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Role of LIF genotypes on basal and day of triggering hormonal profile in women undergoing IUI.

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ABSTRACT

Female infertility is caused by ovulation issues, tubal disorders, uterine abnormalities, endometriosis, and peritoneal factors. For years, intrauterine insemination has been used to treat infertility alone or with ovarian stimulation. Obtaining adhesion ligands and losing inhibitory components are necessary for pregnancy. LIF promotes and differentiates proliferation and differentiation in embryonic, hematopoietic, and endothelial cells. LIF also controls prostaglandins (PGs), which mediate implantation and decidualization. The measuring hormone profile on cycle day 2 before control ovarian stimulation including FSH, LH, E2, Prolactin, TSH, and AMH. Serum LH, E2. The progesterone level was estimated on the day of triggering. The effect of LIF gene genotypes on hormone level. The hormone levels of 100 intrauterine inseminated women were measured on the second day of the cycle and the day of ovulation. DNA extraction for LIF gene variation detection utilizing sequencing and HRM. The current study found no significant differences between pregnant and non-pregnant groups for hormone levels, genotype, and genetic effect on female hormone levels with ($P \geq 0.05$). The LIF gene did not affect hormone levels in infertile females may be due to the tiny samples or the complex signaling chain that happens during implantation.

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KEYWORD

Intrauterine Insemination; Leukemia Inhibitory Factor; LH; FSH; TSH.

1. Introduction

The main problem for reproductive medicine has always been infertility. It is defined as not being able to prove a pregnancy after 12 months of regular, unprotected sexual activity (Fernando Zegers-Hochschild et al., 2017 [1]). In different countries, the main causes of infertility are different. About 30% of infertility is caused by female factors, 30% by male factors, 25% by things that can't be explained, and 15% by both male and female factors or other things (Deshpande and Gupta, 2019 [2]). Intrauterine insemination, or IUI, is a way to treat infertility. It is a type of artificial insemination. When the ovary releases one or more oocytes to be fertilized, the washed and concentrated sperm is put directly into the uterus. The hormonal assay involves measuring the plasma levels of hormones, such as luteinizing hormone (LH), to find out if a woman is ovulating and if she has a pituitary gland disorder (Pillai et al., 2017 [3]), follicle-stimulating hormone (FSH) to find out about her ovarian reserve, and prolactin level to confirm an anovulation cycle, since a high level of prolactin (PR) (Khine, Taniguchi and Harada, 2016 [4]). Also, thyroid stimulating hormone (TSH) to check for problems with the thyroid gland. Changes in the highly regulated local activity of TSH and thyroid hormones directly interfere with embryo attachment and early implantation (Wu Z.et al., 2019 [5]). This is because TSH receptors are found in the luminal epithelium, and glandular and luminal epithelium increased statistically significantly on LH days 6 to 9, which is when pinopodes show up (Sayem et al.,2018 [6]). Progesterone measurements in the second half of the cycle help confirm ovulation (La Marca and Mastellari, 2020 [7]). Leukemia inhibitory factor (LIF) is a type of cytokine that belongs to the

interleukin-6 (IL-6) family, which also includes IL-6 and IL-11. LIF is linked to the following things that happen during implantation: the endometrium becoming receptive, the embryo attaching to the endometrium, stromal decidualization, trophoblast invasion, blastocyst growth and development, and uterine leukocyte infiltration (Zahraa Alzaidi et al. 2021 [8]). The LIF gene has been cited to play an important role in embryo development and implantation. This pleiotropic cytokine affects a range of events in implantation, such as endometrial receptivity, embryo-endometrial interaction, stromal decidualization, trophoblast invasion, and blastocyst development. Lower levels of LIF have been found in the blood and endometrial tissue of women with recurrent pregnancy loss (Neena Malhotra et al. 2020 [9]) .

Few studies have looked at how LIF gene changes affect a person's ability to have children. However, infertile women have a higher rate of mutations near the start codon of exon one and in exon 3, which are important for controlling how LIF works in the body. This has been linked to unexplained infertility and recurrent implantation failure after IVF (A. Oliveira., et al 2016 [10]). The single nucleotide polymorphism (SNP) thymine (T)/guanine (G) in the untranslated region three ′ (3′ UTR) (rs929271/c.1414T > G) is a polymorphism of the LIF gene (Liu et al., 2023 [11]). Due to the importance of the LIF gene in the development and implantation of embryos and its main role in affecting the implantation of the embryo in the endometrium and the development of the blastocyst, the low level of this gene in some women was linked to recurrent fetal loss. Our study was designed to find out the role of genetic variations of the LIF gene in women who had IUI and how they affected hormone levels on the second day of the cycle.

2. Patients and Methods

The study looked at 100 infertile women who attended to infertility clinic of High Institute of Infertility Diagnosis and Assisted Reproductive Technologies/ Al-Nahrain University from 2021 to 2023 to try to get pregnant. They were divided into two groups according to pregnancy where: 26 patients had a positive pregnancy, and the other 74 had a negative pregnancy.

Inclusion criteria:

Male partner (no apparent infertility issues and normal seminal fluid analysis). Female partner (age 18-39, Unexplained infertility, Female without uterine abnormality, and woman with patent tubes diagnosed by HSG.

Exclusion Criteria:

Women with anatomical abnormality of the uterus, Cases of adenomyosis and endometriosis, any abnormality in the endometrium, Females with tubal obstruction and any Endocrinopathy

1-Hormone Profile

All female participants in this study had baseline hormonal measurements done on cycle day two before control ovarian stimulation, including FSH, LH, E2, Prolactin, TSH, and AMH. Serum LH, E2, and progesterone levels were estimated at the day of triggering (day 11-14) of ovulation by human chorionic gonadotropin injection (HCG) 5000-10000 I.U. The Vidas® costume kit analyzed hormones. The mini VIDAS system uses an enzyme immunoassay sandwich technique with ELISA technology for fluorescence detection .

2- DNA Extraction and assessment

Each participant was given five ml of peripheral venous blood in an EDTA tube on IUI day. DNA was extracted and purified from whole blood using the QIAamp DNA Blood Mini Kit® (Cat. No. 51104, Qiagen, GmbH). A Nabi UV/Vis NANO spectrophotometer (MicroDigital Co®, Korea) measured sample concentration and purity after DNA elution.

Concentration ranges from 2 to 1500 ng/µl, while purity ranges from 1.7 to 1.9 based on the A260/280 absorbance ratio.

(Table 1): Primers of LIF gene.

Gene	SNP	Sequence 5´----3´	Product size	source
LIF	rs929271	5'GTGACTTGCTTTAGGGTGTC3'	111(bp)	Heydarzadeh and Mehmannavaz
		5'CCAAGAACAGTGTGAACCAG3'		

2- Polymerase Chain Reaction (PCR)

The PCRs had a final volume of 25 µl, with 20 ng of DNA, 10µl of PCR master mix (Maxime PCR PreMix kit, Cat. No. 25025, iNtRON® Biotechnology), 10µl of each primer, and molecular grade water. The MultiGeneOptiMax Gradient Thermal Cycler® (Lab net, USA) program in (Table 2) was used:

(Table 2): Thermal profile of conventional PCR to amplify the region of (rs929271) of LIF gene

Phase of Reaction	Tm (°C)	Time	Number of cycles
Initial Denaturation	94	5 min	1
Denaturation -2	94	45 sec	40
Annealing	62	45 sec	
Extension-1	72	45 sec	
Extension -2	72	7 min	1

3- DNA Sequence and high-resolution Melting (HRM)

Direct sequencing: Samples were sent to Macrogen® Company for direct sequencing, which is the best way to

find out a person's genotype (Seoul, Korea). High-resolution melting is a new way to look at nucleic acid sequences after PCR to find genetic differences. The principle of HRM technology depends on temperature to separate the nitrogenous bases and using a fluorescent dye that can bind to dsDNA called Eva green.

4- Statistical analysis

The chi-square was used to figure out the OR (odds ratio) and the 95 percent CIs (confidence intervals), as well as to figure out how SNP and pregnancy are related to each other. Fisher's exact test was used to figure out how important the link was. The unpaired T-test (Student's T-test) was used to look at quantitative (numerical) parameters. Chromas 2.6.6 software was used to look at and analyze the sequencing results (Technelysium Pty Ltd, Australia)

3. Results

Mean± SE of age for females with negative pregnancy (was 31.88 ±0.44) while females with Positive pregnancy was (31.08 ±0.81) with on significant difference (P = 0.3). (r = 0.99, p = 0.348). The results of FSH, LH, E2, AMH, and Prolactin hormones showed that there were no statistically significant differences between pregnant and non-pregnant females for the six hormones involved in the current study in cycle two day with a P-value of 0.6, 0.1, 0.4, 0.5, 0.9 and 0.7, respectively, as shown in (Table 4). (Table 5) explains the results of the hormones on the trigger day, showing that there were no significant differences between both groups of the current study for the three hormones, LH, estrogen, and progesterone, respectively. The mean ± SD for LH hormone was (6.51± 2.08), estrogen level was (213.27± 122.31) and progesterone was (0.93±0.15) for female with negative pregnancy while the level of the hormone for female with positive pregnancy was (6.68±1.88), (225.84±169.69), and (0.93± 0.12) for LH estrogen and

progesterone respectively. The P-value was 0.7, 0.6, and 0.8 for the three hormones respectively. The rs929271 is located on chromosome 22 with allele variation (G/ T) in the 3´ UTR region (non-coding region) .

The results of the genotype and allele frequencies of the LIF gene in (Table 6) showed that the percentage of mutant genotypes was 50% and 47.3%, with an odds ratio of 0.5 and a P-value (0.7) for females with positive and negative pregnancies, respectively. The percentage of wild genotypes was 7.7% and 13.5% for both groups, respectively. The results showed that there were no significant differences for G and T alleles between the two groups of the current study, according to a P value that was 0.5. (Table 7) shows the mean and SD of the level of hormones at cycle day two and their association with the three genotypes of the LIF gene GG, GT, and TT, where the results showed that there were no significant differences between the genotypes of the gene in the serum level of hormones for FSH, LH, E2, AMH, TSH, and Prolactin with a P-value of 0.1, 0.3, 0.2, 0.2, 0.3 and 0.7, respectively. (Table 8) showed the results of the distribution of the level of hormones for the three genotypes of the LIF gene on the day of the trigger revealed that there were no significant differences between the three genotypes GG, GT, and TT, where the P value for each of LH, E2, and progesterone was 0.9, 0.2 and 0.2, respectively.

(Table 3): Age categories according to pregnancy

Age groups	**Pregnancy**	
	Negative	Positive
24-28	14	5
29-33	34	15
34-39	26	6
mean± SD	31.88 ±3.82	31.08 ±4.15
Std. Error of Mean	0.44	0.81
p-value	0.3	
Chi-square	0.2	

(Table 4): Hormones concentration at cycle Day 2 including FSH, LH, E2, AMH, TSH, and Prolactin.

Pregnancy		FSH	LH (1)	E2 (1)	AMH	TSH	prolactin
Negative 74	Mean	5.87	5.05	37.57	2.48	2.12	19.19
	SD.	1.73	1.94	7.471 30	1.66	0.71	6.10
Positive 26	Mean	5.67	4.49	36.29	2.23	2.11	18.79
	Std. Deviation	1.98	1.22	6.73	1.54	0.72	5.51
	p-value	**0.6**	**0.1**	**0.4**	**0.5**	**0.9**	**0.7**

(Table 5): Hormonal Profile on the Day of Trigger

Genotype Rs LIF	Pregnancy		P-value	OR	CI 95%
	Negative (74)	**Positive** (26)			
GG	10 (13.5%)	2 (7.7%)	--	1.00	(Reference)
GT	29 (39.2%)	11(42.3%)	0.4	0.5	0.09 -2.79
TT	35 (47.3%)	13 (50%)	0.7	0.5	0.10 - 2.79
Total	74	26	--	--	--
Allele Frequency					
G	0.33 (49)	0.3 (15)	--	1.00	(Reference)
T	0.67 (99)	0.7 (37)	0.5	0.8	0.41 - 1.63

(Table 7): Hormonal parameter at cycle Day 2 according to LIF gene distribution

Rs LIF		FSH IU/ml	LH IU/ml	E2. pg/ml	AMH ng/ml	TSH IU/ml	Prolactin ng/ml
GG	Mean	6.01	4.62	34.01	2.05	1.93	18.79
	SD	1.09	1.99	6.41	1.21	0.47	7.93
GT	Mean	5.3750	5.2275	37.4775	2.7125	2.24	19.66
	SD	2.16413	2.12699	8.36861	1.76928	0.80	5.09
TT	Mean	6.1417	4.7060	37.8396	2.2496	2.07	18.68
	SD	1.52718	1.38337	6.37671	1.56982	0.68	6.11
p-value		0.1	0.3	0.2	0.2	0.3	0.7

(Table 8): Hormonal parameter on the day of a trigger according to LIF gene distribution

Rs. LIF		LH IU/ml	E2 pg/ml	Progesterone ng/ml
GG	Mean	6.52	167.06	0.90
	SD	2.06	55.37	0.07
GT	Mean	6.62	241.99	0.96
	SD	2.53	156.76	0.14
TT	Mean	6.50	207.68	0.91
	SD	1.524	127.48	0.15
p-value		0.9	0.2	0.2

4. Discussion

The results of the hormones at cycle Day 2 showed that there were no significant differences between pregnant and non-pregnant women for the sex hormones. This may be because all the women in this study were in the fertile age group with no significant endocrine pathology, which did not show significant differences between the two groups. Clear differences may appear if there was a control group that was not fertile. The results of the current study were consistent with the previous study by Ragheb et al. 2019, in some hormones like E2, TSH, and LH with a p-value of 0.7, 0.5, and 0.1 respectively; on the other hand, the above study does not agree with ours regarding prolactin and FSH hormones that showed a significant difference between a pregnant and non-pregnant female with P- value 0.001 and 0.04 sequentially (Ragheb et al., 2019 [12]). This could be because all of the women in this study were able to have children, so there were no big differences between the two groups. If there was a control group that didn't have children, there might be clear differences. When FSH stimulation is used with IUI, more Oocytes are released, which makes it more likely that a woman will get pregnant. FSH is also used to produce Controlled Ovarian Hyperstimulation, which results in producing more follicles for In Vitro Fertilization in humans (Santi et al., 2017 [13]). Hypothyroidism in females leads to an elevated level of the hormone prolactin, which decreases. The levels of follicle-stimulating hormone (FSH) luteinizing hormone (LH) and finally cause infertility. Obesity acts upon the reproductive cycle by decreasing estrogen metabolism and stimulating menstrual disturbance along with ovulation (Sharma et al., 2022 [14]). Zheng et al., 2022 [15]) found that the basal E2 level was much lower in pregnant women than in women who were not pregnant. The AMH level was

significantly higher than that of the non-pregnant group. The duration of infertility years in the pregnancy group was shorter (3.40 ± 2.10 VS 4.07 ± 2.90).

On the day of the trigger, the hormones LH, estrogen, and progesterone were tested, and the results showed that there were no significant differences between the two groups. The high concentration of E2 on trigger day may be linked to little or no production of progesterone and an increase in the LH level, which is in line with a previous study, and there is evidence to suggest that the high estradiol levels usually caused by ovulation induction with gonadotropins are linked to adequate progesterone levels in the luteal phase (PCASRM., 2020 [16]). The results of the hormonal profile at cycle day 2 based on the genetic distribution of the LIF gene showed that there were no significant differences between the genotypes of the LIF gene (GG, GT, and TT) in terms of the serum levels of Hormones FSH, LH, E2, AMH, TSH, and Prolactin. This could be because all of the women in this study were unable to have children, so there were no significant differences between the two groups. If there was a control group that didn't have children, there might be clear differences. Another study similarly found that the mean serum concentration of FSH, LH, E2, TSH, and prolactin level has no significant difference between the pregnant women (polycystic and non-polycystic) and non-pregnant women at cycle day 2 in ICSI (Zena F. Hussein et al 2018 [17]).

On the day of the trigger, there was no difference in the serum levels of progesterone and LH based on the LIF genotype. However, the E2 hormone showed a clear increase in the heterozygous genotype, followed by the mutant genotype, and then the wild genotype, which had the lowest level. As (Yung-Liang Liu, 2023 [18]), patients in POSEIDON group 1 or 2

had an unexpectedly poor or suboptimal ovarian response to standard ovarian stimulation, even though their ovarian reserve parameters were good. The results showed that young patients had more of the LIF (rs929271) TG/GG genotypes and the G allele. Results showed that there were significant differences between the genotypes of LIF (rs929271) in poor-responder groups and young (age 35 years) and normal responders; the TG/GG and G alleles were more common in group 1 in normal responders (Matheus Roque et al 2021 [19]).

6- Conclusion

The current study revealed that there were no significant differences between the levels of the six hormones (LH. AMH, FSH, E, PRL, and TSH) between the two groups of this study, as well as the absence of statistically significant differences for the level of progesterone, estrogen, and LH between both groups of women who underwent IUI. The levels of these hormones were distributed according to the genotype of the LIF gene. There were no significant differences between the levels of hormones for each genotype of the gene, whether it was Wild, Heterozygous, or Mutant. This means that even though this gene is important and linked to female fertility, it did not show a clear effect on the levels of hormones, which may be due to the limitations of this study due to the small sample size may be a reason for the absence of statistically significant differences.

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Author Contribution

Sarab K. Hammood performed the study, and Manal T. Al-Obaidi, and Salih Ibrahem, supervised the work .

Conflict of Interest

The authors declare no conflict of interest .

Ethical Clearance

The study was approved by the Ethical Approval Committee.

Financial Disclosure

There is no financial disclosure.

Ethical Clearance

The study was approved by the Ethical Approval Committee

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