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THE CONTRIBUTION OF SEVERAL FACTORS IN INTERPRETING THE POTENTIAL MOLECULAR AND IMMUNOLOGICAL CAUSE OF MULTIPLE IMPLANT FAILURE IN IRAQI WOMEN

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Abstract:
An unsuccessful attempt of a fertilized egg or embryo to deposit itself in the uterine endometrial layer is known as implantation failure, resulting in an unsuccessful pregnancy. Recurring implantation failure (RIF) describes the inability to implant high-quality embryos more than once. Embryo chromosomal defects, endometrial disorders, and immune system concerns are among the many possible causes. The purpose of this study was to investigate the function of the endometrial expression of leukaemia inhibitory factor (LIF), glycoprotein 130 (gp130) receptor, IL-1β, and HB-EGF molecules in patients of primary unexplained infertility, as well as the release of these molecules throughout the window of implantation. The study was conducted on 70 women diagnosed with primary unexplained infertility for at least two years and repeated implantation failures following IVF-ET, along with 50 normal fertile women who participated as a control group. Tissue from the endometrium and uterine flushing were collected. The expression of LIF, gp130 IL-1β, and HB-EGF mRNA was tested by RNA extraction and PCR. The control group were significantly higher than those in the patient group ($p<0.05$) concerning LIF, gp130 IL-1β. In conclusion, Endometrial LIF, gp130 IL-1β mRNA expression may serve as a molecular indicator of infertility with no known cause.
Keywords: Leukemia inhibitory factor, Glycoprotein 130, RIF, IL-1β, HB-EGF

introduction: 1-

A crucial step of pregnancy in mammals is implantation, which determines whether the pregnancy will be successful and impacts the health of the progeny[1]. During implantation, the blastocyst implants itself into the endometrial stroma and vasculature after developing a tight connection to the endometrial epithelial cells[2]. Three distinct phases of blastocyst adherence have been identified by histological investigation of human uteri throughout early pregnancy: apposition, adhesion, and invasion[3]. Excluding extrinsic influences such as nutrition, pollution, endocrine disruptors, diseases, and stress). Several Endogenous factors (such as cytokines, growth factors, and adhesive molecules have been discovered as potential components that could influence the progression of the developing embryo through the oviduct and the cellular connection between the endometrium and the formed blastocyst[4]. A suitable definition of recurrent implantation failure (RIF) has yet to be reached in the medical community. The majority of failed pregnancies occur in the first trimester, leading many to believe that embryo implantation errors are the primary cause of these complications[5]. Interleukin-1β (IL-1β) and heparin-binding epidermal growth factor (HB-EGF) are two examples of the cytokines and growth factors that express in the embryo and the uteri which have been identified to behave in a synergistic

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manner during the stage of mother-fetal crosstalk by stimulates trophoblast invasive activity necessary for placentation, according to research findings[6]. On the other hand, leukemia inhibitory factor (LIF) and mucin 1 are considered to be two of the major significant signaling vectors. LIF receptor (LIF-R) is expressed by the blastocyst, which indicates the significance of LIF in embryo-maternal crosstalk. Endometrium and oocyst are also considered to be expression sites for LIF-R[7]. The activation of signal transduction pathways is caused by the binding of LIF to LIF-R and glycoprotein (gp130)[8]. An attempt was made in this study to classify some of the distinct reasons for multiple implantation failures, with the expectation that this would make it possible for couples who experience implantation failure following embryo transfer to obtain the proper medical attention.

2-Patients and Methods:

2-1 Patient selection

The study was conducted on 70 women diagnosed with primary unexplained infertility for at least two years and repeated implantation failures following IVF-ET at the Higher Institute for the Diagnosis of Infertility and Assisted Reproduction Techniques Al-Nahrain University (Baghdad/ Iraq) from April 2023 to May 2024, along with 50 normal fertile women participated as a control group. No patient received steroid hormone treatment during the last three months, and their regular menstrual cycles were 28–30 days long. The average age of women unable to conceive was (35.1± 6.2) years, with a range of (27–40) years. No endometriosis, tubal or ovarian pathology, or ovulatory abnormalities were detected by hysterosalpingography or laparoscopy in any of the individuals included in the study. Results show normal levels of progesterone in the blood, prolactin in the plasma, thyroid function within normal range, and semen analysis within normal range according to World Health Organization (WHO) standards. All participants were asked to provide their age, menstruation history, BMI, duration of infertility, and reasons for infertility, as well as the oocyte retrieval number, embryo transfer number, high-quality embryo retrieval number, and embryo freezing number were documented. The control group consisted of fertile and ovulatory women with at least one full-term live birth without any previous diagnostic pregnancy loss during their reproductive years, with a primary age of (33.6±7.6) years and a range of (25-38).

2-2 Methods

Following informed agreement, participants had endometrial tissue and uterine flushing samples taken from the wall of the uterus cavity via a "Pipelle catheter." Specimen collection occurred entirely inside the implantation window, which begins with LH surge+7 and ends at LH+11. In preparation for the tests, the tissue and flushing samples were kept at a temperature of -20°C. Tissues were utilized to extract RNA and conduct PCR tests for LIF, gp130, IL-1β, and HB-EGF mRNA. The enzyme-linked immunosorbent assay (ELISA) was performed to assess the production of LIF, gp130, IL-1β, and HB-EGF from uterine flushing samples. The Quantikine ELISA kit was developed by R&D Systems Inc and was supplied by Bender Medsystems, USA was used for this purpose

2-3 Procedures for RNA extraction and RT-PCR

Trizol (Qiagen, USA) extracted total RNA (around 50-100 mg) from endometrial tissue, as mentioned before by [9]. The pellet that contained RNA was treated with around 1–3 U of RNase-free DNase for every μg of RNA, then left to incubate at 37 °C for 30 minutes. After that, it was rinsed with 75% ethanol. Reverse transcription was performed on a total RNA sample of 2 (μg) in a reaction mixture of

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20 (µl) using a standard kit (cDNA kit, Applied Biosystems, USA). following these steps: annealing for 10 minutes at 27°C, the production of cDNA for 30 minutes at 46°C, and finally, activating the enzyme for 5 minutes at 96°C.

2-4 cDNA amplification

Amplification of cDNA by real-time PCR was used to determine the LIF, gp130 mRNA IL-1β and HB-EGF expression amounts. In order to design probes and primer sequences for every gene, the ProbeFinder was utilized (Table 1). As a housekeeping reference, the β-actin gene was chosen. The Reaction procedure: 45˚C for 25 min, 93˚C x5 min (95˚C 20 sec, 58˚C x20 sec ,72˚C x25 sec) for 45 cycles, 90˚C x5 min, 58˚C x35 sec and 95˚C x35 sec. The data were evaluated with the assistance of the Real-time detector (ABI-8400).

Table (1): Primer sequences employed in the investigation

Gene	Forward primer	Reverse Primer
IL-1β	AAG GCT GGC TTA TTG CAG TGG C	TGT AGT GGT CGT CGG AGA TT
HB-EGF	ACA AGG ACG AGC ACG CGA AAA G	CGA TCA CCA GCA GAC AGA CAG ATG
LIF	CAC CCT CAC TGA ACC ACA GAG	CCC TGT GGG CAT GTT TCA TAC
gp130	ATA CTG GAG TGA CTG GAG	CAT CTT GTG AGA GTC ACT
β-actin	CAC AAG TCA CAC TTC ACA	CTA TCA CCT CGT GCT CT

2-5 statistical analysis

The statistical analysis was conducted using SPSS 15.0, developed for Windows by SPSS Inc. and based in Chicago, IL, USA. Mean ± SD is how measurement data following a normal distribution is displayed. Following a one-way analysis of variance in SPSS, the groups were compared using Student's t-test. An indication of a statistically significant difference was given by a p-value less than 0.05.

3-Results:

The two groups of patients did not differ significantly ($p>0.05$) regarding age, duration of infertility, or body mass index. Table (2) shows that the baseline statistics were consistent and similar.

Table (2) Comparative analysis of basic conditions between the patient group and the control group

	Patients group (n=70)	Control group (n=50)	t-value	p-value
Age (Years)	(35.1± 6.2)	(33.6±7.6)	0.71	0.832
Duration of fertility	(4.8±3.1)	-	-	-
Sampling day	(21.66±0.85)	(21.60±0.82)	0.22	0.454
NO. of previous IVF	1–5	1-2	0.12	0.245
BMI	(23.05±3.45)	(21.77±2.44)	0.59	0.790

BMI, body mass index.; IVF, *in vitro* fertilization

There was no statistically significant difference between the two groups in the HB-EGF levels ($p>0.05$). When comparing the levels of IL-1β in the control group to those in the patient's group, it was found that the control group had a considerably higher level ($p<0.05$)

Table (3): The levels of LIF, gp130, IL-1β, and HB-EGF in the endometrial tissue and uterine flushes

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of both the control and patient groups

	Patients group (n=70)	**Control group (n=50)**	**P value**
LIF pg/ml	80±9.98	320±34.6	**0.008**
gp130 pg/ml	40±67	580±68	**<0.001***
IL-1β (ng/ml)	15.32±7.37	17.26±7.49	**0.01**
HB-EGF (pg/ml)	312.25±255.13	272.28±195.06	0.092

LIF, Leukemia inhibitory factor; gb130, Glycoprotein 130; IL-1β, interleukin-1β; HB-EGF, heparin-binding epidermal growth factor; **Bold line**: Represent significant result; *: Highly significant result

In terms of mRNA expression of HB-EGF, there was no significant result between the patient and control group; otherwise, IL-1β was statically significant between the two groups. On the other side (LIF and gp130) were highly significant.

Table (4): Expression mRNA level of (LIF, gp130, IL-1β, and HB-EGF) in the endometrial tissue and uterine flushes of both the control and patient groups

	Patients group (n=70)	**Control group (n=50)**	**P value**
LIF	4.8±6.5	47.5±29.3	**<0.001***
gp130	41.7±28.4	164±87	**<0.001***
IL-1β	0.28±0.07	0.39±0.19	**0.01**
HB-EGF	0.48±0.12	0.51±0.26	0.08

LIF, Leukemia inhibitory factor; gb130, Glycoprotein 130; IL-1β, interleukin-1β; HB-EGF, heparin-binding epidermal growth factor, **Bold line:** Represent significant result; *: Highly significant result

4- Discussion:

One of the most critical factors that can affect a pregnancy's outcome is the implantation process[10]. Several molecules, such as integrins, colony-stimulating factors, growth factors, and cytokines, influence the uterine receptivity to the blastocyst's planned implantation[11]. The LIF cytokine is a member of the IL-6 family of cytokines, which are able to communicate with the cell surface through specific receptors. These receptors all include the same gp130 component, which is an essential signal transduction protein. Decreased LIF levels in uterine flushing fluid throughout the late luteal phase may be indicative of implantation failure, according to a noninvasive method that measures LIF secretion in these samples[12]. According to the findings of the current investigation, the amount of LIF that infertile women secreted within the window of implantation in their uterine flushing samples was significantly insufficient. Prior research indicated that the endometrium released the highest concentration of IL-6 cytokine-receptor family member gp130 during implantation. According to the study, endometrial gp130 expression was significantly lower in infertile women than in control women[13].The interleukin-1 (IL-1) is a polypeptide that is composed of three components: two receptor agonists, namely IL-1α and IL-1β, and one receptor antagonist, namely IL-1rα. Over the past few years, there has been a growing interest in the role that IL-1β plays in reproductive activity[14]. It is believed that this role is connected with endometrial receptivity. Through both systemic and local actions, IL-1β plays a role in regulating the reproductive process of females by stimulating P production, which in turn

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control's reproductive function, and by inducing local expression in uterine, ovarian, and follicular fluid[15]. The study indicated that the levels of IL-1β were noticeably higher in the control group compared to the infertile patients' group, suggesting that IL-1β could play a role in the expansion, development, maturation, and induction of ovulation of follicles, which in turn can enhance the rate of fertilization and the quality of the embryo, leading to an improvement in the rate of implantation. The expression of IL-1β within the patient group was significantly lower than the control group. According to earlier research, IL-1β, IL-1RtI, and IL-1rα can be identified in several human tissues, including the endometrium, endometrial fluid, embryo, and the connection between the endometrium and the embryo[16]. These molecules have the ability to limit gamete implantation in rats by blocking the action of IL-1RtI in such tissues. The exact mechanism by which IL-1 controls endometrial tolerance remains unclear. There are several members of the epidermal growth factor family, and HB-EGF is one of them. plays a function in facilitating the molecular crosstalk between the embryo and the intima by binding to the HB-EGF receptor on the outer layer of the embryo and the endometrium[17]. This occurs within the catalysis of heparan sulfate proteoglycan[18]. Compared to the patient group, the control group exhibited increased endometrial HB-EGF mRNA expression, according to the RT-qPCR results of this investigation. This implies a possible close relationship between embryo implantation and considerably elevated HB-EGF mRNA in the endometrium.

5- Conclusion:

The inability to conceive after 2 to 6 rounds of in vitro fertilization is known as recurrent implantation failure. Embryonic and parental genetics are two of the many potential causes of an unsuccessful implantation attempt. There is a genetic basis for implantation failure, as indicated by the levels of LIF, gp130, IL-1β, and HB-EGF gene expression and the disparities between the infertile and the control groups. Future studies in this field may enhance fertility rates and pave the way for previously unexplored clinical consequences.

Abbreviation:

RIF: Recurring implantation failure
LIF: Leukaemia inhibitory factor
gp130: glycoprotein 130
IL-1β: Interleukin-1β
HB-EGF: heparin-binding epidermal growth factor
WHO: World Health Organization

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Authors' contributions

Zahraa ALzaidi, Reem QM Wafeeq, and **Safa A. AL-Gebori** performed the study

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Ethics approval and consent to participate

This study was approved by the Ethics Committee of Higher Institute for the Diagnosis of Infertility and Assisted Reproduction Techniques Al-Nahrain University (Baghdad/ Iraq). The patients or guardians signed informed consent.

Competing interests

The authors declare that they have no competing interests

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