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Genetic Polymorphisms of PLCζ and PAWP Genes in Infertile Men that Undergo ICSI Protocol

Dhuha Salim Namaa[1] , Saad S. Al-Dujaily [2] , Ismail H. Aziz[3] , Bushra J. Al- Musawi[4]

[1] Forensic DNA Center Research and Training, Al-Nahrian University, Baghdad-Iraq.
[2]Department of Clinical Reproductive Physiology, High Institute for infertility Diagnosis and ART, Al-Nahrian University, Baghdad-Iraq.
[3] Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad-Iraq.
[4] Kamal Al-Samarai IVF Hospital, Ministry of Health, Baghdad- Iraq.

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Abstract: Intracytoplasmic sperm injection (ICSI) is a technique that *in vitro* fertilization clinics use to remedy a myriad of male infertility status. The sperm activation of the egg is the first critical step of embryogenesis included both completion of meiosis and progression into mitotic cycles. phospholipase C-zeta isoform ζ (PLCζ) and post acrosomal WW binding protein (PAWP) genes as considering physiological triggers that stimulate embryogenesis in human. The target of this study is to determine the single nucleotide polymorphism (SNP) in PLCζ (exon 11, 13) and PAWP (intro 1) genes sequence, and examine if these SNP can lead to defects in egg activation and embryo formation. Semen analysis was done for (62) men, the samples were collected from infertile men undergoing ICSI protocol and divided into 5 groups according to the percentage of fertilization rate (FR%). Evaluation of seminal fluid analysis was accounted as recommended by WHO criteria (WHO, 2010). PCR reaction was performed for semen samples to amplification the target region, exon 13; SNP I489P:rs 10505830, and exon 11; SNP H398P rs: 1359777791) of PLCζ gene, as well as PAWP gene (intro 1), and selection (30) PCR product samples for sequencing analysis. In conclusion, the results showed the PLCζ sequencing was not noted the target SNPs in this study. Whereas, PAWP genes sequence revealed that one SNP; C/G/T rs: 710194; was located on chromosome 22:41996556 bp; position 1454 in intro one, which present in all sample.

Keywords: Oocyte activation, Fertilization failure, PAWP, PLCζ, ICSI.

Corresponding author: (Email: dhuha.salim@yahoo.com).

Introduction

Intracytoplasmic sperm injection (ICSI) is a widely applied treatment choice for couples presenting *in vitro* fertilization (IVF) clinics (1), when compared to traditional IVF treatments, this is remarkably effective in treating many cases of male infertility. In these cases of ICSI failure, the main reason showed up to be a decrease in oocyte activation (2). Throughout mammalian fertilization, sperm induces a remarkable series of oocyte Ca^{2+} intracellular oscillations that extend a chain of biochemical events called oocyte activation (3). Defects in this process underlie some cases of human male infertility and may have a relation for more general problems of an

embryo (4). In humans, oocyte activation begins soon after gamete fusion and continues beyond the completion of meiosis. Following the gamete fusion , fertilizing sperm releases a soluble 'sperm factor' into the oocyte (5), Sperm-specific phospholipase C isoform ζ (PLCζ) (6) and post acrosomal WW binding protein (PAWP) are two candidates for sperm-borne oocyte activating factors (SOAFs) . The human PLCζ gene localized in chromosome 12, which consists of 15 exons and can an expression in both the testis and spermatozoa.(7) After PLCζ introduced into the ooplasm and hydrolyses Phosphatidylinositol 4,5-bisphosphate (PIP2) containing vesicles, generating Inositol trisphosphate (IP3) and Diacylglycerol DAG (8). IP3 diffuses across the ooplasm to the endoplasmic reticulum (ER) membrane where it binds to IP3Rs causing the release of Ca^{2+} via membrane channels, in addition Diacylglycerol DAG where activates the protein kinase C pathway PKC which is believed to translate Ca^{2+} signals into cellular responses (9,10).

The PAWP which is a testis-specific protein localized in perinuclear theca of the matured mammalian sperm head only during latter phases of spermiogenesis and can induce calcium oscillation in the oocyte (11); PAWP expectedly did not hydrolyze PIP2, but importantly was unable to modulate *PLCζ* enzymatic activity *in vitro* (12).The goal of this research is, to establish if fertilization failure in ICSI is associated with target SNP of both PLCζ and PAWP genes and impact on the number of embryos and qualities embryos in the couples undergo ICSI protocol.

Materials and Methods

Study design

The study was conducted in the Kamal Al-Samurai IVF Center, Ministry-of Health in Baghdad-Iraq and Forensic DNA Research and Training Center, Al-Nahrain University, through a period extended from December 2018 to August 2019.The protocol involved sixty two infertile men undergoing ICSI procedure. Their ages ranged from (23-41) years. Patients were classified intentionally according to fertilization rate; **Control group:** twenty five infertile men undergoes ICSI technique, (successful oocyte division after fertilization). **Patients group:** thirty seven infertile men and healthy female undergo ICSI technique, arrest oocyte division after fertilization in different percentage. (Depended on the percentage of embryo division for each case).

Sample collection

Freshly ejaculated semen samples were obtained by masturbation after (3-5) days of sexual abstinence. Then specimens were analyzed for sperm concentration, progressive motility and normal morphology according to WHO criteria (WHO, 2010).

Genomic DNA extraction of *PLCζ* and *PAWP*

Collected the seminal fluid samples (200µl) into a sterile 1.5 mL micro

centrifuge tube, after doing centrifuge added (50µl) Tris EDTA (TE) to the pellet. Extraction of DNA by using Organic DNA extrication procedure was depended on chemical material; the concentration and purity of extracted DNA were detected by using Nano Drop™ Q3000 Spectrophotometer in order to detect the integrity of samples. PCR procedure were performed using specific primers for *PLCζ*, *PAWP* were designed according to National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/genbank, which is synthesized by Alpha DNA Ltd (Canada). Primers sequencing that used for PCR are *PLCζ* Exon11**,** **[(F:**CACCATTAGTGCCTGACCCC), R:AGGGAAAATGCTGCTTTTGTTTTG)], *PLCζ* Exon13, [(F:TCAATGTTTGTGGGAGCTGA) (R:GGACATAATGGAAAACCCTTG, and *PAWP*, (F:CCTTTCCCTGAAGCCCTGTA) (**R**:GAATGGAGTGAGAAGCAGCG), were base pair (bp) for each genes **(**636, 370, 800) respectively.

The Amplification of specific region of the *PLCζ exon 11, exon* 13 and *PAWP* genes using GoTaq® Green Master Mix Kit (promega).The reaction mix setup, were applied as recommended by the manufacturer. PCR program was summarized in Table (1) with modification.

Table (1): PCR Programs

Steps		Temp(°C)	Time (m:s)	Cycle
Initial Denaturation		95 °C	05:00	1
Denaturation		95 °C	00:15	35
Annealing	***PLCζ exon 11* gene**	60 °C	00:30	
	***PLCζ exon 13* gene**	60 °C		
	***PAWP* gene**	62 °C		
Extension		72 °C	00:30	
Final Extension		72 °C	05:00	1
Hold		4 °C	∞	

DNA sequencing by Sanger sequencing

The selected samples (n= 30) from PCR products and primers, were sent to Macrogen company (South Korea), for sequencing analysis to detect any mutation in the samples of this study The results then analyzed by using Bioedite software.

Statistical analysis

Data were statistically analyzed by utilizing SPSS for Windows, version 21(SPSS Inc. Chicago, IL, United States). Data have appeared as mean ± standard error of mean (SEM) Categorical variables were analyzed by Chi-square test. Tukey test, test LSD, and Bonfferoni Post Hoc test for multiple comparisons were applied after ANOVA tests.

Results and Discussion

In the present study, patients groups were classified according to the percentage of fertilization rate (the FR

score was as the following: group A (90-100)%FR included only the control fertile couples. Patients infertile groups included a FR% B:(70-80) , C:(40-60), (D:<30), (E:no-fertilization). The confirmed sperm function parameters showed in Table (2).

Today male fertility condition is based on an evaluation of conventional sperm parameters despite the inability to differentiate between infertile and fertile males (13,14).The seminal fluid analysis remains the cornerstone of a male fertility assessment. Therefore, it does not provide a systematic explanation of the subcellular changes that occur in the spermatozoa of infertile men, which necessitates a more analysis and understanding at the molecular level (14).

Table (2): The microscopic semen parameters for the studied Fertilization rate groups.

Certain sperm function Parameters	Control group (A) n = 25	Fertilization rate groups n = 37				P-value ‡
		Group(B) n= 7	Group(C) n= 18	Group(D) n= 7	Group (E) n= 5	
Sperm count (million/ejaculated)	33.04±4.96	47.86±13.35	43.35±5.825	12.57±3.69	31±9.69	0.053 (NS)
Progressive motility (%)	20.60±2.77	22.86±7.14	25.8±5.09	14.29±4.14	21±7.14	0.62 (NS)
Non progressive motility (%)	21.6±2.33	17.86±3.912	20.35±2.59	14.7±4.06	18.4±5.39	0.323 (NS)
Immotile (%)	59.64±4.41	62.14±10.34	54.9±5.62	76.43±7.45	67±10.9	0.552 (NS)
Morphologically abnormal sperm (%)	79.12±3.37	87.71±7.77	78.24±2.58	87.43±4.56	86.40±6.72	0.48 (NS)

Data were expressed as mean ± SE; Statistical analyses were performed by ANOVA followed by Post Hoc test (Tukey's test) for multiple comparisons.
‡ ANOVA significance test (2-tailed); NS, no significant difference.

The reason for decreasing quality in male reproductive function remains to be elucidated. As the most common type of male infertility is idiopathic – exhibiting one or more abnormal semen parameters with no identifiable cause (15). Environmental or lifestyle factors that causative of male factors infertility (MFI), such as environmental exposures, sexually transmitted diseases, medications, overproduced reactive oxygen species (ROS) which was causes high levels oxidative stress (OS). The main abnormalities that cause abnormal sperm morphology may be due to genetic, high-temperature exposure to the testes, and interaction with chemical or radioactive substances, as well as lifestyle and infections. (16).

The present study exhibited no significant association between percentage of type male factor (normal, OAT oligoasthenoteratospermic, ASth: asthenozoospermic ,AT: asthenoteratospermic, OA: oligoasthenozoospermic. and the percentage of fertilization rates groups; (person Chi –square = 0.177) Figure (1).

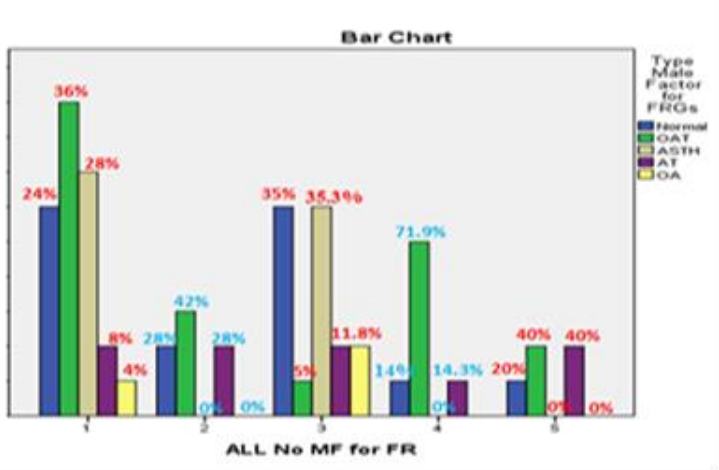


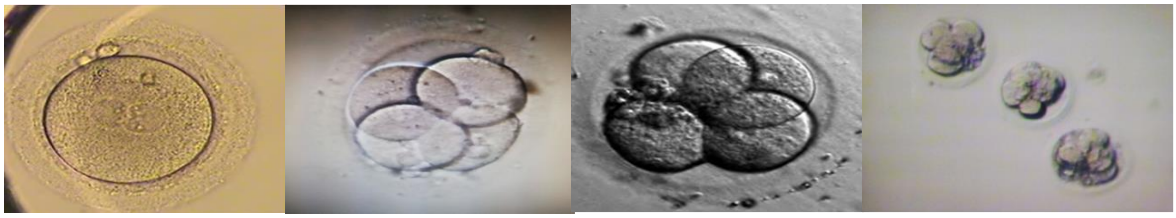
Figure (1): Percentage of each type of male infertility factor apportionments in fertilization rate groups.
Data were expressed as counts with percentages; † Statistical analyses performed by the Chi-squared test (person); NS, no significant difference. (A)1:(90-100)FR%; (B) 2: (70-80)FR%, (C) 3: (40-60)FR%, (D) 4: (<30 FR)%, (E) 5: (non-FR)%; Normal: control group.

For couples who suffer from a decrease in fertilization rate, ICSI assists to lesser male factor infertility (17). Mostly expects that morphologically abnormal sperm have an effect on the grading of embryonic quality indirectly. Because of, acrosome organelle covering the head of the sperm and containing enzymes that responsible for digest the egg cell coating, thus permitting the sperm to enter the oocyte as well as, which play an important role in egg activation, such as *PLCζ* and *PAWP* gene.

It is clear from the outcome of the present research that couples undergo ICSI procedures who had normal or poor semen parameters did not affect the percentage of fertilization rate. However, studies have shown that intra-cytoplasmic insemination with sperm containing acrosomal defects did not lead to successful fertilization even in the absence of fertilization barriers, since the oocyte could not be efficiently activation (18, 19).

Normally fertilized oocytes with two pronuclei after oocyte microinjection express the evidence fertilization or the first step of egg activation.

The grading of Embryos (G1, G2, and G3) for couples that undergo ICSI protocol shows in images (2 A, B, C, D).



(A) (B) (C) (D)

Image (1): Cleavage stage of embryos. (A) normally fertilized oocytes with two pronuclei and two extruded polar bodies (B) Grad 1 (4-cell) embryo C: Grad 2 (4-cell) embryo with <10-15 % fragmentation,. D: Grad 3 (4-6cell) embryo with 20-25 % fragmentation.X400 magnification.

Conventional embryo evaluation requires the manual grading of human embryos at the blastocyst stage (embryo on day 5) on the basis of morphological examination by skilled embryologists (20).

Although this selection method is widely used in clinical practice, the evaluation of embryos based on a static picture is a simplistic, subjective assessment of embryo quality (21,22).

The results of embryos profile for patients that undergo ICSI protocol, were shown in Table (3).

Table (3): Results of Embryo profile for patients that undergo ICSI protocol.

Parameters	C group (A) n = 25	Fertilization rate groups n = 37			P-value
		Group(B) n= 7	Group(C) n= 18	Group(D) n= 7	
NO. Embryos	6.12±0.827	6.14±0.634	3.99±0.585	3.57±0.719	0.13 (NS)
Grading of Embryos G1	2.56±0.32 a	2±0.577 a	1.59±0.272 a	0.79±0.286 b	0.01*
G2	2.48±0.49	3.14±0.143	1.94±0.489	1.86±0.508	0.546 (NS)
G3	0.8±0.26	1±0.318	0.4±0.211	1±0.436	0.493 (NS)
NO. Embryo transfer	3.56±0.224	3.29±0.184	2.76±0.278	3±0.436	0.135 (NS)

G, Grad of embryo; a, b: Different letters mean there is significant difference;* P<0.05 = Significant values; NS, no significant.

In fact, there is some disagreement as to the impact of sperm DNA damage on fertilization rates. Some studies have indicated that low fertilization rates have been associated with high levels of sperm DNA damage. (23). Nevertheless, other investigators have shown that sperm DNA damage has a slight effect at the fertilization stage, in the study of WW Zheng *et al 2018,* was found that sperm DNA damage is negatively associated with fertilization rates. Pronuclear formation and early embryo formation do not seem to be dependent on the integrity of sperm DNA, because the embryonic genome is expressed only after the second cell division. In general, if the type and degree of DNA damage can be repaired by the oocyte, embryo development can be accomplished even in the presence of sperm DNA fragmentation(24).

PCR assay to detection mutation of *PLCζ &PAWP* genes

Total DNA was successfully extracted from all semen samples. PCR reaction was performed for all samples to amplification the target region of *PLCζ,* which were performed using a specific primers in exon (11,13) for the *PLCζ* gene and intron one for *PAWAP* gene. Agarose gel electrophoresis was used to confirm this amplification for these regions. The following figure demonstrates of agarose gel electrophoresis of the *PLCζ* gene PCR amplified products, exon 13; Mutation I489P :rs 10505830, exon 11; SNP H398P rs: 1359777791, and intron one for *PAWAP* gene. Using the red safe stain which was showed the fragment size (370, 636, and 801) bp respectively of all samples was amplified successfully with a single band. Figure (2; A, B ,C).

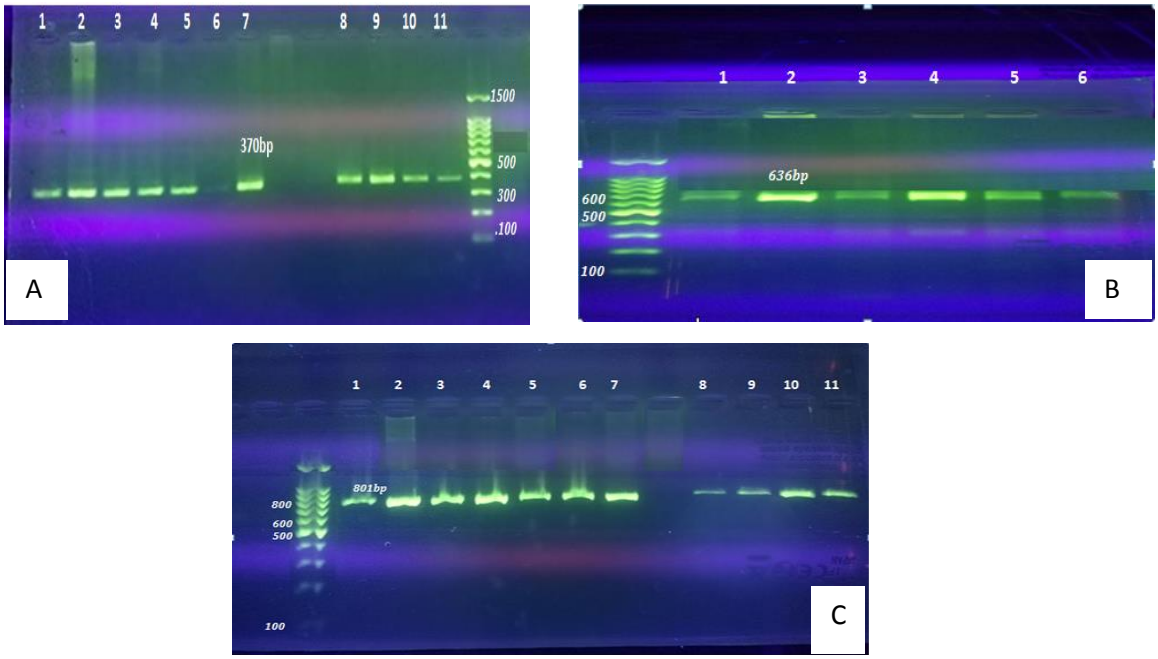


Figure (2): PCR products of: (A) *PLCζ* gene-exon 13 of the molecular size 370 bp. (B): *PLCζ* gene-exon 11 of the molecular size 363 bp. (C): *PAWP* gene- of the molecular size 801 bp. Lane 1:100pb DNA marker. Bands were separated on 1.5% agarose gel at 5 v/cm² for 90 min.

Sequencing results of *PLCζ* & *PAWP*

The DNA sequencing results for (30)samples have been analyzed. As for, *PLCζ* gene exon11 (SNP;H398P, rs:1359777791)and exon 13 of *PLCζ* SNP I489P :rs 10505830) , The results found no mutation in these regions when alignment the sequence with original sequencing of this gene using NCBI nucleotide alignment tool. Nucleotide in which DNA sequence obtained in this study were compared with human reference *PLCζ* (NCBI Reference Sequence: NG_052826.1) that available in website (https://www.ncbi.nlm.nih.gov/nuccore/NG_052826.1), (Figure 3 A,B).

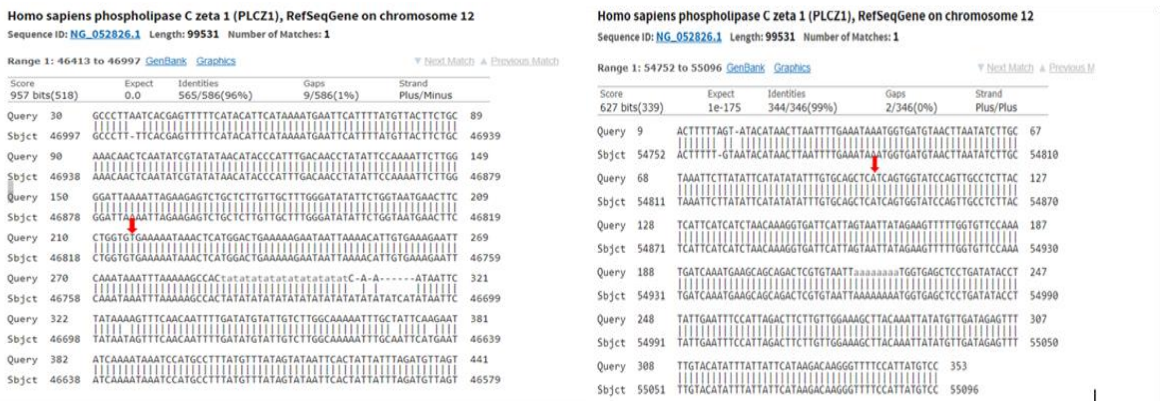


Figure (3): DNA sequence alignment of *PLCζ* gene. A: exon 11, B: exon 13 obtained from Gene Bank. Query represented of samples; Subject represented of database of National Center Biotechnology Information (NCBI).

In addition, the analysis of *PAWP (WBP2NL)* genes sequence intron one was revealed that one SNP; C/G/T rs: 710194; was located on chromosome 22:41996556 bp; position 1454 in intro one (non-coding area). Therefore was no significant impact of the SNP rs: 710194 genotype on the expression fold of *PAWP* in infertile men, Which was found in all studying sequencing samples when alignment the sequence with original sequencing for this gene using NCBI nucleotide alignment tool. Nucleotide BLAST (Basic Local Alignment Search Tool) in which DNA sequences obtained in this study were compared with the human reference *PAWP (WBP2NL)* gene (NCBI Reference Sequence: NG_937830.3) NCBI as shown in Figure (4).

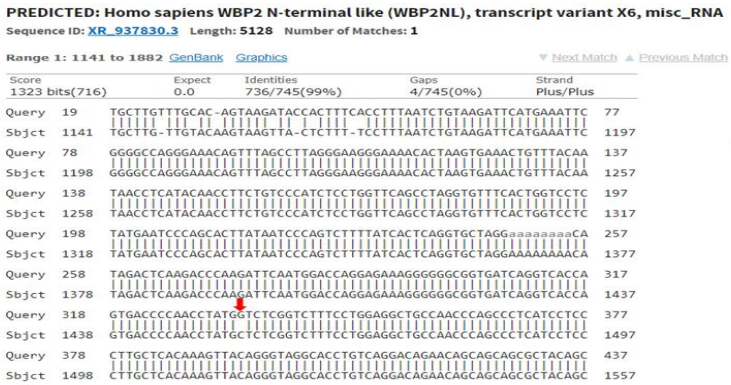


Figure (4): DNA sequence alignment of *PAWP (WBP2NL)* gene, obtained from Gene Bank. Query represented of samples; Subject represented of database of National Center Biotechnology Information (NCBI).

It was concluded from the results of this research that, male factors are not considering the evidence for successful ICSI outcomes for patients undergo this protocol and evaluation of sperm fertility quality dependent on morphology and DNA integrity in cases of ICSI protocol. In addition, no mutation in target regions of *PLCζ* when alignment the sequence with original sequencing of this gene using NCBI nucleotide alignment tool. Based on the results, we suggestions doing Next-generation sequencing (NGS) of PLCZ genes to identify potential SNP in the Iraqi population.

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