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Single Nucleotide Polymorphism (SNP) rs2229569 with L Selectin Gene Expression in Iraqi Female during *in vitro* Fertilization Program.

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Abstract

Infertility is recognized as one of the leading reproductive health problems in various regions of the world. In vitro fertilization (IVF) is one of the most effective treatments for infertility. In the current research, the role of the selectin -L (*SELL*) gene, especially the rs2229569 polymorphism, has been determined in terms of implantation and expression level. This work involved 67 females who underwent an in vitro fertilization cycle, divided into two major groups: the implantation failure female group and the implantation success female group. Blood samples were collected from the females. After DNA extraction from blood sample then amplification by polymerase chain reaction, samples were sent for sequence analyses. The SNP rs2229569 was detected and that recorded three genotypes. The failure group had a deviation from H.W.E., and the success group was in agreement with H.W.E. law according to Chi square values (X2 = 11.60, 1.58). Significant differences in the failure group refers to the effects of infertility in the study population. Odds ratio of rs2229569 showed that the T allele was (2.09), so T allele may be considered a risk allele for the failure implantation. Finally, the *SELL* gene expression had a downregulation in female with failed implantation compared to female in successful implantation groups.

Keywords: Gene expression, Gene polymorphism rs2229569, Infertility, In vitro Fertilization, Selectin L.

Introduction

Infertility is a common problem and unique medical condition with important psychologic, economic, demographic, and medical implications [1]. It involves a couple rather than a single individual. Infertility is defined as the inability to achieve a clinical pregnancy after trying for 12 months with regular, unprotected sexual activity [2]. Primary infertility affects women who have never had a baby, while secondary infertility affects women who have already given birth at least once[3]. Recent research has found that 15% of married couples around the world are at increased risk of poor reproductive health and mental health due to infertility and invasive treatments [4].

Many factors, including hormones, prostaglandins, and adhesion molecules, work together to regulate the intricate crosstalk between the endometrium and the blastocyst necessary for successful implantation.[5, 6]. Adhesion molecules are a class of membrane-associated glycoproteins found on the surface of cells that play key roles in cellular processes such as recognition, adhesion, migration, and differentiation[7]. Different structural and

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functional properties categorize them into four groups: cadherins, integrins, selectins, and immunoglobulin (Ig)-like proteins [8]. In various inflammatory factors the level of soluble platelet selectin, as one of the cell adhesion molecules, is increased[9]. When the endometrium is most receptive to embryo implantation, it is called the "window of implantation," and it occurs during the mid-luteal phase in humans.[10, 11]. In vitro fertilization (IVF) is a way to help women get pregnant when their fallopian tubes are damaged or blocked[12]. During the window of implantation, the endometrium expresses several genes that enable the process of implantation to occur, such as selectins[13].

The selectins, which include P-selectin, L-selectin, and E-selectin [14], are a family of cell adhesion molecules. The *SELL* gene that encodes the L-selectin protein in humans is located in a tandem arrangement with other members of the family on the long arm of chromosome 1 (1q24.2). L-selectin consists of nine exons and eight introns (https://www.ncbi.nlm.nih.gov/) [15]. The surface of vascular endothelial cells express adhesion molecules, enabling leukocytes in adhering to other tissues in that region[16]. The binding of *SELL*, expressed by the trophoblast, to oligosaccharide based ligands expressed by the endometrium[17] is critical for the initial attachment of an embryo to the endometrium. Thus, L-selectin-ligand interactions in the uterus may serve as a link in the chain leading to the essential first attachment for implantation[18]. According to previous information, the present study has been designed to evaluate the relation between *SELL* gene and IVF outcome.

Materials and Methods

Study Design

The study consisted of 67 women under IVF cycle divided into two major groups including 41 implantation failure female group and 26 implantation success females group. Their age range was 18-44 years, from Rooh ALhayat Center for IVF, AL Farah Center for IVF and AL Nada Center for IVF in Baghdad, Iraq.

Collection of Blood Samples:

Following an hour after embryo transfer, the blood samples were drawn from each female (success 26 and failure 41 females) as two ml of blood was added directly into an EDTA containing tube for genotyping study. 250 µL added to 750 µL of GENEzol for gene expression.

Genomic DNA Extraction and Genotyping:

DNA was isolated using 2 mL of whole blood collected in tubes of EDTA using a purification kit for the genomic DNA (Geneaid). The amplification of DNA represented in fragment of exon 5 region of SELL gene (forward and reverse). Then the polymorphism of the *SELL* gene was detected using the PCR-sequencing method. PCR amplifications were detected in a total volume of 25 µL consisted of 5 µL genomic DNA (30 to 53.3 µg/mL), 13 µL D.W., 5µL master mix [1 U DNA polymerase, 1000 µM dNTP , Reaction Buffer with 1.5 mM Mgcl2(1 x)] and 1 µL of each primer as follow L selectin forward (10 pmole / µl) were 5′-TTTGAATCCTAGCCCTGCCAC-3′; and reverse (10 pmole / µl) were 5′-AAGCCCCAGAGTAATGCTTGA -3′ both primers were designed by second author used primer designing tool in NCBI. The program of PCR were shown in **Table1.** The 832bp PCR fragment was confirmed to be present after separation on a 1% agarose gel with ethidium bromide staining. The sequencing technique was used to identify the *SELL* gene polymorphism.

Table 1. The PCR program of *SELL*

No	Step	Temperature ˚C	Time	No of cycle
1	Initial denaturation	94	5 min	1
2	Denaturation	94	45 sec	
3	Annealing	58	45 sec	35
4	Extension	72	2 min	
5	Final extension	72	5 min	1

DNA Sequencin

After amplification, the PCR products were analyzed of exon 5 region for *SELL* gene (forward and reverse) of all failure and success implantation group. The 61 samples (35 failure group and 26 success group) were sent to Macrogen Corporation

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– Korea for sequencing by using automated DNA sequence Macrogen.

Real Time -PCR (RT–PCR)

250 µL of blood was added to 750 µL of GENEzol and used for gene expression. RNA was isolated using the GENzolTM TriRNA Pure Kit, and then RNA was converted to complementary DNA (cDNA) using the Accu PowerRRT RocketScriptTM PreMix Kit from Bioneer, Korea, and Oligo dT20 as primer. *SELL* gene expression was detected by real-time PCR (RT-PCR). This technique was carried out by a dye that used the real-time fluorescence of a cDNA binding dye (SYBR Green) to measure cDNA amplification. L-selectin Primer for Real time PCR was forward TGA TTC AGT GTG AGC CTT TG and reverse CTT GAC AGG TTG GTT CTG [19]. The required volume of each component was 25 µL: (5 µL SYPER Green), 13 µL nuclease-free water, 1 µL of each forward and reverse primer, and 5 µL cDNA. The absolute target quantities were calculated using the human reference gene (junctional cadherin complex regulator) (JHY). The primer of reference gene was forward GTCCAGGGGTATTACAGGCAA and reverse TCAGGAATCAGCCCAAGACG were designed by present study . The threshold cycle was used to quantitatively measure the levels of gene expression [20].

Statistical Analysis

The results of genomic DNA amplification were analyzed using BioEdit software. Online Hardy-Weinberg equilibrium H.W.E. Calculator to test whether the observed genotype was applied with H.W.E. WINPEPI software was used to calculate the significance and odds ratios of genotyping and allele frequencies of the studied genes.

Results

The present study examined the *SELL* gene polymorphism by sequencing method in infertile female who underwent IVF programs, including failure and success implantation groups. Sanger's sequencing was performed on the samples of amplified PCR-products for exon5 on *SELL* gene. The sequences were blast to a reference sequence of *SELL* gene in the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) as shown in **Fig. 1 and Fig. 2,** and the samples analyzed by BIO Edit as shown in **Fig. 3**.

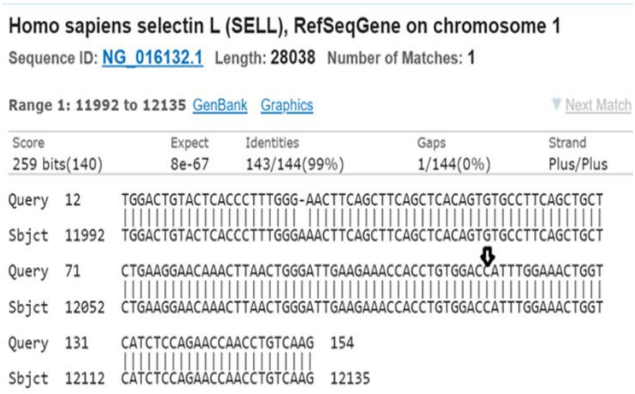


Figure1. A representative sequence alignment of *SELL* at exon 5 amplification results with NCBI Blast. Arrow is for normal homozygous genotype rs2229569.

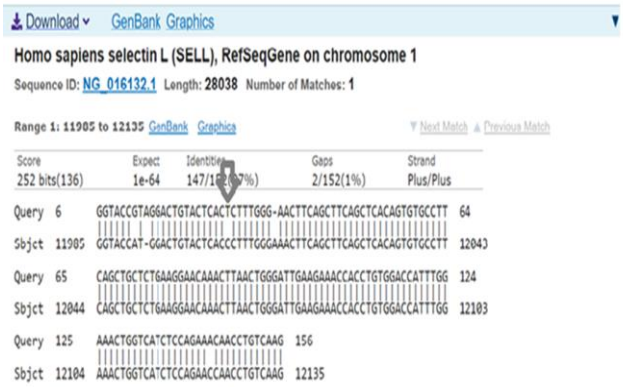
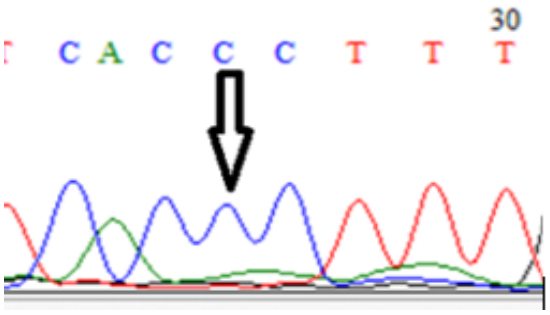


Figure2. A representative sequence alignment of *SELL* at exon 5 amplification results with NCBI Blast. Arrow is for heterozygote genotype of rs2229569.



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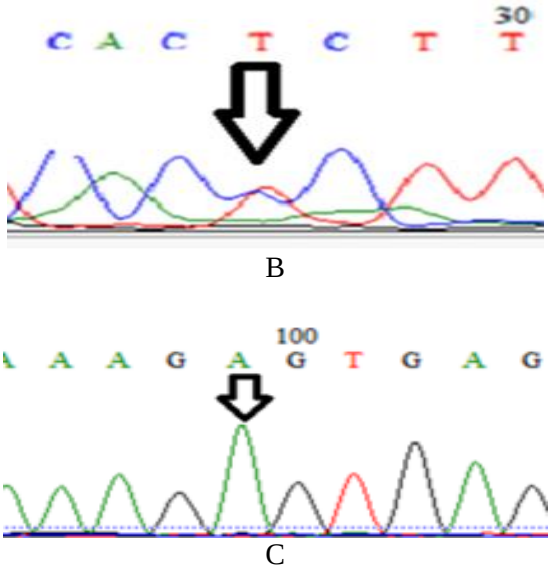

Figure 3. A and B and C shows the sequence of DNA samples of the rs2229569 analyzed by BIO Edit software. In A, the normal homozygote genotype C\C. B, the heterozygote C\T and C, the mutant homozygote TT genotype.

The frequency of genotypes and alleles of the SNP of *SELL* T>C rs2229569 using H.W. E were shown in **Table 2**. The results in **Table 2.** Showed significantly higher (P>0.05) of genotypes frequency for failure implantation group but showed non-significant in success group. This indicates that the failure group had deviation from H.W.E while success group agreed with H.W.E. The Expected frequencies: CC homozygous genotype recorded 21.61vs.20.35, while the heterozygous genotype CT had expected frequency 11.79vs 5.31and TT genotype had 1.61vs. 0.35 as expected frequency among two study groups at receptively. Homozygote genotype CC was more frequent in both groups failure and Success group (25+21=46) which made it common genotype in Iraqi female population.

Comparison of the frequencies of allele and Genotype of SELL gene polymorphism (rs2229569 T>C) between failure and success groups was shown in Table 3. The present data in Table3 showed that the genotypes (CC, CT, TT) recorded different between groups. Frequency of homozygous genotype CC in success group (80.77%) was higher than in failure group (71.42%), while the heterozygous genotype CT was (15.38%) in success group and (14.29%) in failure group. Homozygous TT genotype was higher in failure group (14.29%) than in success groups (3.85%). According to odds ratio TT recorded 4.17 therefore TT genotype may be considered an etiological fraction with positive association with failure of IVF while other genotype CC and CT may be protective genotype with odds ratio 0.6 and 0.9. The frequency of C allele was (78.57%)in the failure group, while in the success group was (88.46%). The frequency of T allele was (21.43%) in the failure group while in the success group was (11.54%). Moreover, T allele could have recorded odds ratio 2.09 which make it risky factor with positive association with failure implantation while C allele was protective 0.48 with negative association with the failure implantation.

Table 2. Frequencies of genotypes and allele of the SNP of *SELL* T>C rs2229569 in exon 5 using H.W.E

***SELL* rs2229569T>C Genotype**		CC	CT	TT	C	T	χ^2	P-value
Failure implantation Female (35)	Observed no(%)	25 71.42%	5 14.29%	5 14.29%	0.78	0.22	11.60	0.003**
	Expected no(%).	21.61 61.73%	11.79 33.67%	1.61 4.6%				
Success implantation Female(26)	Observed no(%)	21 80.77%	4 15.38%	1 3.85%	0.88	0.12	1.58	0.50NS
	Expected no(%)	20.35 78.26%	5.31 20.41%	0.35 1.33%				
Total Observed (%)		46 75.40%	9 14.76%	6 9.84%				
P value		0.555 NS	0.738 NS	0.102 NS				

*** (P≤0.01) significant, ** (P≤0.01) highly significant, NS: Non-Significant.**

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Table 3. Comparison of the genotype and allele frequencies of L-selectin gene polymorphism (rs2229569 T>C) between failure and success groups

***SELL* polymorphism rs2229569T>C**	Frequencies (%) failure group (n=35)	success group (n=26)	Odds Ratio	Etiological or Preventive Fraction%	Fisher's exact probability	CI 95%
CC	25 (71.42%)	21 (80.77%)	0.6	40.05	0.5	0.16to 2.03
CT	5 (14.29%)	4(15.38%)	0.92	8.3	0.8	0.21to 4.25
TT	5(14.29%)	1 (3.85 %)	4.17	76.0	0.1	0.52to 80.82
		Alleles Distribution				
C	55(78.57%)	46 (88.46%)	0.48	52.2	0.2	0.16to 1.32
T	15 (21.43%)	6(11.54%)	2.09	52.2	0.1	0.76to 6.26

CI: confidence intervals

Results of Real Time PCR of *SELL*

Sixty-one 61 female underwent IVF included 35 failure group implantation,26 success group selected for L selectin gene expression. Quantitative real- time PCR was validated and performed. *SELL* gene expression was quantified into two groups: the failure group and the success group with used the reference gene (*JHY*). As shown in **Table 4,** the mean ct of selectin L gene for the failure, and success group were respectively (18.62, and 18.11), while the means of Ct for (JHY) gene expression for the failure, and success were respectively (26.37and 26.71). The present study result shows that the fold change in *SELL* was down regulation in the failure group (0.56 ±0.17) while upregulation in the success group was (1.00 ±0.00) and significant difference in the fold gene expression between these two groups.

Table 4. Comparison between Failure and Success folding of *SELL* gene

Study Group	**Mean of ct of *SELL***	**Mean of ct of JHY**	**Mean Δ ct**	**Mean Δ ct of calibrator**	**Δ Δ ct**	**2- Δ Δct**	**Experimental group\success group**	**Fold of gene expression mean±Std**
Failure group	18.62	26.37	-7.75	7.2	-14.95	31651.8	31651.8\ 57052.4	0.56 ±0.17*
Success group	18.11	26.71	-8.6	7.2	-15.8	57052.4	57052.4\57052.4	1.00 ±0.00

*** (P≤0.01) significant, ** (P≤0.01) highly significant, NS: Non-Significant.**

Discussion

This study analyzed the L selectin gene polymorphism with expression level during an in vitro fertilization program for rs2229569 in exon five of the *SELL* gene. The failure implantation group showed deviations from H.W.E. in all genotypes. Significant differences in the failure group refer to the effects of infertility in the study population, in which infertility in the failure group leads to a deviate from HWE, and *SELL* gene polymorphism may be considered a factor related to infertility. Other Iraqi studies have emphasized that the *SELL* gene may play a role in type 2 diabetes mellitus in the Iraqi population[21]. Therefore, it is possible that *SELL* defects contribute to implantation failures and infertility.

The present study found that the genotypes of *SELL* gene non- significantly varied between failure and success group.TT genotype which could be considered an etiological genotype, while CC could be a protective allele related to success of implantation according to odds ratio. The rs2229569 T allele was most common among the failure group. This allele was present in about 21.43 % in failure group, but only 11.54% in the success group, according to Fisher's probability (2.09) may be consider etiological allele and could be related with the failure of implantation. This observation may highlight the role of the *SELL* polymorphism in IVF outcome. A different studies emphasize that *different genes* are associated with infertility and endometriosis one of them is *SELL* [22-24]. The present study agrees with study in Iran that showed E-Selectin mutant genotype frequency was significantly higher in Coronary Patients

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pathogenesis [25]. The polymorphism of *SELL* in present study results from transition amino acid proline to serine, this genetic variant determines synthesis of protein with other amino acid serine; this leads to a change in the effectiveness of protein domain 1.

The results of the present study showed that *SELL* gene expression was downregulated in the failure groups (0.56) compared with the success groups (1.00) according to fold gene value. These findings demonstrate that downregulation of *SELL* gene expression may be associated with failure of embryo implantation. The present study agrees with other studies about Iraqi females showing that downregulation of Integrin Beta3 gene expression was associated with implantation failure [26,27]. Another study demonstrated that the level of l-selectin ligand was significantly more available in pregnant female[28]. Another local study shows that *SELE* gene probability is associated with development of breast cancer [29]. It is well known that selectins play crucial roles during implantation as the primary adhesion molecules at the maternal-fetal interface[30, 31]. Higher rates of embryo implantation and successful pregnancies have been linked to higher levels of L-selectin ligand in the secretory endometrium, suggesting a role for L-selectin ligand in facilitating endometrial receptivity and mediating the maternal-fetal interface[32-35].All these studies goes with the present study also emphasized a finding that there are many factors effect on IVF outcome and L selectin could be one of them. In order to improve this conclusion another studies with large sample requires.

Conclusion

In conclusion, this study demonstrates that the TT genotype and T allele of rs2229569 may be associated with the failure of implantation therefore it may be considering as the risky allele in female under IVF program. In addition, the present study shows that downregulation of SELL gene expression may be associated with the failure of embryo implantation.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval. The study included human participants and was approved by the ethics committees of the intended hospitals. Participants gave their free, informed consent to take part in this research.
- Ethical Clearance: The project was approved by the local ethical committee at University of Baghdad.

Authors' Contribution Statement

A. M. S. A designed the whole project and primers following the research work, analyzed data and wrote the manuscript. R. H. R. collected the samples and writing the draft of the manuscript. A. H. A. read the physiological part. All authors read and approved the final manuscript.

Journal Declaration:

Dr. Asmaa M. Salih Almohaidi is an Editor for the journal but did not participate in the peer review process other than as an author. The authors declare no other conflict of interest.

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تعدد الأشكال للقاعدة المفردة rs2229569 مع التعبير الجيني للسلكتين L في الاناث العراقيات اثناء برنامج الاخصاب خارج الجسم الحي

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الخلاصة

يُعرف العقم بأنه أحد مشاكل الصحة الإنجابية الرائدة في مناطق مختلفة من العالم. يعتبر الإخصاب في المختبرأحد أكثر علاجات العقم فعالية. في الدراسة الحالية ، تم تحديد دور جين *SELL* ، وخاصة تعدد الأشكال rs2229569 ، من حيث الانغراس ومستوى التعبير. اشتملت الدراسة على 67 انثى خضعن لدورة إخصاب في المختبر ، مقسمة إلى مجموعتين رئيسيتين: مجموعة الإناث التي فشلت عملية الزرع ومجموعة الإناث الناجحة في عملية الزرع. تم جمع عينات الدم من مجموعتي الاناث. بعد استخلاص الدنا من الدم وتضخيم الحمض النووي عن طريق تفاعل البلمرة المتسلسل ، تم إرسال العينات لتحليل التسلسل ، وتم تحديد SNP rs2229569 التي اظهرت ثلاثة أنماط وراثية. كانت مجموعة الفشل منحرفه عن H.W.E.، وكانت مجموعة النجاح في اتفاق مع H.W.E.وفقًا لقيم مربع كاي (X2 = 11.60 1.58)، تشير الفروق المعنوية في مجموعة الفشل إلى تأثير العقم في مجتمع الدراسة. تُظهر نسبة الأرجحية لـ rs2229569 أن أليل T كان (2.09) ، لذلك قد يمثل T allele أليل المخاطرة لعمليةفشل الزرع .أخيرًا ، أظهر جين SELL مستوى تعبيرًا منخفضًا لدى الاناث المصابات بفشل الزرع مقارنة بالاناث في مجموعات الزرع الناجحة.

الكلمات المفتاحية: التعبير الجيني ,تعدد الأشكال للقاعدة الواحدة rs2229569, العقم ,الاخصاب في المختبر,سلكتين L.