LIF concentration was determined by the absorbance at 450 nm on the EL800 Universal Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT). Venous blood (2 ml) was collected on the third day of the menstrual cycle after fasting. If subjects had oligomenorrhoea or amenorrhoea, fasting venous blood could be collected at any time. The concentrations of serum oestradiol, FSH, LH, progesterone, testosterone and prolactin (PRL), and follicular fluid oestradiol and progesterone, were measured by chemiluminescence (Roche, Switzerland). The lower limit of detection for oestradiol, FSH, LH, progesterone, testosterone and PRL were 18.4 pmol/l, 0.1 IU/l, 0.1 IU/l, 0.095 nmol/l, 0.087 nmol/l and 0.996 mIU/mL, respectively.

**Luteal support and judgment of pregnancy outcome**

After fertilization, progesterone (Abbott, the Netherlands) was administered by intramuscular injection at a dose of 60 mg per day and continued for 14 days for corpus luteum support. If pregnancy was confirmed, progesterone administration was continued until the 10th week of pregnancy. Biochemical pregnancy was defined as blood HCG >10 IU/L on the 14th day of transplantation. Clinical pregnancy was confirmed with ultrasound examination of the number of gestational sacs and fetal heartbeat on approximately the 64th day after embryo transfer.

**Statistical Analysis**

Differences between groups were evaluated for statistical significance by Student's t test or Mann-Whitney rank sum test, depending on whether the data were normally distributed. Receiver operating characteristic (ROC) curve analysis was used to analyze the cut off value of LIF concentration and their sensitivity/specificity. Significant P

values were set at <0.05. The data were expressed as mean ± standard deviation (SD). The data between two samples were compared by Mann–Whitney U-test. The relationship between the parameters was analysed by Pearson correlation analysis. All calculated P-values were two-sided, and $P < 0.05$ was used to determine statistical significance. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, USA) and SPSS 18.0 software (SPSS Inc, Chicago, IL, USA).

## Results

### Clinicopathological characteristics of 208 infertile women with tubal problems

The cause of infertility in all patients was tubal obstruction. Because 39 pg/mL is the median value, we choose 39 pg/mL as the demarcation point for the two groups. The patients were divided into two groups according to LIF levels: low LIF group (<39 pg/mL, 108 cases) and high LIF group (≥39 pg/mL, 108 cases).The average age in the high LIF group was 31.7±5.0 years old, and 33.9±4.7 years old in the low LIF group. There were no significant differences in basal FSH level, LH level, E2 level and endometrial thickness between high LIF and low LIF groups. The ovulation induction programs were long protocol. There were 28 cases of primary infertility and 76 cases of secondary infertility in the high LIF group, and 20 cases of primary infertility and 84 cases of secondary infertility in the low LIF group. The pregnancy rate in the high LIF group (57.7%) was obviously higher than that in the low LIF group (44.2%) **(Table 1)**.

### Comparison of LIF level in the culture medium between high quality embryos and inferior embryos

Total 373 samples of embryo culture medium were collected from all subjects (pregnant and non-pregnant subjects). According to the Gardner blastocyst scoring

criteria, the culture medium was divided into high quality embryo group (4BC and above, n=198) and inferior embryo group (less than 4BC, n=175). In the pregnant subjects, LIF level in the high-quality embryo group (67.1±8.0 pg/mL) was higher than that in the inferior embryo (62.5±8.6 pg/mL) (Fig. 1A), but the difference was not statistically significant. Similarly, in non-pregnant group, the LIF level in the high-quality embryo group (48.1±8.5 pg/mL) was higher than that in the inferior embryo group (43.9± 9.2 pg/mL) (Fig. 1B), but the difference was also not statistically significant.

**Predictive potential of LIF level in embryo culture medium for clinical pregnancy outcomes**

After 10 weeks of transplantation, the pregnancy outcome of patients was examined by B-ultrasound. According to the presence of gestational sac by B-ultrasound, the patients were divided into pregnant and non-pregnant groups. We found that the level of LIF in embryo culture medium from pregnant group was 52.4±7.8 pg/mL, which was significantly higher than that from non-pregnant group with LIF level of 37.5±9.5 pg/mL($P<0.05$) **(Fig. 2A)**. Meanwhile, we found that the level of β-HCG in embryo culture medium from pregnant group was 1.44±0.49 IU/L, which was also significantly higher than that from non-pregnant group with its level of 1.18±0.27 IU/L ($P<0.05$) **(Fig. 2B)**. To evaluate the potential of LIF level and β-HCG level in embryo culture medium in predicting clinical pregnancy, receiver operating characteristic (ROC) curves were plotted, and the area under the curve (AUC) values were determined with the highest specificity and sensitivity, which were used to set the optimal prediction point, Optimal prediction point, sensitivity, specificity and AUC value of the potential of LIF level in embryo culture medium in predicting clinical pregnancy were 36.68 pg/mL, 82.6%,

65.5% and 0.762 (95%CI: 0.671–0.853) respectively **(Fig. 2C)**. The AUC of the potential of β-HCG level in embryo culture medium in predicting clinical pregnancy were 0.819 (95%CI: 0.729–0.908). The AUC of the potential of LIF level combined with β-HCG level in embryo culture medium in predicting clinical pregnancy were 0.834 (95%CI: 0.752–0.915) respectively. Our research shows that the combination of LIF level combined with β-HCG level is more predictive of pregnancy than their individual predictive power.

**Univariate and multivariate logistic regression analyses for clinical pregnancy outcome of patients who underwent IVF-ET**

Using univariate and multivariate analyses, we found no statistically significant correlations between basal FSH level, LH level, endometrial thickness, E2 and pregnancy outcomes (**Table 2**). We found that age (p=0.041) and LIF level in embryo culture medium (p=0.02) were important predictive factors for clinical pregnancy outcomes (**Table 3)**.

## Discussion

In IVF-ET, how to select the most development potential embryos is the key for successful pregnancy. At present, the morphology screening of embryos is widely used. However, there are some limitations for morphology screening. For example, high quality of embryo morphology may not represent absolute good development potential embryos. There is a need to find additional or complementary evaluations for embryo quality or developmental potential. Researchers have explored a variety of effective methods to select the best potential embryos to improve clinical pregnancy outcomes for IVF-ET. Using morphological monitoring, numerous morphological features related

to embryo quality have been described, such as cell cycle timing, compaction times and cell shape (Papanikolaou *et al*., 2008; Somfai *et al*., 2010). Analysis of the embryonic secretome (proteins that are produced and secreted by the developing embryo) could provide a non-invasive approach for embryo assessment (Seli *et al*., 2010). Identification of a biomarker in human embryo culture medium that relates to embryo quality and implantation potentia could be an invaluable tool in conjunction with morphological parameters during embryo selection for transfer (Katz-Jaffe *et al*., 2009). Assessment of embryonic proteome has been of particular interest and provides a snapshot of cellular function and embryo physiology. In the present study, we assessed the production of LIF in embryo culture media from individual human blastocyst. The results showed that LIF level in embryo culture medium had a good correlation with clinical pregnancy rate. The embryos for the patients who became pregnant showed higher level of LIF in their culture medium compared with the embryos for the patients who were not pregnant.

The success of ART pregnancy is closely related to follicular development, number of ovum, fertilization, embryo development and implantation. The most critical process of them is embryo implantation into the endometrium. LIF is a secretory glycoprotein, which is a member of interleukin (IL)-6 family. It was named LIF because it can inhibit the growth of myeloid leukemia cell line M1 and promote differentiation (Aghajanova, 2010). It has been demonstrated that LIF has ability to inhibit the differentiation and maintain the proliferation of embryonic stem cells. LIF is involved in all aspects of reproductive activities, including follicular development, embryo implantation, growth, development, and differentiation. Meanwhile, LIF was found to be associated with the occurrence of the infertility, recurrent abortion and other diseases. LIF transcripts are

expressed in many tissues during embryonic development. Perrier *et al*. (2004) found the expression of LIF gene in mouse embryonic germ cells from 3.5 to 12.5 days. Another study found that the best effect of LIF during blastocyst formation is to promote the embryonic development (Tsai *et al*., 2000). In the present study, LIF was detected in the blastocyst culture medium by ELISA with high sensitivity, which further confirmed the preimplantation embryos have the ability to synthesize and secrete LIF, which may promote embryo development via autocrine pathway. However, additional studies are required for further development of a quick, high-throughput system to adequately determine embryonic LIF production from individual embryos prior to clinical application. This may provide the laboratory embryologist or infertility physician an opportunity to determine the quality of embryo, thus improving pregnancy outcome and minimizing multiple gestations. Embryo quality is an important factor that affects a patient's pregnancy, but high embryo quality does not mean that the patient will be able to become pregnant, because pregnancy is also affected by many other factors, such as hormone levels, endometrial receptivity, Synchronization of endometrium and embryo development.

Studies have shown that medium LIF can promote embryonic development, accelerate embryonic hatch, improve embryonic developmental potential (Grazul-Bilska *et al*., 2006). Kauma *et al*. (1995) found that the addition of LIF to mouse embryo culture medium could promote the development of preimplantation mouse embryos. However, the effect of LIF on embryonic development is different at different time points of LIF addition. The effect of LIF on embryo development was the strongest during blastocyst formation. LIF plays roles in embryogenesis in two ways: inhibit the differentiation of inner cellmass to maintain the pluripotency of embryos; and stimulate

the proliferation of trophoblast ectoderm. A key step inmammalian pregnancy is blastocyst implantation. Embryo implantation is a complex physiological process. Successful implantation is the result of the coordination between endometriosis and invasive blastocyst. Recent studies (Choi *et al*., 2016) have shown that LIF is a prerequisite for blastocyst implantation. Moreover, LIF can play an important role in influencing endometrial receptivity and maternal-fetal interface during early pregnancy. β-HCG concentration may be applied as an indicator of potential developmental progress of the embryo (Keane *et al*., 2016). In our study, we also found that pregnancy predictions in combination with LIF and β-HCG are significantly better than their individual predictive power.

LIF plays an important role in embryo development and differentiation. Researchers such as Fukunaga and others added LIF to the rhesus monkey ESC differentiation culture fluid, and found that LIF can increase the number of cells with positive alkaline phosphatase activity. The addition of LIF to the in vitro differentiation culture fluid can promote the differentiation of rhesus monkey ESC into germ cells (Fukunaga *et al*., 2010). Dunglison's research shows that adding 1000 IU/mL LIF to serum-free culture medium can increase the formation rate of human embryos from 18.4% to 43.6%. LIF can significantly improve the embryo cleavage rate and embryo quality. (Dunglison *et al*., 1996). Choi's research found that the addition of recombinant human LIF to the in vitro culture medium of porcine oocytes and parthenogenetic embryos can promote egg maturation and embryo development (Choi *et al*., 2013). De Matos and other studies have confirmed that LIF can induce the cumulus expansion of human and mouse cumulus complexes in vitro. The addition of recombinant FSH and LIF to the in vitro culture medium can significantly improve the quality of mouse oocytes and the eggs of

mouse embryos. Cracking rate, blastocyst formation rate and delivery rate (De Matos et al., 2008). Fry and other studies have shown that adding LIF to the in vitro culture fluid of sheep blastocyst stage embryos can increase the embryo hatching rate by 6 times, the embryo survival rate by 4 times, and can greatly improve the success rate of transplantation (Fry *et al*., 1992). Mitchell and colleagues divided the 2-cell stage mouse embryos into two groups for in vitro culture, one group added 5000 u/mL anti-polyclonal antibody, and the other group added 5000 u/mL LIF. In the uterine cavity of pseudo-pregnant rats, it was found that the embryo implantation rate and pregnancy rate of the LIF group were significantly increased compared with the control group (Mitchell *et al*., 2002). Many factors can reduce the expression level of LIF. For example, studies have shown that when the level of Th2 type cytokines (such as IL-4) increases, it will promote the production of LIF, and when the level of Th1 type cytokines increases, the synthesis of LIF and The secretory process is inhibited (Markert *et al*., 2011). MicroRNA (miRNA) regulates gene expression in a post-transcriptional manner, which hybridizes the target mRNAs with complementary sequence and subsequently leads to translation repression or mRNA degradation. miR-223-3p suppresses pinopodes formation and LIF protein expression, which may lead to diminished embryo implantation (Dong *et al*., 2016). However, the detailed mechanism for regulating the expression level of LIF is still not completely clear, and further research is needed.

In summary，quantitative detection of LIF in blastocyst culture medium is expected to be a parameter for selecting high-quality embryos for transfer in addition to morphology in IVF clinics. Moreover, blastocyst-derived LIF may be used an indicator for embryo quality and the prediction of pregnancy outcome. However, we judge the degree of

tubal obstruction by performing hysterosalpingography on the patient, but this method has certain limitations. In the process of hysterosalpingography, tubal spasm, inadequate operation experience of some clinicians, intubation is not in place, radiologists cannot judge in time, and problems are not corrected in time, etc. are the reasons for false positives, in order to minimize the false positive rate, we choose senior gynecologists with rich HSG experience to perform the operation in the imaging process, and senior radiologists with rich experience in HSG reading to interpret, try to avoid the influence of the above factors, so as to minimize the false positives rate to obtain objective and accurate diagnostic results. At the same time, the findings in this study should be validated in a large cohorts of subjects who undergo IVF-ET, from multiple IVF clinics in different cities.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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## Figure legends

**Fig. 1** LIF level in high quality embryo culture medium and inferior quality embryo culture medium. **A** LIF level in high quality and inferior quality embryo culture medium of pregnancy group; **B** LIF level in high quality and inferior quality embryo culture medium of non-pregnancy group.

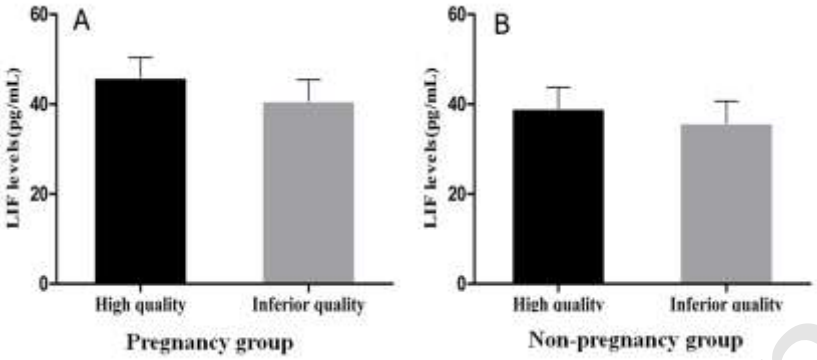
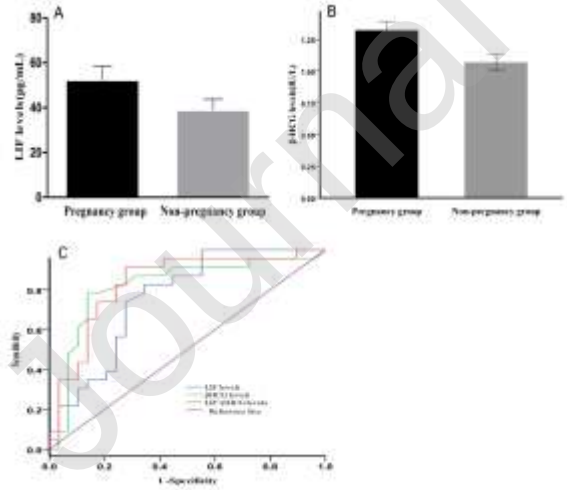



**Fig. 2 A**. LIF level in embryo culture supernatant between Pregnancy and Non-pregnancy group. **B**. β-HCG level in embryo culture supernatant between Pregnancy and Non-pregnancy group. **C**. ROC analysis of LIF and β-HCG to assess the predictive powers of clinical pregnancy.



## Tables

**Table 1. Characteristics of the 208 infertile women with tubal factors**

| | High | Low | P-value |
|---|---|---|---|
| **Number of patients** | 104 | 104 | ns |
| **Average age (years) (mean±SD)** | 31.8±5.0 | 33.9±4.7 | ns |
| **Basal FSH levels (UI/l) (mean±SD)** | 8.0±3.4 | 7.7±1.8 | ns |
| **Basal LH levels (UI/l) (mean±SD)** | 5.4±2.4 | 5.1±2.0 | ns |
| **E2 (UI)(mean±SD)** | 896.5±284.0 | 815.3±289.4 | ns |
| **Endometrium (mm) (mean±SD)** | 1.1±0.2 | 1.1±0.2 | ns |
| **Primary** | 28 | 20 | |
| **Secondary** | 76 | 84 | |
| **Pregnancy** | 60 | 45 | |
| **Pregnancy rate (%)** | 57.7% | 44.2% | <0.05 |
| **LIF** | 65.4±8.3 | 46.6±9.3 | <0.05 |

**Table 2 Univariate analysis of various factors in patients with pregnancy outcome**

| Factors | B | SE | Wald | HR | 95% CI | | *P* value |
|---|---|---|---|---|---|---|---|
| | | | | | Low | Upper | |
| **Age** | 0.148 | 0.068 | 4.768 | 0.863 | 0.756 | 0.985 | 0.029 |
| **Basal FSH** | 0.020 | 0.105 | 0.036 | 0.98 | 0.799 | 1.203 | 0.85 |
| **Basal LH** | 0.099 | 0.134 | 0.551 | 0.905 | 0.697 | 1.177 | 0.905 |
| **Endometrial thickness** | 1.822 | 1.493 | 1.489 | 6.182 | 0.332 | 11.525 | 0.609 |
| **E2** | 0.232 | 0.001 | 0.058 | 1.00 | 0.998 | 1.002 | 0.81 |
| **LIF** | 0.034 | 0.013 | 6.42 | 1.034 | 1.008 | 1.061 | 0.011 |

**Table 3 Multivariant analysis of various factors in patients with pregnancy outcome**

| Factors | B | SE | Wald | HR | 95% CI | | *P* value |
|---|---|---|---|---|---|---|---|
| | | | | | Low | Upper | |
| **Age** | 0.135 | 0.071 | 3.554 | 0.874 | 0.760 | 1.005 | 0.041 |
| **LIF** | 0.033 | 0.014 | 5.401 | 1.034 | 1.005 | 1.063 | 0.02 |