Evaluation of *TNP1* and *PRM1* gene expression in male infertility patients with low or high sperm DNA fragmentation

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Abstract

Objective: The transition nuclear protein 1 (*TNP1*) gene is a member of the TNP family and is abundantly expressed during spermatogenesis. Protamine 1 (*PRM1*), another sperm nuclear protein, is abundant in many species. The present study aimed to evaluate transition nuclear protein 1 (*TNP1*) and protamine 1 (*PRM1*) gene expression in infertile male patients with low and high sperm DNA fragmentation (SDF).

Material and Methods: Semen samples (n=100) were obtained from male participants undertaking treatment with intracytoplasmic sperm injection. The expression levels of *TNP1* and *PRM1* were measured using real-time quantitative polymerase chain reaction. The data were compared with statistical tests, (independent samples T- or Mann-Whitney U) as appropriate. A $p<0.05$ was considered significant.

Results: Patients with low-SDF exhibited a significantly lower sperm concentration compared to those with high-SDF ($p=0.002$). There was significant down regulation of *TNP1* ($p=0.036$) and *PRM1* ($p=0.04$) in patients exhibiting high-SDF levels compared to those with low-SDF levels. A significant moderate positive correlation was observed between the relative expression levels of *TNP1* and *PRM1* ($r=0.459$, $p<0.001$).

Conclusion: In the present study *TNP1* and *PRM1* were differentially expressed in male patients being treated for infertility and who had low or high-SDF. (J Turk Ger Gynecol Assoc. 2025; 26: 7-14)

Keywords: Male infertility, *TNP1*, *PRM1*

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Introduction

Infertility is an important health problem in all countries. Almost 140 million people (around 15-18%) worldwide face infertility (1). Causative factors may be genetic, immunologic, hormonal, anatomic, teratogenic, infectious, smoking, exposure to radiation and others. It is a multifactorial condition and 25% of infertile couples have more than one factor (2). The cause of infertility may involve the female partner (1/3), male partner (1/3) or both (1/3). Around 15% of male infertility is caused by genetic factors (2,3), which can influence the spermatogenesis process, and may cause increased sperm anomalies (4).

Spermatogenesis, which begins after puberty, includes mitotic, meiotic and cellular reorganization. As a result of spermatogenesis, spermatozoa are formed, which enable the transmission of the male genetic material. During spermatogenesis, cell cycle checkpoints remove spermatocytes that harbor damaged DNA. Spermatocytes with undamaged DNA are allowed to continue spermatogenesis (5).

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Spermatozoa are highly differentiated cells consisting of three parts: the tail, the mid-piece and the head. Head of the sperm delivers the haploid genome into the oocyte during fertilization. Sperm cells are critical for the oocyte genome and vision (6). Chromatin condensation is important to minimize damage to sperm DNA during its passage through the male and female and reproductive tracts (7).

Another factor that has a significant place in male infertility is sperm DNA fragmentation (SDF) (8). Apoptosis, protamination disorders, and reactive oxygen species stand out as significant contributors to sperm DNA damage (9). SDF tends to increase with age, almost doubling in the 60s compared to the 20s (10). SDF can cause early pregnancy loss, and also may be an indicator in patients who cannot achieve pregnancy after assisted reproductive technology treatment or have a low fertilization rate (11). Although fertilization ability would not be lost in the case of SDF, it can negatively affect developmental stages of the embryo and result in genetic anomalies in the offspring (12,13). During the spermatogenesis process, the formation of a compact DNA occurs when transition proteins and protamines (PRM) replace histone proteins (14). In this multistage process, somatic cell histones undergo replacement with sperm PRM (15). This process begins with the substitution of histones by transition nuclear proteins (TNP) in round spermatids, followed by the subsequent replacement of both TNPs with PRM in elongating spermatids. As a result of this histone-protamine exchange, chromatin becomes highly condensed while remaining transcriptionally inactive (16).

PRM compress sperm DNA to almost six times greater compactness than somatic cell DNA, ensuring that the sperm nucleus remains protected and stable (17). This compact DNA combined with the mobile structure of the cell, provides an excellent vehicle for the genetic material it will carry to form the zygote (18).

The *TNP1* gene is a member of the *TNP* family and is abundantly expressed during spermatogenesis. Positioned on chromosome 2, *TNP1* encodes transition proteins (TP1 and TP2) (19). TP proteins are important since they reduce breaks during DNA condensation (20). Thus, *TNP1* plays a role in chromatin condensation. Defects in TPs may lead to atypical condensation of sperm chromatin, impaired motility of spermatozoa and increased frequency of breaks in sperm DNA strands (21,22). In another study, *TNP1* expression levels were examined for increased sperm DNA damage due to smoking and it was observed that *TNP1* gene expression was downregulated in cases with high sperm DNA damage (23).

PRMs, another sperm nuclear protein, is abundant in many species (24). The genes for PRMs (*PRM1* and *PRM2*), are located on chromosome 16p13.13, which forms a multigenic cluster (25). The *PRM* genes code the P1 and P2 proteins. P1 and P2 are synthesized as mature and precursor, respectively (26). PRM, which are rich in cysteine and arginine, have a special structure responsible for sperm chromatin condensation (27). Functions, such as production of a condensed genome with a hydrodynamic and compact nucleus, increase in transcription factors and other proteins during spermatid development, participation in paternal genome imprinting, being part of a checkpoint during spermatogenesis and playing a role in fertilization are some of functions of PRMs (15). The role of *PRM1* in male infertility is not fully understood (28). A recent study found that the level of *PRM1* gene expression is associated with reproduction (29).

Moreover, protamination is an important epigenetic regulatory process (30). Protamination results in the removal of histone-bearing epigenetic signals. This implies a role for protamination in the regulation of spermatozoa epigenetically. Epigenetic factors are active in the post-fertilization regulation of transcription. Histone modification and DNA methylation are potential mechanisms that may be involved in epigenetic regulation (23). Kläver et al. (31) found that there was a significant correlation between male infertility and increased levels of DNA methylation profiles in repressed genes.

For successful fertilization to take place, both sperm and egg need to possess the capacity to initiate key molecular mechanisms that can lead to viable embryonic development. In this regard, numerous studies have indicated that over 80% of fertilization failures in assisted reproductive treatments are linked to sperm-related issues (32). Studies have examined the impact of sperm protamine deficiency in fertilization failure (33,34) and the relationship between protamine imbalances in the sperm nucleus (35) and sperm DNA damage (36). In the present study, the goal was to evaluate *PRM1* and *TNP1* gene expression in cases with and without sperm DNA damage to understand the relationship between protamine expression and male infertility. Thus, the present study evaluated *TNP1* and *PRM1* gene expression in male infertility patients with low and high-*SDF*. The null hypothesis was that there was no significant difference in *TNP1* and *PRM1* gene expression levels between low and high-SDF groups.

Material and Methods

Participants were male infertility patients who applied to a genetic diseases evaluation center (Microgen, Ankara, Türkiye). Semen samples were taken from each subject. Semen samples were obtained from male participants undertaking treatment with intracytoplasmic sperm injection (Gen-Art IVF Center). Participants included in the current study were between the ages of 25 and 49 years. Informed consent form was given by all participants. A physical examination was performed on the participants. Responses were recorded with a structured

questionnaire covering the participants' medical history, occupation, and lifestyle.

The duration of abstinence from ejaculation for all participants was between 3 and 7 days. Samples were obtained by masturbation. The semen sample was diluted with 1X PBS Solution in a 5 mL Polystyrene Round Bottom tube to obtain a sperm concentration of $3x10^6$/mL. The samples were centrifuged at 2400 rpm for 5 minutes. 1X PBS Solution (1 mL) was added to the pellet and centrifuged again at the same time and speed. Then 1 mL of 4% paraformaldehyde solution stored at -20 °C was added to the pellet. The sample was fixed at +4 °C.

Criteria for inclusion and exclusion of patients

Criteria for inclusion of the current study were male infertility patients aged between 25 and 49 years. Patients with a history of cancer treatment, genetic disorders, hypogonadism or cryptorchidism were excluded.

Ethical approval and sample size determination

The protocol of the current study received approval from the ethics board of Biruni University Non-Interventional Clinical Research Ethics Committee (approval number: 2023/77-28, date: 06.01.2023). Based on data from the study of Amor et al. (23), 98 patients were found to be sufficient for the present study (power: 0.80%, significance level: 0.05, effect size: 0.506). Considering potential drop-outs, it was planned to enroll 100 patients.

TUNEL assay

The procedural steps for this test were conducted in accordance with the manufacturer's instructions (TUNEL kit; Roche Diagnostics GmbH, Germany). Following the washing and fixation protocol, 50,000 cells were analyzed (37). The patients were divided into two groups. Low-*SDF* group (n=50): having <16.8% TUNEL positivity and high-*SDF* group (n=50): having ≥16.8% TUNEL positivity.

Isolation of RNA

First, β-mercaptoethanol (240 μL) was added to each specimen. Vortex was used for mixing specimens. The specimens were then incubated at room temperature for 15 minutes with the lid closed. After incubation, 400 μL of 100% ethanol was added and the specimen was mixed by vortex for 40 s. Samples mixed with vortex were withdrawn without removing tissues and transferred to spin columns with brief patient information. Spin columns were centrifuged at 13000 rpm for 1min 30 s.

After centrifugation, the collection tubes were emptied and 400 μL RNA wash buffer was added onto the spin columns. By using purified semen specimens, total RNA isolations were performed according to manufacturer's recommendations of ZYMO RESEARCH Quick-RNA™ MiniPrep Kit. The quantity and purity of the isolated RNA were assessed using the NanoDrop spectrophotometer (Thermo Scientific, USA). Absorbance spectrophotometric ratio was at 260/280 nm.

Reverse transcription and quantitative PCR

Manufacturers' recommendations were followed when performing all procedures. The expression levels of *TNP1* and *PRM1* were measured using a real-time quantitative polymerase chain reaction (RT-qPCR) technique. The MiScript (Qiagen, Hilden, Germany) reverse transcription kit was used to convert total RNA to *cDNA* in a 10 μL reaction volume. Take 10 μL of each RNA sample and place it in 0.2 PCR tubes. The specimens were denatured for 5 minutes at 65 °C and then immediately placed on ice for 2 minutes to stop the process. Then, the master mix procedure was undertaken (LightCycler® 480 Roche Device). Each run included a no-reverse transcriptase control and a no-template control. Whole qPCR tests were conducted in duplicate, and the Ct values were normalized to GAPDH. MiScript reverse transcription kit was used to convert total RNA into c*DNA* (a reaction volume of 30 μL) (Qiagen, Hilden, Germany). Briefly, a mixture was made using 300 ng of isolated RNA, RNase-free water, miScript Reverse transcriptase mix, miScript HiFlex Buffer (5×), and miScript nucleic mix. The mixture was incubated in a thermocycler at 37 °C for 60 minutes, followed by incubation at 95 °C for 5 minutes to deactivate the transcriptase mix.

qPCR was conducted using the StepOnePlus™ System (7500 Fast Applied Biosystems, USA). The generated c*DNA* served as the template for qPCR analysis, conducted with SYBR Green and QuantiTect primer assay (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. Each run included a no-template control and a no-reverse transcriptase control. Whole qPCR tests were conducted in triplicate, and the Ct values were standardized to GAPDH. Relative RNA quantity in the samples was assessed individually using the comparative ΔCt method, with the threshold cycle (Ct) indicating the cycle number at which the fluorescence curve intersects the qPCR threshold.

Statistical analysis

All statistical analysis were performed by using IBM SPSS 22 (Chicago, IL, USA) at 95% confidence level ($p<0.05$). Data were firstly analyzed with Kolmogorov-Smirnov test to understand whether the data normally distributed or not. Independent samples t-test were used to analyze age data. Mann-Whitney U test were used to analyze *TNP1*, *PRM1*, sperm concentrations and DNA damage percentage data. The Spearman correlation test was used to determine whether there was a correlation between the relative expression of the *TNP1* and *PRM1*.

Results

The consort flow diagram for patient selection can be seen in Figure 1. A total of 374 patients were eligible for the study, however, 100 met inclusion criteria. Laboratory processing failure occurred in seven of these patients. One of these was in the low-*SDF* group and six were in the high-*SDF* group.

There were no significant differences between the groups in terms of age. Table 1 presents sperm concentration, percentage of DNA damage, relative amount of *TNP1* and *PRM1* by group. In patients with low-*SDF*, sperm concentration was significantly lower than those with high-*SDF* (p=0.002). Moreover, there was significant down regulation of *TNP1* (p=0.036) and *PRM1* (p=0.04) in patients exhibiting high-SDF levels compared to those with low-SDF levels. A significant moderate positive correlation was observed between the relative expression levels of *TNP1* and *PRM1* (r=0.459, p<0.001).

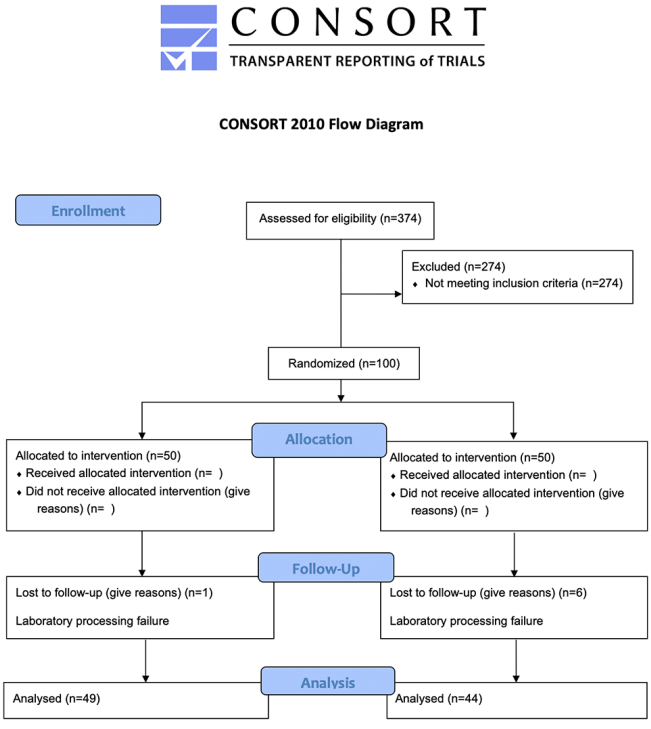

Figure 1. Consort flow diagram

Discussion

The present study aimed to evaluate *TNP1* and *PRM1* gene expression in male infertility patients with low and high-*SDF*. The relative amounts of the mRNA of *TNP1* and *PRM1* genes were differentially expressed between low and high-*SDF* groups. Thus, the null hypothesis was rejected. *TNP1* gene expression was down-regulated in patients with high-*SDF* compared to patients with low-*SDF*. Yu et al (38) conducted a study on mice by deleting the *tnp1* gene. Deletion of this gene affected sperm morphology and motility and also caused male infertility.

The general assumption was that *TNP*s are integrated into chromatin after histones are removed and that they act as intermediates between histones and PRM, since they constitute a significant portion of the genome in elongating spermatids (39). Several studies have highlighted the importance of *TNP1* in male infertility (40-42). Firstly, *TNP1* is essential for producing healthy sperm (42). Secondly, a considerable portion of men lacking *TNP1* are diagnosed as infertile due to severely diminished sperm motility (38). Knockdown of *TNP1* can cause abnormal sperm morphology and decreased progressive motility, leading to male infertility (38). Besides their role in *DNA* compaction, *TP1* and *TP2* are implicated in repairing DNA breaks that naturally occur during chromatin remodeling in the spermatid nucleus (43,44). There are studies showing that the disappearance of single strand breaks is consistent with the presence of transition proteins in elongating spermatids (45). TP1 is also able to stimulate intermolecular ligation of double-stranded breaks in DNA (46).

Histone and protamine substitution results in a transcriptionally dense and silent chromatin (16). Many studies have shown that an abnormality in protamine expression has a direct relationship with male infertility (36,47-49). A direct relationship between sperm protamine deficiency and fertilization failure has also been demonstrated in studies (34,50-52). Successful

Table 1. Comparison of the studied parameters between low and high sperm DNA fragmentation groups

	Patients with low sperm DNA fragmentation	**Patients with high sperm DNA fragmentation**	**p**
	(n=49)	**(n=44)**	**NA**
Age (years)	36.02±5.30	35.52±4.66	0.634
Relative amount of *TNP1*	-0.12±3.76	1.60±5.02	**0.036**
Relative amount of *PRM1*	-2.64±2.89	-0.37±4.31	**0.04**
Sperm concentration	47489795±35278724	33900000±48246392	**0.002**
Percentage of DNA damage	8.28±3.44	23.01±7.18	**<0.001**
NA: Not applicable, DNA: Deoxyribonucleic acid, TNP1: Transition nuclear protein 1, PRM1: Protamine 1			

fertilization necessitates the coordinated initiation of a series of events between the sperm and egg components, crucial for normal embryonic development. Both the sperm and egg must possess structural and genetic competence for successful fertilization to take place. The initiation of oocyte activation, and the fusion of oocyte and sperm chromosomes after fertilization, is mediated by sperm nuclear decondensation factors (53,54).

Fertilization failure is a frequent occurrence, observed in around 30% of oocytes following intracytoplasmic sperm injection in assisted reproductive treatments. Although more than 80% of unsuccessfully fertilized oocytes were released into the sperm using the microinjection technique, fertilization failure was attributed to the lack of biochemical mechanisms that initiate activation in the oocytes (55). Despite these deficiencies, the process may not start, or even if it starts, it may not proceed normally. It is caused by incomplete activation of sperm factors (56-58). Sperm morphology, sperm nuclear morphology, some changes in sperm chromatin and acrosomal defects are some of the sperm morphology-related factors in fertilization failure (34,59,60).

Recent studies suggest that the presence of unrepaired DNA damage surpassing a critical threshold in embryos, whether generated *in vivo* or *in vitro*, could explain the developmental halt observed post-implantation in embryos with a normal karyotype. Such damage is thought to occur during or after implantation and is characterized as a late paternal effect (61,62). In addition, there are indications suggesting a heightened level of DNA damage in sperm samples where a blastocyst cannot be obtained (63).

Dysregulated DNA methylation is very common in male infertility cases. There is a negative correlation between methylation and sperm DNA damage (64). Post-translationally modified histones are present in the sperm head fraction. This preserves epigenetic memory and facilitates epigenetic reprogramming of the zygote. Moreover, the process of protamination, which includes protamine phosphorylation and acetylation, may also play a role in this epigenetic process (65). While approximately 85% of histones are replaced with PRM during spermiogenesis in humans, epigenetic modifications, such as acetylation and methylation, are considered to be prevented by the remaining histones (66,67).

These findings underscore the multifaceted role of sperm cells beyond their primary function of transferring paternal DNA to the oocyte. The distinctive chromatin structure, which harbors epigenetic markers on genes implicated in transcription regulation and developmental processes, implies a significant involvement of the paternal genome in these crucial processes (67). In consideration of these insights, using sperm with protamine abnormalities during intracytoplasmic sperm injection may pose significant concerns for the developing embryo (68). Consequently, such circumstances may potentially lead to epigenetic alterations (69).

Another important role of protamine is to condense DNA, safeguarding its integrity by rendering the father's genetic contribution inaccessible to nucleases and mutagens (70). Consequently, anomalous protamine expression and dysregulated sperm chromatin might disturb processes associated with these functions, potentially affecting the transmission of paternal genetic material, resultig in negative impacts on embryo development. Indeed, deviations in the protamine composition within sperm nuclei have been associated with increased vulnerability to DNA damage (35).

Smoking and hookah use have been shown to cause increased *SDF* (23,71). Although direct comparison cannot be made between these studies and the results of the present study, these earlier findings should be noted. Amor et al. (23) reported that the expression of *TNP1* and *PRM1* were down-regulated in the spermatozoa of heavy smokers compared to non-smokers. Tofighi Niaki et al. (71) reported a trend in the decrease of *PRM1* expression among hookah smokers compared to controls (3.49±5.41 and 1.22±1.96), although the reduction did not reach significance.

Finally, we found that the relative level of *TNP1* expression moderately positively correlated with *PRM1* expression. This finding is in accordance with the findings of a previous study (23).

Numerous reports indicate a direct association between abnormalities in protamine expression and male infertility (36). Studies have demonstrated that round-headed spermatozoa from infertile patients contain lower levels of protamine and higher levels of histone and intermediate proteins compared to normal spermatozoa (71). This suggests that chromatin organization and acrosome formation occur at late stages of spermiogenesis.

The most common cause of male infertility is abnormalities in chromatin condensation, demonstrating how important chromatin condensation is (72). First, *TNP1* and *TNP*2 are replaced by histones, and then *PRM1* and *PRM2* are replaced by *TNP*s. These processes ensure the compression of the chromatin that will occupy the sperm head (73). In the current study, *TNP1* and *PRM1* genes, which have very important functions during chromatin condensation, were studied in patients with low and high-*SDF*. Sperm *DNA* damage can result from incomplete chromatin condensation. Sperm *DNA* damage serves as an indicator of low fertilization rates and an inability to achieve pregnancy. It can lead to early pregnancy loss and diminish the likelihood of clinical pregnancy in *in vitro* fertilization procedures (11,74). The results of the current study uncovered a significant down-regulation in *TNP1* and *PRM1* gene expression in individuals with high-*SDF* compared to those with low-*SDF* levels. This down-regulation may lead

to incomplete chromatin condensation, thereby contributing to sperm DNA damage. Benchaib et al. (75) and Borini et al. (37) used the same technique for sperm preparation as was used in the present study, and threshold values for the TUNEL assay were used as 20%. However, in a recent study by Sharma et al. (76), it was concluded that high specificity and positive predictive value was obtained at a cutoff point of 16.8%. Thus, threshold value of 16.8% for TUNEL assay was chosen in the present study.

Conclusion

In the present study *TNP1* and *PRM1* were differentially expressed in male patients being treated for infertility and who had low or high-SDF. Both of these genes were significantly down-regulated, based on mRNA levels, in male infertility patients with high levels of SDF compared to infertile peers with low-SDF levels.

Ethics

Ethics Committee Approval: *The protocol of the current study received approval from the ethics board of Biruni University Non-Interventional Clinical Research Ethics Committee (approval number: 2023/77-28, date: 06.01.2023).*

Informed Consent: *Informed consent form was given by all participants.*

Footnotes

Author Contributions: *Surgical and Medical Practices: Y.Ş., E.S.A., S.A., V.B., Concept: Y.Ş., E.S.A., S.A., V.B., Design: Y.Ş., E.S.A., S.A., V.B., Data Collection or Processing: Y.Ş., E.S.A., S.A., V.B., Analysis or Interpretation: Y.Ş., E.S.A., S.A., V.B., Literature Search: Y.Ş., E.S.A., S.A., V.B., Writing: Y.Ş., E.S.A., S.A., V.B.*

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