Iraqi Journal of Biotechnology, September 2018, Vol. 17, No. 2, 57-67

Role of leukemia inhibitory factor(LIF) gene variation on implantation rate following IVF program in PCOS and non PCOS women

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Received: June 10, 2018 **/ Accepted:** July 19, 2018

Abstract: Implantation of the blastocysts into the maternal uterus is a crucial step in mammalian reproduction, which is controlled by a number of complex molecules like hormones, cytokines, and growth factors and their cross talk. A network of these molecules plays a crucial role in preparing receptive endometrial and blastocysts. This study aimed to found out the role of Leukemia inhibitory factor (LIF) , gene expression, concentration and genetic polymorphisms in the endometrial that may interfere with implantation process of polycystic ovary syndrome(PCOS) and non-PCOS women. A convenient blood (6ml)sample of 80 infertile women undergoing *in vitro* fertilization (IVF) program were intentionally divided according to the cause of infertility into 40 healthy women their husbands complaining from male infertility factors, and 40 infertile women with polycystic ovary syndrome. Leukemia inhibitory factor(LIFs) were measured on ovulatory and luteal phase of cycle (CD14-CD16,17) at the day of ovarian pickup and embryo transfer by using quantitive polymerase chain reaction(qPCR), Elisa technique ,polymerase chain reaction(PCR) and sequencing to determine any genetics polymorphisms in *LIF*gene study. Results of the present study indicate that no mutation was detected in LIF gene in the healthy and PCOS women. The *LIF* gene expression in addition to levels of serum leukemia inhibitory factors are more valuable in predicting the pregnancy out come in infertile PCOS women than in non-PCOS women .This result can be utilized to be used as predictors of implantation window for successful implantation and pregnancy.

Keywords: implantation, leukemia inhibitory factor, PCOS, IVF program .

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Introduction:

Infertility is defined as the failure to achieve a clinical pregnancy after 12 months or more of regularly unprotected sexual intercourse(1). The infertility is either primary when never the couple having had a live birth or secondary infertility which is failure to realize a live birth after having had alive birth or abortion(2). The cause may be related to a problem with the man, woman or both(3). In females ,one of the most infertility problem is PCOS which described as endocrine disorder that may associated with hyper-androgenism and chronic anovulation (4). Failure of PCOS treatment may solute by (IVF) programs. The first succeful fertilization of human eggs in the (IVF) procedure was in 1978. The fact of the first child-birth by this

process was a real milestone because it gave hope to the infertile couples as it offered a possible solution to the problem(5). The success in IVF programs depends on sperm fertilization to mature oocyte, embryonic development and implantation. The implantation process involves complex and synchronized molecular and cellular events between the uterus and the implanting embryo(6). Implantation can occur during only a very short time period, known as the ‘‘window of implantation,’ during this window, the embryo fuses itself to the endometrium, giving it access to the maternal blood supply. This process is enhanced by many markers and factors(7). Implantation failure is related to either maternal factors or embryonic causes. Maternal factors include uterine anatomic abnormalities, thrombophilia, non-receptive endometrium and immunological factors(8).

Many factors may interfere with implantation process. Leukaemia inhibitory factor (LIF) is one of these factors which is a glycoprotein that plays an important role in implantation, but also has a variety of functions in different organ systems. Primarily, LIF protein structure was identified as a factor of differentiation for hematopoietic cells, but has since been shown to support diverse physiological functions(9). LIF was first identified from its ability to induce differentiation of myeloid leukemia cells into macrophage-like cells, but LIF is in fact produced and secreted by a variety of cell types, including epithelial and stromal cells in the endometrium(10). The purpose of this study was to evaluate the role of *LIF* gene in successful implantation human embryos undergoing IVF programs of PCOS and non-PCOS women and study gene expression and genetic polymorphism of LIF in the endometrium that may interfere with predication implantation process.

Materials and methods:

Sample collection:

Venous blood samples (6ml) were collected from each woman for both groups. Each blood sample was divided into two tubes:

1- EDTA tubes for molecular studies.
2- Gel plain tube for serological test: the serum obtained by putting the blood samples in gel plain tube, the tubes centrifuged at 5000rpm for five minutes, serum was collected and kept in freezer until used.

Convenient blood sample of 80 infertile women undergoing IVF program were intentionally divided according to the cause of infertility into 40 healthy women their husbands complaining from male infertility factors, and 40 infertile women complaining from polycystic ovary syndrome. Blood samples were taken from the patients of IVF Department in the Kamal Al-Samarraee Infertility Treatment Hospital , Baghdad-Iraq. The LIFs were measured on ovulatory and luteal phase of cycle (CD14-CD16,17) at the day of ovarian pickup and embryo transfer respectively. Every participant woman was interviewed and asked to answer information including hormones, age, type of infertility and duration of infertility, the blood sample were taken in oocyte pick up and embryo transfer

Hormonal analysis:

Hormonal measuerments (LH, FSH, TSH, E2 and Prolactin) were

performed by using Automated Immune Assay (AIA) by the VIDAS auto analyzer, (BioMérieux Company, France). VIDAS hormonal assay is an automated quantitative test for use on the VIDAS instrument for the quantitative measurement of human serum using the enzyme linked fluorescent assay (ELFA) in Hormonal Laboratory at Kamal Al-Samarraee Infertility Treatment Hospital.

ELISA assay:

Measurement the concentration of *LIF* (ng/ml), serum level by using Enzyme-linked immunosorbent assay Elisa kit (cusabio).

RNA extraction:

RNA was extracted from blood samples by using AccuZol^{TM} kit (Bioneer Company) A total RNA 10pg (18 µl) was reversely transcribed to a complementary DNA (cDNA) by using AccuPower^{R}RocketScrript^{TM} RT Premix kit (Bioneer). The procedure was carried out in a reaction volume of 20µl according to the manufacturer with modifications, PCR Program for cDNA synthesis program, as follow Primer: annealing 30 ºC for10 minutes, cDNA synthesis 42 ºC for 30minutes, heat inactivation 95 ºC for 5minute.

Quantitative RT- PCR (qRT–PCR):

The expression level of *LIF* gene was estimated by Two Step RT- QPCR to confirm the expression of target gene, quantitative real time qRT-PCR SYBR Green assay was used. This assay was performed using a syber green master mix (GoTag qPCR Master Mix, Promega, USA) , in 10 µl reaction volume as illustrated in Table (1).

Table (1) Thermal Cycling Protocol al Cycling Protocol.

Steps	°C	m:s	Cycle
Hold	95	05:00	1
Denaturation	95	00:15	45
Annealing	55	00:30	
Extension	72	00:30	

Primers used for Quantitative Real Time PCR:

Primers used for LIF(ID: 3976) gene amplification in this listed in table(2), primer was designed according to National Center for Biotechnology Information(NCBI) http://www.ncbi.nlm.nih.gov/genbank, and stored lyophilized at (-20°C). Table (2).

Table (2): specific primer for *LIF*

Primer Name	Seqences	Annealing	Size
Forward primer	5′-CCAACAACCTGGACAAGCTA-3′	63	498 bp
Reverse primer	5′-GGGGTTGAGGATCTTCTGGT-3`		
LIF F	5`-GCCCCAAGTGTTCGTGTGTCTG-3`		
LIF R	5`-GCGATGCCCATCTTCAGACAACT-3`		

Gene Expression Calculation:

Stander curve was performed as following eleven of 0.2 ml tube prepared, 90 µl of nuclease free water was added to each tube then made a serial dilution by adding 10 µl from

sample of $41X10^{10}$ 1/ µl copy number to the first tube and made a serial dilution by transferred 10 µl from first tube to second tube and so on.

The standard curve reaction started from the third tube ($41X10^{8}$ 1/ µl copy No.) to the tube number eleven (41 1/ µl copy No.).

DNA extraction:

DNA extraction was done by using gSYNC^{TM} DNA extraction kit (Geneaid,Taiwan). PCR was performed using Accu-power$^{(R)}$PCR pre-mix (Bioneer ,south korea) DNA purity was measured by Quantus Florometer the: PCR was carried out in Veriti^{TM} thermo-cycler (Applied Biosystem) using the standard cycle procedure. Initial denaturation at95 ºC for 5 minute, then 30 cycles of 30 seconds of denaturation at 95 ºC ,30 seconds of annealing at 63 ºC, (Table 2).

PCR products sequencing:

The PCR products (80 samples) and primers were sent to macrogen company (South Korea), for sequencing analysis to detect any mutation in the samples of this study The results were analyzed using genious software**.**

Results and Discussion:

The age distribution, duration of the infertility women:

The age distribution and duration of infertility of all studied groups shown in table 4. The mean age of the PCOS women was (29.700±5.547) years and the mean age for the non- PCOS women was (31.725±6.543) years. There was no significant (P>0.05) difference between the two studied groups.The mean of infertility duration in PCOS women (6.825±3.713) was similar to the infertility duration in non-PCOS women (6.825±4.419) and the p value was (0.999). These results were in agreement with other studies (11,12). It is clear that the mean age of all women enrolled in the present study is tend to be similar to the studies mentioned earlier indicating that infertile women in our community seeking medical and this probably due to early marriage. On the other hand the mean age of infertile PCOS women in the present study is comparable to some studies (13, 14).

Table (4): mean age and duration infertility women classified into PCOS and non- PCOS groups

	Group	N	Mean	SD	P value
Age	**PCOS**	40	29.700	5.67	0.290
	Non- PCOS	40	31.725	6.60	
Duration	**PCOS**	40	6.825	3.10	0.999
	Non PCOS	40	6.825	2.03	

*** N= number of cases, SD= standard deviation, PCOS= polycystic ovary syndrome.**

Type of infertility and pregnancy status:

The type of infertility in women with primary infertility (no=31, the percentage =77.5%) and women with secondary infertility (no=9,the percentage = 22.5%) shown in table 5. There was no significant (P=0.605) difference between PCOS and non – PCOS women, regarding the type of infertility. The result of the current

work showed that the rate of primary infertility more than that of secondary infertility in the entire samples of women and also in women with or without PCOS. The overall rate was approximately two thirds with primary infertility and one third with secondary infertility. These results are comparable to the results obtained from a study dealing with prevalence of primary and secondary infertility in which the rate of primary infertility is more frequent than that of secondary infertility(14) .Also the positive pregnancy rate was 5 (12.5%) for PCOS women and 5 (12.5.5%) for non-PCOS women .Thus both infertility (P=0.605) and pregnancy frequency (P=0.631) were no significantly related to the PCOS and non PCOS groups. The infertility type and pregnancy statues were no significantly related to the PCOS and non PCOS groups this result close to result that recorded by (14).

Table (5): Type of infertility and pregnancy state in PCOS and Non-PCOS groups

		Group			
		PCOS		Non PCOS	
		N. patients	%	N. patients	%
Infertility	Primary	30	75.0%	32	80%
	Secondary	10	25.0%	8	20.0%
Pregnancy	Positive	5	25.0%	11	27.5%
	Negative	35	75.0%	29	72.5%

*** N= number of cases, PCOS= polycystic ovary syndrome.**

Correlation between reproductive hormones of pregnant PCOS and Non-PCOS women:

In PCOS group, the mean of FSH in pregnant women who have PCOS (6.98μIU/ml±1.81) was shown no significant difference (P=0.86) compared to non- PCOS women (5.58μIU/ml ±1.35).The level of FSH in pregnant PCOS and non PCOS women shown no significant (p=0.88 and p=0.78, respectively) differences than corresponding groups of non-pregnant women as illustrated in table 6. This result consistence with the results of previous studies (15, 16). The findings of (17) hypothalamus induces a change in gonadotropin releasing hormone (GnRH) pulse frequency leading to increased release of follicle stimulating hormone (FSH) from the pituitary gland in PCOS.

There was a no statistically significant (p=0.06) difference in the mean of LH between the pregnant women of PCOS group (6.51μIU/ ml±2.01) compared to non-PCOS group (3.82 μIU ±1.2). This compatible with other studies (15,16).There was a significant statistical(p=0.04)difference in the mean of LH between them non-pregnant women of PCOS group (8.86μIU/ml±3.78) compared to non-PCOS group (5.34 μIU ±2.03) as revealed in table 6. These findings is consistence with studies done by (17,18,19) who noticed elevated LH concentrations (above the 95th percentile of normal) can be observed in approximately 60% of women with PCOS. The findings of Sirmans and Pate (17) regarding the combined hormonal contraceptive (CHC) are a good treatment option for those patients that do not wish to become pregnant,

and they are often considered first line for the treatment of PCOS-related hirsutism and acne. CHCs promote negative feedback on the production of LH, causing a decreased synthesis of androgens by the ovaries. Other mechanisms by which CHCs reduce androgens decreasing circulating levels of free androgen by increasing the production of sex hormone-binding globulin in the liver; decreasing adrenal androgen secretion.

Regarding E2 hormone, there was no significant difference (P=0.09) increase between the pregnant women complaining from PCOS and non PCOS group (table 6). At the same time a statistical significant elevation was noticed in the E2 hormone of pregnant women with and without PCOS compared to non-pregnant of both women groups. The association between the high E_2 and premature progesterone elevation suggest that at least one of the mechanisms that play a role in the premature increase of plasma progesterone is linked to the high response of the ovary to ovarian stimulation (20). An excess in the number of follicles and consecutively an excess of proliferating granulosa cells can lead to an increased progesterone production (21). Estradiol hormone was higher but reach the significant level in women with PCOS than in non-PCOS women. The mean serum E2 and LH levels increased along with E2 exposure, whereas the mean serum. The mean serum E2 level on the day of hCG administration gradually increased along with E2 exposure (22) however, the granulosa cells of patient with PCOS are functionally robust and exhibit increased estrogen responses to FSH stimulation compare to those of normal women, which may, in part, account for increased risk of ovarian hyperstimulation syndrome in PCOS women undergoing ovulation induction with gonadotropin therapy (23).The results of the current study similar to other studies (22,14).

The mean of prolactin hormone in PCOS pregnant women (10.04 ng\ml±3.22) was significant (p=0.005) compared to non- PCOS women (4.16 ng\ml±1.19).There was no significant differences in the mean of prolactin hormones in pregnant women compared to non-pregnant women in PCOS group (p= 0.390).On other hand, there was a significant differences increase the mean of prolactin hormones in pregnant women compared to non-pregnant women of non- PCOS group (p= 0.005) as shown in table 6. This result came closed to (24) and similar to (22) who reported the increased E2 levels due to controlled ovarian hyperstimulation may compromise endometrial receptivity for embryo implantation. The mean of TSH in PCOS women (2.68±0.99) was shown no significant (p=0.446) difference compared to non-PCOS pregnant (1.91±0.72) .However, there was a significant (p=0.041) elevation of TSH in non- pregnant PCOS women compared to non –PCOS. The mean of TSH in pregnant non-PCOS women (1.91±0.72) was shown significant (p=0.035) difference compared to non- pregnant non-PCOS pregnant (2.96±1.01) as shown in table 6, These results are in agreement with (25) and (26) who found increased levels of TSH in PCOS women compared with controls(27).

Table (6): Correlation between reproductive hormones PCOS and Non-PCOS women

Level in serum	Pregnancy state	Groups				P value
		PCOS		Non PCOS		
		Mean	SD	Mean	SD	
FSH (mIU/ml)	Pregnant	6.98	1.81	5.58	1.35	0.86
	Non pregnant	7.14	1.93	7.07	1.56	0.931
	P value	0.88		0.78		
S.LH (mIU/ml)	Pregnant	6.51	2.01	3.82	1.2	0.06
	Non pregnant	8.86	3.78	5.34	2.03	0.04*
	P value	0.61		0.371		
.E2-dHCG (pg/ml)	Pregnant	1887.74	712.02	1196.00	599.10	0.09
	Non pregnant	449.40	199.91	306.03	100.47	0.02*
	P value	0.001*		0.004*		
Prolactin (ng/ml)	Pregnant	10.04	3.22	4.16	1.19	0.005*
	Non pregnant	7.94	2.67	7.59	2.11	0.69
	P value	0.39		0.005*		
TSH (mmol/L)	Pregnant	2.68	0.99	1.91	0.72	0.46
	Non pregnant	2.13	0.89	2.96	1.01	0.041*
	P value	0.297		0.035*		

*** P<0.05 *= Significant**

LIF concentration in PCOS vs. non PCOS women in oocytes picks up stage and embryo transfer:

The mean of LIF in the serum of pregnant and non –pregnant of two groups at the time of oocyte pick up and embryo transfer (shown in tables 7and 8). There was a significant (p=0.044) increase in the mean of LIF at the time of oocyte pick up in the pregnant of PCOS group compared to non-pregnant (0.158±0.028, 0.14±0.02 respectively) and non PCOS pregnant women (0.219±0.037) compare to non-pregnant (0.166±0.04) and the P value was a significant (p=0.044) increase in the mean of LIF concentration of pregnant non-PCOS women was noticed compare to PCOS women.

On other hand, the mean of LIF in the serum of pregnant women of PCOS at the time of embryo transfer was highly compared with non-pregnant women (0.195±0.02, 0.164±0.029 respectively), however, statistically no significant (P=0.092) differences was observed between them, there was no significant (P=0.43) difference in the concentration of LIF in pregnant PCOS (0.195±0.02) and pregnant non-PCOS groups (0.226±0.08). The concentration of LIF in the pregnant non-PCOS at the time of ET was shown no significant (P=0.95) differences compared to non-pregnant women of the corresponding group as shown in table 8. LIF production measure endometrial cultures from idiopathic female factor infertile women are reduced compared with fertile women (28).Similar to the results of current study, it has been found that LIF can also be detected in uterine flushing, and its level is significantly lower in women with unexplained infertility (29).

Endometrium of infertile women produces significantly less LIF during

the period of receptivity.(30) .This results explain LIF plays a central role in the control of implantation and when the gene lacking function their blastocysts fail to implant and do not give rise to the development of clinical gestation (30). *LIF* plays a critical role in the process of blastocyst implantation. Therefore, the aberrant LIF production is linked to implantation failure (31).The same observation was noticed by (32) when reported that LIF concentrations were lowered in both serum and follicular fluid of infertile compared with the healthy one.

Table (7): LIF concentration in PCOS vs. non PCOS women in oocytes picks up stage.

Pregnancy status	Group		P value
	PCOS	Non PCOS	
	Mean± SD	Mean± SD	0.044*
Pregnant	0.158 ±0.028	0.219±0.037	
Non pregnant	0.14±0.02	0.166±0.04	**0.35**
Pvalue	0.46	0.09	

*** P<0.05 *= Significant Analyses were performed by: - Independent samples t-test.**

Table (8): LIF concentration in PCOS vs. non PCOS women at time of embryo transfer.

Pregnancy Status	Group		P value
	PCOS	Non PCOS	
	Mean±SD	Mean± SD	
Pregnant	0.195±0.02	0.226±0.08	**0.43**
Non pregnant	0.164±0.029	0.223±0.04	**0.057**
P value	0.092	0.95	

*** Analyses were performed by: Independent samples t-test**

LIF expression in PCOS and non-PCOS women at the time of oocytes pick up and embryo transfer:

The mean of LIF expression of pregnant and non –pregnant of two groups at the time of oocyte pick up (shown in tables 9 and 10). There was no a significant (p=0.061) increase in the mean of LIF at the time of oocyte pick up in the pregnant of PCOS group compared to non-pregnant (24.79±4.32, 23.95±1.80respectively) and non PCOS pregnant women (25.90±1.67) compare to non-pregnant (23.71±2.68) and the P Value was no significant (p=0.061) increase in the mean of LIF expression of pregnant non-PCOS women was noticed compare to PCOS women (The expression of LIF in the pregnant non-PCOS at the oocyte pick up was shown significant (P=0.045) differences compared to non-pregnant women of the corresponding group as shown in table 9. On other hand , the mean of LIF expression of pregnant women of PCOS at the time of embryo transfer was highly compared with non-pregnant women (24.79±4.32, 23.95±1.80 respectively). However ,statically no significant (P=0.71) differences was observed between them.There was no significant (P=0.061) difference in the expression of LIF in pregnant PCOS (24.79±4.32) and pregnant non-PCOS groups(25.90±1.67). The expression of LIF in the pregnant non-PCOS at the time of ET was shown no significant (P=0.17) differences compared to non-pregnant women of the corresponding group as shown in table 9.

LIF regulates multiple processes prior to and during implantation such as uterine transformation into a receptive state, decidualization, blastocyst growth

and development, embryo-endometrial interaction, trophoblast invasion, and immune modulation the same results obtained by other researchers (32,33) . It has been noticed that the LIF may also be involved in immune tolerance through regulation of HLA-G, a class1 MHC molecule especially expressed by invasion cytotrophoblast cells (34). The LIF secreted from the uterus is regarded an important factor in embryo implantation, and the maximal expression of LIF in endometrial is during implantation window (35.36, 37), therefore the LIF expression was highly level in pregnant women compared to non-pregnant.

Table (9): LIF expression in PCOS vs. non-PCOS at the time of oocyte pick up

Pregnancy state	Groups		P value
	PCOS	Non PCOS	
	Mean±SD	Mean±SD	0.86
Pregnant	27.72±3.99	28.14±3.13	
Non pregnant	25.14±3.27	24.15±0.54	0.521
P value	0.297	0.045	

Table (10): LIF expression of PCOS vs. non-PCOS at the time of embryo transfer

Pregnancy state	Groups		P value
	PCOS	Non PCOS	
	Mean±SD	Mean±SD	0.061
Pregnant	24.79±4.32	25.90±1.67	
Non pregnant	23.95±1.80	23.71±2.68	
P value	0.71	0.17	0.88

*** Analyses were performed by: Independent samples t-test.**

Sequencing LIF gene:

No mutation was found in this region study(exon one) when alignment the sequence with original sequencing for this gene in NCBI. Other study found potentially functional mutations in the LIF gene do infrequently occur in women with unexplained infertility and may play a role in the etiology of infertility.(29, 38, 39, 40, 41) However, routine screening for LIF mutations or polymorphisms in these women is not justified for the low prevalence of gene alterations the role of LIF gene mutations in unexplained infertility and implantation failures in IVF patients is not clear yet(35).

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