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The Deficiency of *PROTAMINE* 1 Gene Expression and Its Effects on Sperm DNA Fragmentation in A Sample of Iraqi Infertile Male

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ABSTRACT

Background: Protamines are the nuclear proteins essential for chromatin compaction during spermatogenesis. Spermatozoa may develop the SDF if the spermatogenesis is dysregulated, which affects male infertility

Objective: This study was conducted to quantify the *PROTAMINE* 1 gene expression in the semen specimen of infertile Iraqi male and estimate sperm DNA fragmentation.

Patients and Material: 50 semen specimens were gathered from infertile males and 50 samples from healthy fertile individual as control group and subjected for semen analysis, determinations the expression of *PROTAMINE* 1 gene and estimation sperm DNA fragmentation.

Results: There were no appreciable differences in the ejaculation volume between the studied groups.the fertile group's mean sperm concentration (m/ml)(49.58 ±2.93)was statistically significantly ($p < 0.05$) higher than the infertile male group(27.08±15.7).The progressive motility of fertile male(57.20 ± 2.27) was significantly($P < 0.001$) higher as compared to other infertile male groups(22.29 ± 1.95).The morphologically normal sperm of control males(52.20 ± 1.91) was a highly significant ($P < 0.001$) as compared to infertile male groups (36.30± 2.20).The fold protamine 1 gene expression of patients group(0.26) had a lower significantly than the healthy group (1).The mean SDF of infertile male group(43.05 ± 2.93) was statistically significant ($P < 0.001$)than the fertile male group(17.97 ± 9.90).

Conclusion: The current study found that increased sperm with breaks DNA was linked to reduced protamine 1 gene expression and negative impacts on sperm parameters.

Keywords: *protamine* 1 gene, Spermatozoa, sperm DNA fragmentation, infertile Iraqi patients, gene expression.

INTRODUCTION

One of the most significant gynecological issues, fail to conceive clinically after a 12 months of routine, sexual activity that is not protected is known as infertility[(1)]. The reasons behind infertility are multiple, including diseases of ovulation process and the uterus in women, in addition to the disorder of sperm production and poor semen quality in men[(2)].

Infertility became in recent years a serious social, mental and physical health problem worldwide [(3,4)]. Millions of couples all over the world suffer from the problem of infertility [(5)]. Factors known to cause male infertility were: decrease the production of sperm cells, progressive motility of sperm, normal sperm morphology [(6)]. Men and women of reproductive age who were infertile estimated at about 15%, and the reason was due to the male in 50% of them [(7)]. There were many factors that affect the fertility of males, the most significant of which were hormonal deficiencies, physical conditions, issues with sexually transmitted diseases, environmental and lifestyle factors, and genetic factors [(8)]. The sperm cell's primary function during fertilization was to transfer the paternal genome to an egg. According to **Kumaresan *et al***. who indicated that the integrity of the sperm's genetic material was critical to the complete success of fertilization and full embryo development[(9)].The replacement of most histones with smaller proteins, called protamines, during spermatogenesis was what causes the spermatozoa cell to have a distinct chromatin structure [(10)].Protamine is manufactured in the last stage of sperm of many animals and plants and includes *PRM*1, *PRM*2, and *PRM*3. Protamines bind to DNA resulting in sperm chromatin condensation and a genetically inactive state[(11)]. The genome-wide histone hyperacetylation was usually what starts the binding process. Next, the spermatid histone variant was loaded on the chromosomes. Finally, Protamines work with transition proteins to bind to DNAs to create a compacted sperm head in the final[(12)].

The follow meiotic differentiation of spermatids completely reorganizes the whole male germ cell genome. A large proportion of Protamines gradually take the place of somatic histones as the spermatid core expands and contracts. Protamines are basic nuclear and small proteins that are unique to sperm and are necessary for the condensation of the haploid genome. During fertilization, this vital step of tight packing shields the sperm genome from the negative effects of physical and chemical factors [(13)]. Protamination is that protamine replaces histones linked to DNA, which leads to a decrease in the

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proportion of histones compared to protamines, occurs during spermatogenesis. In turn, sperm DNA Protamination is necessary for nuclear condensation, and spermatozoa may develop the SDF if this process is dysregulated. However, in the spermatozoa cell of infertile males, level breaks deoxyribonucleic acid of sperm may be increased [14]. The Sperm Chromatin Structure Assay was the only assay that assesses the amount of uncondensed chromatin, deoxyribonucleic damage severity, the proportion (broken) DNA of sperm cell[15]. There were three main mechanisms that lead to SDF : (1) Endonucleases' action caused spermatozoa that were supposed to undergo apoptosis to fail to do so and to be released with fragmented DNA(2) faulty chromatin maturation, where DNA nicks were not repaired during the usual operation of sperm cell chromatin condensation, resulting in persistent breaks and less dense deoxyribonucleic acid that was more vulnerable to breakage from external causes and (3)Reactive species of oxygen can break DNA during oxidative stress as spermatozoa travel directly in the testes [16,17]. Leukocytospermia, which origins from bacteriospermia, can impair male fertile abilityfrom a variety of processes, including interference with spermatogenesis, decline of sperm function, and genital tract dysfunction[18].

Mismatched bases, base loss, base alterations, pyrimidine dimers, DNA crosslink, single strand breaks and double strand breaks were a few examples of DNA damage. Any of these changes have the potential to cause sperm DNA fragmentation (SDF) and compromise the success of ART or natural conception. A recent scientometric analysis found that lifestyle variables, varicocele, and as thenozoospermia have been sources of original data for fragmented DNA of sperm research over the previous twenty years [19]. Increased SDF levels have been linked to illnesses such inflammation of the male accessory gland, varicocele, and old age of the father, cancer, chronic sickness, ecological toxin exposure, and lifestyle variables, as well as male infertility [20]. A high level of SDF reduced the likelihood of conceiving naturally and was linked to lower pregnancy rates following IUI, IVF, and to decreasing following ICSI [14]. Despite having a very complex structure, the genetic material of sperm was susceptible to break, and according to **Esmailkhani *et al*.** over 80% of idiopathic infertility patients have a connection to DNA integrity[18].

PATIENTS AND METHODS

PATIENTS

The research was planned as a prospective study. The sample size was 100 fertile and infertile male during their visit to Kamal Al- Samaraie IVF Hospital, Baghdad-Iraq, with average ages between (22-48)years old. The selected 50 male were primary infertile male condition according to their seminal analysis. The seminal analysis was done in accordance with the manual of WHO 2010. In addition to the patients samples,50 healthy fertile male as a groupof control, this study also included them. The duration of the study was from April 2020 until April 2021.

RNA extraction from the semen sample

All samples had total RNA extracted using the TransZolUpreagentin accordance with the manufacturer's instructions (TransZolUpPlus RNA Kit):

-Phase segregation

The semen specimen was stored in a room-temperature for five minutes in order to distinguish the nucleoprotein complex. 200µlof chloroform and 750µl of TRIzol LS reagent were combined. The tube was centrifuged to separate the contents after being gently shaken by hand for 30 seconds at room temperature. The mixture separated into an upper aqueous phase colorless, lower colorless chloroform -phenol phase, and an interphase. The RNA was still in the aqueous phase which transferred to the micro centrifuge tube for the RNA isolation technique. The RNA extracted from the samples was reverse-transcribed to cDNA Using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Trans Gen Bio-Tech, China). The procedure carried out in (20 µl)the total RNA volume and reaction volume were both (20 µl).

Quantitative Real Time PCR (qRT–PCR) runs:

The Quantitative Real Time PCR (qRT–PCR) was carried out utilizing the Qiagen Rotor gene Q Real-time PCR System (Australia).

The 2xqPCR Master Mix Kits were used to measure the fold changes and expression levels of the *PROTAMINE1* and *GAPDH* genes. The forward and reverse primers for the target *PROTAMINE 1* gene and housekeeping gene*GAPDH* (reference gene: glyceraldehydes-3-phosphate dehydrogenase), were designed and are shown in table (1).

Table (1):Primers utilized in the current research.

Symbols of gene	(5′→3′ direction)
PRM1	**F-CCGCCAGAGACGAAGATGT**
	R-TACACCTCAGCCTGTACCTG
GAPDH	**F-GAAATCCATCACCATCTTCCAGGC**
	R-GAGCCCCAGCCTTCTCCATG

10µl qPCR Green master mix, for RT-qPCR in Two Steps, 1 µl of each primer (10 µM), 2 µlcDNA, and 6 µl nuclease-free water were added to the reaction mix, which

was adjusted to a final size of 20 µl as advised by the production company. The mixture was put in a real-time thermocycler(Qiagen Rotor gene QRT-PCR System (Australia), which was designed for the next optimized phases: One cycle of Enzyme activation(30 sec at 94ºC), then 40 cycles of denaturation (5 sec at 94ºC), annealing (15 sec at 58ºC), and extension (20 sec at 72 ºC), and lastly one cycle of melting curve at 1min /95 ºC-30 second /55 ºC-30second/95 ºC. This process was carried out on samples. The term was written as $2^{-\Delta Ct}$, which stands for relative fold change. As a consequence, a folding variation in the level of expression of a certain gene represented the data, standardized to an endogenous control (housekeeping gene) and compared to a calibrator, which is the specific gene in healthy subjects.

Detection of sperm Deoxyribonucleic acid fragmentation (SDF):

The fragmented DNA of sperm was assessed by the Acridine orange test method [21]. Each of the samples was smeared on the slide and left for 15-20 minutes in the air until completely dried. After that, these dried slides were immersed in the fixation (Carnoy's solution) for 3 hours until the second day at 4 °C (the fixation solution is prepared immediately).

After that, the slides were removed from the fixation solution and left in the air until completely dry, followed by the process of dyeing these slides with a solution of the Acridine orange dye prepared immediately as well, as it was prepared daily, and the pH of the stain was finally adjusted to 2.5. The stock solution was maintained at 4 °C in the dark as 1-2 ml of this dye was spread on the slides placed on the slide holder, and the dye was left on the slides for 10 minutes. Minutes after that, the slides were gently washed with distilled water well, then these slides were left to dry, and at this step the slides were ready for reading Using a 40X objective lens on a fluorescence microscope which is equipped with a 460-490 nanometers excitement filter and also equipped with a 520 nanometers septum filter.

Ethical approval

The College of Science Research Ethical Committee of Baghdad University approved this project. Every patient gave their informed permission. This research was conducted in accordance with the Declaration of Helsinki, the code of ethics for human research endorsed by the World Medical Association.

Statistical analysis

The mean and the standard error were taken into consideration using (IBM SPSS) version 28.0 of the mean. The probability was also investigated using an ANOVA table and a student T-test. The probability was calculated using chi-square analysis of Pearson for non-parametric information, to ascertain the connection between the investigated parameters; a Pearson's correlation was performed.

RESULTS

Basic seminal parameters distribution of the infertile and healthy male groups

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

Table (2): Semen microscopically analysis for all groups

Parameters	Control group (no=50)	Patient group (no=50)	*P* value
Volume (ml)	2.89±1.01	2.72±1.21	***NS***
Sperm concentration (million/ml)	49.58 ±2.93	27.08±15.7	**0.001**
Total motility (%)	57.20 ± 2.27	22.29 ± 1.95	**0.001**
Morphologically normal sperm (%)	52.20 ± 1.91	36.30± 2.20	**0.001**

Data were shown as mean ± SE; and T-test analytical statistics was used.

There were no appreciable differences in the ejaculation volume between the studied groups. But the fertile group's mean sperm concentration (m/ml)(49.58 ±2.93)was statistically significantly ($p < 0.05$) higher than the infertile male groups(27.08±15.7).The progressive motility (percentage) of fertile male(57.20 ± 2.27) was significantly higher as compared to other infertile male groups(22.29 ± 1.95).Table (2)also revealed a highly significant rise in morphologically normal sperm of control males(52.20 ± 1.91) as compared to infertile male groups (36.30± 2.20).These findings found that highly significant difference in microscopic semen parameters in fertile men when compared with patients group.

Expression of *PRM* 1 in semen samples:

The results showed in table (3) significant difference of the mean± SE Ct values of *protamine 1* cDNA amplification inpatients group samples (24.08 ± 0.44) compared to the fertile group's corresponding Ct values (22.16±0.25). Average Ct values in the patients group were greater than the healthy group.

Table (3) Folding of protamine 1 gene expression according to 2-ΔCtMethod

Study groups	Mean of *protamine 1* Ct	Mean of *GAPD* Ct	Mean of ΔCt	$2^{-\Delta Ct}$	Experimental group/ Control group	Folding of gene expression
Group 1: Control (no.=50)	**22.16**	**15.55**	**6.61**	**0.01023**	**0.01023/0.01023**	**1**
Group 2: (no.=50)	**24.08**	**15.57**	**8.51**	**0.00274**	**0.00274/0.01023**	**0.26**

To determine the folds of *protamine 1*gene expression in reference to the housekeeping gene, every group's $2^{-\Delta Ct}$results have been versus the control groups. The patientsgrouphad a lower fold of *protamine 1* gene expression than the healthy group.In the patients group, the folding numberwere (0.26). These findings imply that *protamine 1* gene expression of patients groupis reduced significantly, as demonstrated in table (3).

Analysis fragmentationof sperm DNA

By using the AO test, which revealed sperm cells with a healthy nucleus and double stranded DNA that was shining green, the extent of sperm chromosome breakage was calculated. The ones with single strands or broken nuclei had an orange-red glow figure (1).

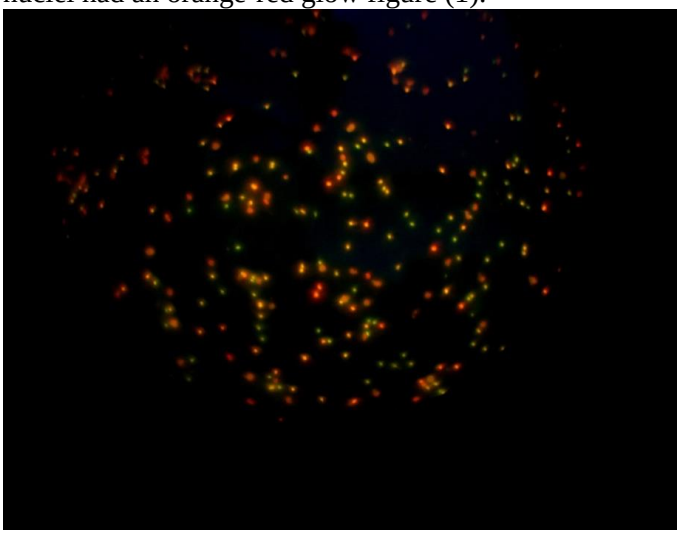

Figure (1): Fluorescence Spermatozoa with green fluorescence were assumed to have normal Genetic material, while sperms with a spectrum of yellow-orange to red fluorescence were thought to have breakage DNA, according to microscopy of Acridine orange-stained cells. (Magnification power 40X).

The analysis fragmented DNA of sperm for the study groups indicated that the mean SDF of infertile male group (43.05 ± 2.93) was statistically more significant than the fertile male group (17.97 ± 9.90).

DISCUSSION

More accurate and therapeutically helpful tests are deemed necessary when taking into account male factor infertility because it is considered that standard spermatozoa cells analysis, and is employed in evaluation fertilizationdegree, is not a good predictor. To identify genetic abnormalities and provide molecular information to clarify the pathophysiology of unexplained infertility, a number of assays have been developed. One of the suggested procedures to evaluate sperm quality is sperm DNA integrity analysis. This procedure can describe the issue of infertility in male and femalealso supports clinical counseling when deciding on the procedure of assisted reproduction. This research assessed the presence protamine of sperm transcripts and sperm DNA break in normozoospermic infertile males. Our research showed that compared to healthy fertile, the patients group had significantly more sperm DNA break and less sperm protamine transcript level.

It is yet unclear what causing the potential decline in male reproductive function quality. It's been proposed that the increased prevalence of abnormalities in male reproduction, particularly impaired sperm motility, may be related to lifestyle factors, such as exposures of environmental and occupational, diseases transmitted by sexually, medications. Large amounts of oxidative stress caused by species oxygen of reactivity overproduction are one of the important causes of low sperm cell motility by generation of abnormalities in sperm chromatin of human males [(22)]. The sperm cell's primary function during fertilization was to transfer the male genome to the egg. For effective fertilizing and subsequent embryo development, sperm DNA integrity is essential [(9)].

In this research, male infertility was associated with a larger percentage fragmented DNA of sperm than were healthy controls. Significantly negative links were found between total sperm cell movement and normal morphology and break DNA of sperm. There has been research on the link between sperm qualities and DNA damage levels. DNA damage was revealed to be strongly linked with normal morphology and progressive motility in sperm by **Yuan *et al.*** [(23)]. Our findings indicated that the mean sperm DNA fragmentation of the infertile male groups was statistically greater than fertile control group (17.97 ± 9.90), which was higher at 43.05 ±2.93. These outcomes are consistent with the research conducted by **Sergerie*et al.*** which showed that the SDF found in the infertile groups was greater than the healthy group (40.9± 14.3% versus 13.1± 7.3%) [(24)]. When it refers to IVF and

ICSI results, an SDF >30%, and particularly>40%, was frequently seen as one of the leading causes of infertility. Additionally, an SDF of greater than 40% is thought to increase the likelihood of spontaneous abortion [15].

The findings of the current study also indicated that, when compared to spermatozoa from healthy fertile controls, spermatozoa from infertile males displayed considerably lower levels of *PRM*1 mRNAs. This result reflected to the original mRNAs discovered in the specimens. Additionally, apparent that the patient groups were linked with a decreased of mRNA scopy's number, which suggests the patient group lower expression when versed to the fertile group. These results showed the patient group has lower levels of *protamine* 1 gene expression. The explanation of this state might be related with numerous causes including age, infection, lifestyle, drugs and medical problem. The role of virulence factor in the pathogenicity of pathogen in particular staph aureus increased the negative impact on seminal parameters[25].

The *PRM*1 transcript levels in this study were linked to sperm morphology and motility. Previous research revealed that fewer spermatozoa in infertile individuals expressed *PRM* 1, and the strong correlations between decreased *PRM* 1 expression and abnormalities in semen tests strongly suggest that *PRM 1* plays a role in male infertility pathophysiology[26]. According to other studies, a low *PRM* gene or protein expression has a detrimental influence on male fertility and lowers the sperm cell quality [27]. *PRM*s are involved in crucial spermatogenic processes, and any variation in their expression could have an effect on sperm parameters or sperm motility or shape, ultimately resulting in infertility [28]. Additionally, our findings showed that low protamine transcript levels were linked to significant sperm DNA damage. Increased fragmented DNA of sperm in ICSI patients was found to be caused by *protamine* insufficiency, according to **Nasr-Esfahani*etal*.**[29]. The correlation between DNA break and protamine of sperm concentration was discussed by Nili et al. in 2009. Fragmentation of DNA was much higher of Spermatozoa deficient in *protamine* than in non-deficient spermatozoa, according to research by **Utsuno*etal*.**[30].According to this research, variations in *protamine*1 transcript levels of sperm may contribute for higher fragmented DNA of sperm that may be a contributing factor to decline in possible fertility of patients. DNA fragmentation results from aberrant protamination and any adjustments to the *protamine*1: *protamine*2 ratios, it might allow the sperm to be more susceptible to stresses. like (ROS) [31]. According to **Simon *et al*.** greater spermatozoa cell with fragmented deoxyribonucleic acid was linked to aberrant protamination, which in turn resulted in lower rates of fertilization and poorer embryo quality[32]. Higher pregnancy potential was seen in IVF patients.When the level of semen protamine is normal, as revealed by **Rogenhofer*et al*.**[33]. The clinical outcomes for ICSI patients may be enhanced by employing the protaminesratio as a superior indication for the evaluation of semen quality, according to **Sarasa*etal*.**[34].

CONCLUSION

In this research, it can be concluded that increased sperm with breaks DNA was linked to reduced protamine 1 gene expression and there is a negative link between breaks DNA, morphology, and movement of sperm cell in male infertility. We can also conclude that spermatozoa's DNA breaks helps to identify undesirable deoxyribonucleic acid sperm for use in ART procedures. Males with high SDF also had reduced IVF pregnancy rates, embryo quality, and fertilization rates.

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