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Expression of Protamine-1 Gene and Association with Interleukin-12 gene in Infertile Men: A Case-Control Study in Iraqi Patients

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Abstract

Approximately 15% of all couples all over the world suffer from difficulty conceiving their first child. The word "infertility" is used to describe this problem. When a couple had regular, unprotected sexual intercourse for a year or longer, it indicates that they have been unsuccessful in their efforts to conceive.

The current investigation aims to find out if there is a relationship between *PROTAMIN-1(PRM-1)* and *INTERLEUKINE-12 (IL-12*) gene expressions and their effect on the development of infertility. The current investigation comprised 100 teratozoospermia patients and 100 healthy fertile controls who had their semen examined. Samples were given by Al-Nahrain University's Biotechnology Research Center and the Kamal Al-Samarie IVF Hospital in Baghdad, Iraq. Determinations Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) was employed to test the expression of the *PRM* gene in semen and blood samples, as well as the *IL-12* gene in blood samples. And the titer of *IL-12* was evaluated in the seminal plasma employing the Enzyme Linked Immune Sorband Assay (ELISA) technique. The difference between the mean threshold cycle (ΔCt) of *PRM-1* and *IL-12* mRNA in the infertile patient group versus the healthy control group was also statistically significant. Evaluating the means of $2^{-\Delta\Delta Ct}$ revealed an important finding, a decrease in the expression of *PRM-1* mRNA in the infertile group in comparison to the control group in the two types of samples. Conversely, there was an increase in the expression of *IL-12* mRNA between the two study groups. In infertile Iraqi men, the expression of the *PRM-1* gene was found to be down-regulated, whereas the expression of the *IL-12* gene was found to be up-regulated. Our data revealed the diagnostic value of these genes and might be considered as predictive biomarkers in infertile people with idiopathic infertility .

Keywords: *PROTAMINE-1* gene, gene expression, RT-PCR, *IL-12*,infertile patients .

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التعبير الجيني لجين *PROTAMINE-1* وارتباطه بالتعبير الجيني لل *INTERLEUKINE-12* للرجال العقيمين في العراق:دراسة الحالات والشواهد

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الخلاصة

يعاني قرابة 15٪ من الازواج حول العالم من صعوبة إلحصول على الذرية. حيث تطلق كلمة "العقم"لوصف حالة الازواج الذين يمارسون الجماع بشكل منتظم وبدون اي موانع حمل ولمدة عام او اكثر دون نجاح هذه الممارسة والحصول على الاطفال.

هدفت الدراسة الحالية الى ايجادالعلاقة بين تعبير (*PROTAMIN 1(PRM* و *INTERLEUKINE-12* (*IL-12*) وتطور حالات العقم. حيث شملت 100 عينة من المرضى الذين يعانون من حالات تشوه الحيوانات المنويه و100 من الرجال الاصحاء الخصبين بعد اجراء الفحص الروتيني للسائل المنوي. جمعت العينات في مركز بحوث التقنيات الاحيائية بجامعة النهرين ومستشفى كمال السامرائي لأطفال الأنابيب في بغداد ، العراق. تم اعتماد تقنية النسخ العكسي لتفاعل البلمرة اللحظي (RT-qPCR) لقياس التعبير الجيني لجين PRM في عينات السائل المنوي والدم ، وجين IL-12 في عينات الدم. اضافة الى حساب مستوى IL-12 في البلازما المنوية باستخدام تقنية الفحص المناعي المرتبط بالإنزيم (ELISA) ، اظهرت النتائج وجود فروق ذات دلالات احصائية في متوسط قيمة حدود العتبة (Ct) لـ *PRM 1* و mRNA بين المجاميع قيد البحث وتوصلت الدراسة الى وجود اختلافات في التعبير الجيني ,حيث انخفض التعبير الجيني لل mRNA *PRM 1* في مجموعة المرضى مقارنة بمجموعة الاصحاء في عينات الدم والسائل المنوي . وعلى العكس من ذلك ،لوحظ هناك ارتفاع في التعبير الجيني لل mRNA *IL-12*.

اظهرت النتائج ان للجينات قيد الدراسة اهمية تشخيصية ومن الممكن اعتبارها كمؤشرات تنبؤية لحالات العقم مجهول السبب .

1-Introduction

Infertility represents a frequent reproductive system challenge that results in the inability to have a healthy child, After one year of unprotected sexual activity [1]. Some researchers have classified infertility causes into four groups: Male factors, feminine factors, complex variables, and idiopathic factors are all considerations to be considered. Other factors that influence men's fertility include environmental factors like: body weight, work stress and smoking. Male infertility is caused by idiopathic causes, which are often linked to epigenetic and genetic factors [2].

Seminal fluid examination, which includes a sperm count as well as an investigation of motility and morphology, is the initial stage in determining male infertility. It provides vital information about a patient's reproductive status. There are a number of problems with each of the above criteria, including azoospermia, asthenozoospermia, and teratozoospermia. When it comes to sperm quality, it's widely accepted that sperm structure is an important semen trait [3]. A wide number of biomarker proteins can be found in semen, as well as a significant amount of essential protein and tissue-specific proteins, both of which serve as dependable predictors of pathogenicity in relation to reproductive status [4]. The main nuclear sperm proteins, for example, are known as Protamines (PRMs). Protamines are divided into two types: Protamine 1 (PRM 1), which is produced by a single gene, and the Protamine 2 (PRM 2) family of proteins

(PRM 2, PRM3 and PRM4), which is produced by a single gene that is transcribed and transformed into a precursor protein [5]. Although protamines have been found for over a century, nothing is understood about their function. Theories include the condensation of the gamete (sperm) nucleus into a dense hydrodynamic pattern, the conservation of the genomic message carried by sperm cells, and participation in mechanisms that retain and repair DNA integrity after or during nucleohistone–nucleoprotamine complex formation [6]. Furthermore, Protamines were a part of the most complex proteins discovered naturally, and evidence suggests that Darwinian selection favored them. Male infertility has been known to change the expression of *PRM1* and *PRM2* [7].

Some abnormal spermatogenesis cases have been reported and DNA breaks have been described as *Protamine* gene mutations that cause sperm chromatin damage or imprinting defects have been documented and identified as *Protamine* gene mutations [8]. Several studies have linked abnormal *Protamine* gene expression in infertile male sperm to low sperm concentration, sperm movement, and shape normality, all of which contribute to a reduction in increasing sperm chromatin deficiency and fertilisation efficiency. Changes in *Protamine* expression in sperm were thought to be one of the major reasons behind male infertility [6].

Cytokines are formed physiologically in the male gonad and they are essential in their routine work. As a result, they should also be present as seminal plasma components in the normal state. The exact origin and control of these mediators in the male reproductive system are currently being investigated. Testes and testicular macrophages create most male cytokines. Leyding and Sertoli cells also produce cytokines. They control sexual function and fertility. IL-12 is a one-of-a-kind cytokine composed of two covalently coupled glycoproteins and two covalently linked components (p35 and p40) that manifests a multitude of biological activities in the immune system, thus affecting many physiological phenomena [7;9]. Several sperm characteristics, including quantity, motility, viability, and shape, have been related to cytokines in seminal plasma. For instance, Naz et al. hypothesised that the content of IL-12 was positively correlated with the quantity and proper shape of sperm [10].

This study focuses on *Protamine,* a nuclear-specific protein that is important in gametogenesis and the detection of male-related disorders. The goal of this research was to determine the PRM and *IL-12* gene expression of idiopathic infertile men compared with fertile men, and then analyse their correlation with idiopathic infertility in Iraqi males.

2. The samples and methods

2.1 Sample collection

The present research was carried out on 200 males through their visit to the Biotechnology Research Centre/Al-Nahrain University and Kamal Al-Samaraie IVF Hospital, Baghdad, Iraq, Patients written consent was obtained before they are involving in this work. The collected samples with an average age of between 20 and 46. According to their seminal examination, one hundred men were found to be infertile with teratozoospermia conditions, which had been unable to conceive for at least 12 months, The seminal analysis was carried out using the criteria WHO, 2010. Additionally, this investigation included one hundred healthy, fertile males (normozoospermia) with a normal reproductive history and physical examination. All of the control groups had children within the preceding year. Semen and blood were collected from fertility and infertility samples to use them in a molecular and immunological study.

Ethics approval

This research was approved by Ethics Committee under reference no. M.B 15 IN 1/482021.

2.2 RNA extraction and RT-qPCR:

The whole RNA was isolated from sperm and peripheral blood samples employing Trizol (TRIzol LS Reagent) to analyze *Protamine-1* and *GAPDH (House Keeping Gene)* mRNA expressions and to assess *IL-12* and *GAPDH* mRNA expressions only in blood samples using qRT-PCR. RNA was extracted from the blood by mixing 0.25 mL of blood with 0.75 mL of TRIzol® LS Reagent in a separate tube for RNA extraction from blood, while from semen samples using a modified TRIzol method, the sperm was treated at 55°C overnight with sperm lysate buffer, and sperm DNA was precipitated by mixing an equal percentage of cold isopropyl alcohol after complete digestion. Both cDNAs were synthesized by RT from total RNA using the "WizScriptTM RT solutions kit (Wizbio/Korea)". *Protamine-1*, *IL-12*, and *GAPDH* mRNA levels were quantified using Real-Time PCR (Cepheid Smart Cycler qPCR) (USA). The expression of the *Protamine-1* and *IL-12* genes was quantified using qRT-PCR analysis, as directed by the manufacturer. The primers utilised to evaluate gene expression are detailed in Table 1.

Canada's Alpha DNA created these primers. To combine cDNA with SYBER Green master mix for qRT-PCR, cDNA with SYBER Green master mix was utilized. Each reaction was repeated. To achieve the desired results, a thermal profile was used to program the cycling procedure. This included a 5-minute denaturation or enzyme activation cycle at 94 °C, followed by 40 cycles of denaturation at 94 °C for 15 seconds, followed by 25 seconds of annealing at 58 °C, and then 30 seconds of extension at 72 °C. The dissociation step lasted 1 minute at 95 °C, followed by 30 seconds of extension at 55 °C.

The calculated mean difference of (2 -ΔΔCt) was utilised to compute the relative quantification of *Protamine-1* and *IL-12* expression in samples of sperm and blood [11].
The term $\mathbf{2^{-\Delta\Delta Ct}}$, which stands for relative fold change, was used. As a consequence, the data was provided as a fold change in a particular gene's level of expression, normalized to an endogenous control (*housekeeping gene*), and compared to a calibrator, which is the specific gene in healthy people (12).

Table 1: Primer sequences utilized for real-time amplification RT-PCR

Genes	Forward primer (5′--->3′)	Reverse primer(3′ --->5′)
GAPDH	GAAATCCCATCACCATCTTCCAGG	GAGCCCCAGCCTTCTCCATG
PRM-1	5-CCGCCAGAGACGAAGATGT-3	5-TACACCTCAGCCTGTACCTG-3
IL-12	5-ATGGGAACATTCCTGGGTGTGT-3	5-CGCAGCCTCCTCCTTGTGG-3

2.3 Measurement of IL-12 level in seminal plasma

Human IL-12 levels were determined by employing a human IL-12 ELISA Kit using the sandwich ELISA technique, as directed by the manufacturer (MyBiosource incorporation, USA). The method and the specifics were previously clarified by [13].

2.4 Statistical analysis

The data were statistically analyzed using SPSS 17 FOR WINDOWS (SPSS Inc., Chicago, IL, United States). Mean and standard deviation were used to organize the data. Using the Shapiro–Wilk normality test, it was determined if the analyzed parameters had a Gaussian distribution. Log transformations were performed on variables whose data distributions did not conform to normality before they were displayed in standard units. The Chi-square test was employed to examine categorical data. The degrees of association between variables were

investigated using Pearson correlation analysis. It was considered significant if the two-tailed p-value was less than 0.05 (p0.05). The Pearson Correlation produces a correlation coefficient (r) which measures the relationships between the studied parameters. The area under the curve (AUC) of the researched parameters is determined using receiver operator characteristics (ROC). In addition, the optimal CV, sensitivity, and specificity were identified. A p-value<0.05 was statistically significant [14].

3. Results

3.1 Basic seminal parameters distribution of infertile and healthy men groups

Certain macroscopic and microscopic semen parameters show some differences between the infertile and healthy control groups, as shown in Table (2).

Table 2: Semen microscopical analysis for all groups.

Parameters	Fertile group (normozoospermia) (no=100)	Infertile group (teratozoospermia) (no=100)	P value‡
Volume (ml)	2.81±1.01 a	3.22±1.21 a	NS
Sperm concentration (million/ml)	77.64±59.22 a	47.93±36.12 b	**0.001**
Total motility (%)	71.36±8.38 a	43.33±19.45 b	**0.001**
Morphologically normal sperm (%)	37.43±15.66 a	11.97±2.26 b	**0.001**
IL-12 (pg/ml)	1897±633 a	3372.7±121 b	**0.001**

T-TEST was used to perform statistical analysis on the data, which were reported as mean SD. Mean in rows carrying similar small letters indicate a non-significant difference, while different small letters indicate a significant difference. NS; Non-Significant difference

The current findings reveal that there was no discernible variation in ejaculation volume across the examined sections. But the mean ~~of~~ sperm concentration (m/ml) in the fertile control group (77.64±59.22) was higher by a statistical significance margin than that of teratozoospermia patients (47.93±36.12). The total motility (%) of the fertile men (71.36±8.38) was significantly ($p \leq 0.05$) higher compared to teratozoospermia patients (43.33±19.45). Table (2) also showed a highly significant ($p<0.05$) elevation in the morphologically normal sperm of control men (37.43±15.66) compared with the teratozoospermia men (11.97±2.26). The mean concentration of seminal plasma IL-12 was measured in all studied groups. The concentration of IL-12 in the patient group was substantially higher than in the healthy group, as shown in the same table.

3.2 Detection of gene expression

1- Expression of PRM 1 in blood samples:

The folding expression ($2^{-\Delta\Delta Ct}$) of blood *PRM 1* mRNA was decreased in infertile groups (0.46) compared with control groups, as shown in Table 3.

Table 3 : Fold of PRM1 Blood expression Depending on $2^{-\Delta\Delta Ct}$ Method

groups	Mean ΔCt Target (ct *PRM1-ct GAPDH*)	Fold of gene expression
Group 1 Infertile men (No=100)	**2.07**	**0.46**
Group 2 Fertile Control men(No=100)	**0.97**	**1.0**

2-Expression of *PRM 1* in semen samples:

The relative expression ($2^{-\Delta\Delta Ct}$) of *PRM 1* mRNA was decreased in infertile patient group as follows (0.14) when compared to the group of fertile control men.

Table 4 : Fold of *PRM1* Semen expression Depending on $2^{-\Delta\Delta Ct}$ Method

groups	Mean ΔCt Target (*ct PRM1-ct GAPDH*)	Fold of gene expression
Group 1 Infertile men (No=100)	**4.02**	**0.14**
Group 2 Fertile Control men (No=100)	**1.21**	**1.0**

3- Expression of *IL-12* in blood samples:

When *IL-12* mRNA expression was analysed, it was shown that infertile patients (2.056) had higher levels of expression than fertile patients. This result was statistically significant.

Table 5 : Fold of *IL-12* Blood expression Depending on $2^{-\Delta\Delta Ct}$ Method

Group	ΔCt (Mean of Ct *IL12* - *Mean of Ct GAPDH*	Fold of gene expression
Group 1 Infertile men (No=100)	**-1.750**	**2.05**
Group 2 Fertile Control men (No=100)	**-0.710**	**1.00**

3.3 Pearson correlation coefficient analysis

Investigating the correlation between some important factors included in this study, a correlation coefficient analysis for fertile men was done. The results are shown in Table (6). The findings displayed that there was a positive correlation (r = 0.35) between the folding expression of *PRM-1* and *IL-12* genes, but this correlation was not significant (p = 0.29).\

Table 6: The correlation coefficient analysis of *PRM-1* and *IL-12* expression between patients group.

		PRM-1 expression fold	*IL-12* expression fold
PRM-1 expression fold	r=	1	.35
	P=	---	.29
IL-12 expression fold	r=	.35	1
	P=	.29	---

3.4 Receiver Operating Characteristic (ROC)

1. The *PRM-1* expression

The ROC was used as a binary classifier system to determine the accuracy of *PRM-1* expression in predicting male infertility. ROC analysis indicated that the *PRM*-1 expression is an excellent marker for predicting male infertility with a predictive cut-off value of 0.73 U/ml, an AUC of 1.00, specificity of 100% and sensitivity of 100%. As displayed in Figure 1.

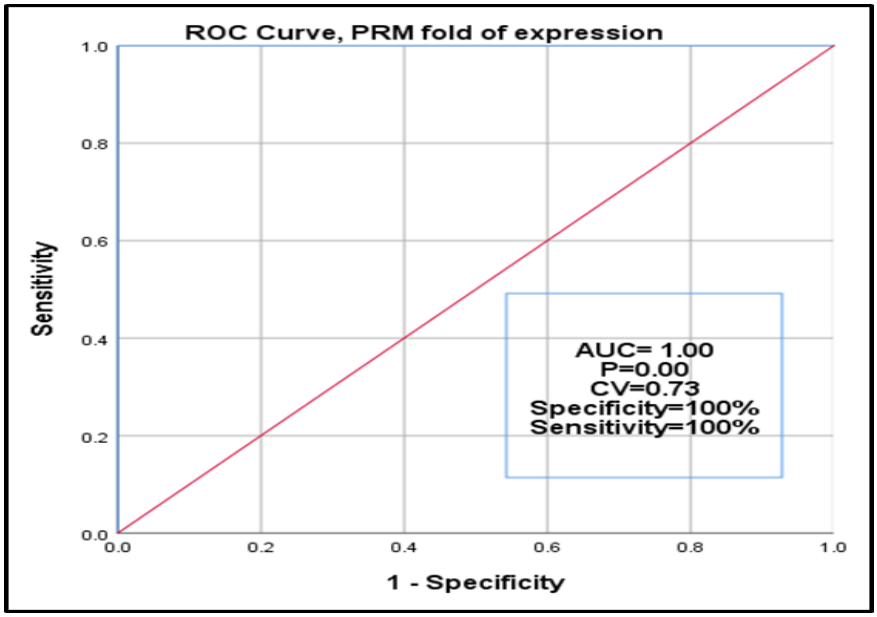

Figure 1: Receiver operating characteristic (ROC) curve for *PRM-1* fold of expression in discriminating between patients group and control group.

2-The *IL-12* expression

According to the Receiver Operating Characteristics analysis, *IL-12* expression is an excellent marker for predicting male infertility, with a predictive cut-off value of 1.25 U/ml, an AUC of 1.00, 100% specificity, and 100% sensitivit**y.**

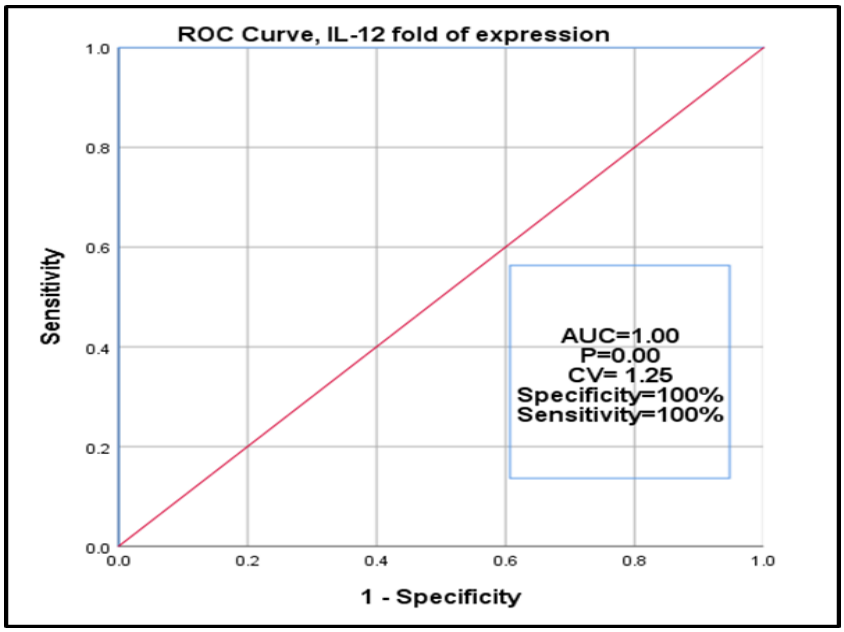

Figure 2: Receiver operating characteristic (ROC) curve for *IL-12* fold of expression in discriminating between patients group and control group.

3-The IL-12 concentration

According to the Receiver Operating Characteristic analysis, IL-12 concentration is a good marker for predicting infertility, with a predictive cut-off value of 2510.50 U/ml, an AUC of 0.98, specificity of 95%, and sensitivity of 95%, as displayed in Figure 3.

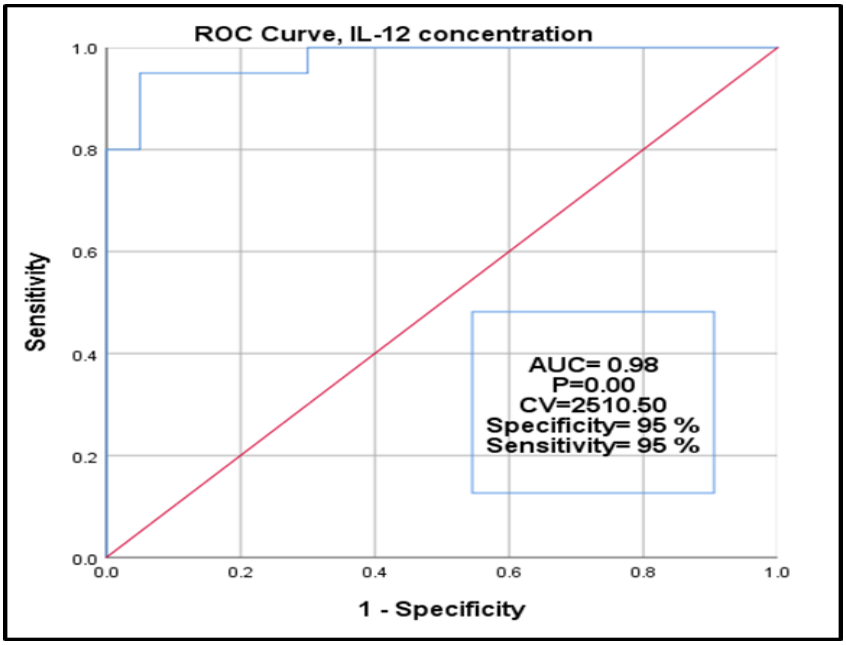

Figure 3: Receiver operating characteristic (ROC) curve for IL-12 concentration in discriminating between patients group and control group.

Discussion:

Male factors were discovered to be the cause of 50% of all infertile instances, accounting for 30% of all cases. The initial stage in determining male infertility was sperm analysis, which included a sperm count as well as a motility and morphology study. Oligozoospermia, asthenozoospermia, teratozoospermia, and azoospermia were all terms used to describe seminal fluid parameter abnormalities. Spermatogenesis is a complicated process in which germ cells are divided during mitosis and meiosis, resulting in the formation of haploid spermatozoa. key semen measure, sperm shape, is regarded as a reliable indication of the effectiveness of spermatogenesis as well as the likelihood of conception [15].

For appropriate germ cell development, highly coordinated gene expression and subtle post-transcriptional control are essential. At least 150 genes are involved in spermatogenesis, the process of male reproduction. Changes in spermatogenesis and its products are affected by changes in a group of genes and expression patterns, resulting in spermatogenesis failure and, as a result, male infertility [16]. In mammals, two Protamine types have been found so far: *PRM1 and PRM2. PRM1* is found in all vertebrate species, but PRM2 is found in select mammalian species, including humans and mice [9;17]. In humans, *PRM1* and *PRM2* deficiency cause sperm morphological problems, motility decreases, and infertility.

Spermatogenesis is a distinct and complex mechanism in which protamine replaces 80 to 85 percent of the histones in human sperm, leaving only 15 to 20% of intact histones in the testes. The formation of intermolecular disulphide bonds in spermatids causes chromatin condensation and morphological alterations, resulting in the formation of mature and differentiated spermatozoa with condensed nuclei and proper head morphology [5]. S-S bonds are definitely important for sperm chromatin stability, and any loss of S-S bonds due to a lack of Protamine renders the chromatin more prone to denaturation [18]. Previous research has found a connection between morphological deficiencies in spermatozoa and the integrity of

their DNA. The results of this study show that there is a possible connection between male infertility and a reduction of *Protamine* expression. These deficiencies can reduce the fertility potential of ejaculated spermatozoa in natural conception by lowering their quality [3].

On the other hand, we focused on the relationship between cytokines like IL-12 released by the testes to influence germ cell proliferation and mesenchymal cell development. These seminal plasma polypeptides regulate sperm quality. Increased cytokine levels may play a role in the male genital tract's immunological response to infection [19]. When a long-term infection or inflammation persists, cytokines may accumulate in the male genital tract, leading to sperm peroxidation and sperm dysfunction. According to a large number of researchers, blood tests for IL-12 in the serum and seminal plasma might serve as an early indicator of genital tract infection and inflammation as well as a call for rapid anti-inflammatory treatment. According to the research, males with aberrant semen parameters had higher quantities of IL-12 in their seminal plasma. This cytokine was found in the semen of men with genital infections, and the study found a favourable correlation between the amount of IL-12 and the quantity of sperm as well as their normal morphology [20]. Reproductive immunology and sperm function are affected by changes in the levels of these cytokines [21].

The results of the current investigation displayed that the gene expression in blood samples for the gene of *IL-12* was significantly increased in the patient group compared to the control group. Jiang et al. (2016)[10] found that males with genital infections had higher amounts of cytokines, such as IL-12, which may play a role in male genital immune defense.

In this work, we evaluated protamine insufficiency and aberrant spermatozoa morphology in infertile males and its correlation with cytokine expression. Previews researches have been conducted to investigate the impact of cytokines and growth factors on sperm function. These findings reveal that IL-12 plays a role in male infertility and that there is a definite correlation between reduced IL-12 and TNF levels and sperm DNA/chromatin damage as well as decreased (progressive and total) sperm motility and morphology [15]. Infertility is associated with abnormal sperm morphology, which in turn is associated with nuclear chromatin integrity.

The present study looked at the expression of the *PROTAMINE 1* gene in blood and sperm samples in addition to the expression of the *IL-12* gene in blood samples from infertile and fertile males. The results of this investigation revealed that the expression of two genes differed considerably between the infertile and control groups. Previous studies showed a lower percentage of spermatozoa expressing *PRM 1* in infertile patients, and the positive associations between lower *PRM 1* expression and defects in semen testing strongly suggest that PRM 1 is involved in male infertility pathogenesis [22].

There is a strong correlation between abnormal *PRM-1* expression and semen concentration, motility, morphology, or fertilisation capacity. The connection between aberrant *PRM-1* expression and abnormal spermatogenesis will be studied using two distinct models. According to the first idea, aberrant *PRM1* expression is suggestive of spermatogenesis abnormalities, most likely due to a malfunctioning transcriptional or translational regulator [23;24]. The second hypothesis to be investigated is that *Protamine* can function as a spermatogenesis checkpoint regulator and that aberrant protamine production activates an apoptotic pathway, resulting in a significant decrease in sperm quality [3;25]. Our recent findings also demonstrated that the histone-to-protamine ratio was substantially linked with specific cytokines (Table 6). The expression of the *IL-12* gene was shown to be positively correlated with the expression of the *PRM* gene, indicating that cytokines are involved in processes of altered protamine

expression and complicated regulatory interactions during histone transition. This might be because cytokines influence histone transition directly or because histone transition impacts sperm quality. This connection is not yet clearly understood [10; 22;26]. Moreover, the findings of the current investigation displayed that the gene expression of the two genes under study, in addition to the concentration of interleukin-12, is an excellent prognostic factor for predicting infertility in a male population.

The results of the current study showed that it is possible to depend on the expression of *PRM* and *IL-12* gene, As predictive biomarkers for male infertility, we recommend that it be among the tests that are relied upon before marriage.

Conclusion: Depending on the current study, the results showed the role of each *PRM-1* and *IL-12* gene on ~~the~~ male infertility cases, in addition to the existence of a common relationship between them in influence. And they can be adopted as predictive biomarkers for male infertility in Iraqi society.

-Conflict of interests:

This research was conducted without conflict of interest among authors, funding agencies, or with any other research group in others institutes.

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