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Investigation of Protamine Genes (PRM1, PRM2) Polymorphism and Its Impact on Sperm Toroid Integrity in Males from Ahmedabad (India)

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Authors' contributions

This work was carried out in collaboration between all authors. Author HH designed the study, wrote the protocol and managed the investigation. Author RN carried out the analyses and wrote the first draft of the manuscript. Author SR helped with literature search and performed the statistical analyses. Author PK managed the clinical aspects of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Although infertility rates have been escalating in the past few years, there are very few studies directed towards its investigation, especially for males in the region of Ahmedabad, India. Hence the present study was aimed at identifying polymorphisms in the protamine genes (*PRM1, PRM2)* and their possible impact on sperm chromatin, in order to determine molecular changes which could impede sperm fertilizing ability.
Methodology: Infertile patients (Group II; n=23) with history of failure of *in-vivo* and *in-vitro* fertilization were included in the study. Normal, fertile age-matched men were selected as controls (Group I; n=25). Sperm Toroid Integrity (STI) assays was used to analyze Protamine-DNA binding

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efficiency. Sequencing was carried out for the protamine genes *"PRM1* and *PRM2*" to screen for relevant single nucleotide polymorphisms (SNPs) that could alter the protein structure and its function of compact DNA binding, possibly impairing sperm fertilizing potential.

Results: A significantly higher percentage of protamine-DNA dissociation and partial toroid disruption was observed in the infertile cases. However, sequencing yielded only a single distinct SNP at nucleotide 239 (rs737008) in *PRM1* gene.

Conclusion: The study revealed therefore that although the sequence of PRM genes and the resultant protamine proteins may not be altered, a loose protamine-DNA association and a disturbed toroid assemblage could render the DNA vulnerable to external pressures, leading to poor sperm function.

Keywords: Protamines; gene PRM1; PRM2; sperm toroid; single nucleotide polymorphisms; male infertility.

1. INTRODUCTION

The problem of impaired fecundity is recently proving to be a cause of grave concern with global reports of declining male fertility [1,2]. Barrett et al. [3] have termed it a global, challenging health problem. Male factor infertility is known to contribute to 40–50% of infertility and as many as 2% of all men exhibit suboptimal sperm parameters [4]. The escalating incidence of infertility has led to a growing dependence on in-vitro technologies which often results in failure of fertilization in culture, culminating in the need for repetitive trials. The emotional and financial stress-load only complicates the issue for both patient and clinician. Hence in-depth research is now imperative to evaluate causes for impaired sperm fertilizing ability which could be specific for individuals of different populations.

In addition, Barroso et al. [5] have emphasized the need for assessing the integrity of the male gamete and have expressed concern regarding transmission of genetic diseases through ICSI. This matter is even more crucial when assisted reproductive techniques (ARTs) are used, since some or all of the stringent processes are bypassed and sperm with damaged DNA may fertilize an ovum through microinjection.

Sperm chromatin is unique in its degree of compaction, structurally and functionally different from that of somatic cells in relation to the level of compaction [6]. For this, basic nuclear proteins protamines (P1 and P2) protect the sperm from micro-environmental stressors during its journey to the site of fertilization. But despite this compact packaging, DNA damage does occur, high levels of which have been reported in infertile men [7]. Disruption of the DNA-Protamine chromatin assembly leaves the DNA vulnerable towards attack, leading to DNA fragmentation and poor sperm function. Research over the last two decades has established that maintenance of sperm nuclear integrity is crucial for the protection of the male genome [8] and the maintenance of sperm vitality. The Protamine genes, *PRM1* and *PRM2* are instrumental in the maintenance of the sperm toroid assembly and thus nuclear integrity and any alteration in these genes could lead to disruption of the compact nuclear organisation which could impact sperm fertilizing ability.

P1 and P2 are arginine-rich sperm nucleoproteins, vital for condensation of nuclear chromatin and protection of the sperm genome from mutation induced by internal and external factors. However, the protamine genes could harbour non- synonymous polymorphisms that could induce conformational changes in the proteins, which would alter their incorporation into sperm chromatin. SNPs have been identified as a risk factor for male infertility and this increases the risk of failed fertilization by 27 – 66% with ARTs. Although the *PRM1* and *PRM2* genes are highly conserved, Tanaka et al. [9] have reported a mutation leading to a single nucleotide replacement induced a nonsense mutation in the *PRM2* gene leading to translation termination, which in turn might result in male infertility due to haplo-insufficiency of *PRM2*. Correspondingly He et al. [10] screened Chinese Han population in which he genotyped 38 SNPs and has noted that the *PRM1* variant rs35576928 (Arg>Ser) was significantly associated with severe oligozoospermia. It was further indicated that this variant in PRM 1 was associated with spermatogenesis defect in that population. Tuttlemann et al. [11] established that the frequency of sequence variations in *PRM1* and *PRM2* genesin the three groups of Caucasian patients with idiopathic teratozoospermia. However none of the *PRM1*/2 variants was found

to be associated with teratozoospermia or individually with other semen parameters. On the other hand, a study on Iranian population [12] showed the relationship among some protamine gene SNPs including *PRM1* (C321A) and *PRM2* (C248T) in 96 idiopathic infertile men with azoospermia or oligozoospermia in which frequency of altered AA and GG genotypes were slightly higher in infertile case group.

Hence, the current investigation was an attempt to detect SNPs of the protamine genes *PRM1* and *PRM2*, from cases of unexplained infertility in the local population of Ahmedabad city (Gujarat, India), which could be correlated with altered protamines and disrupted toroid organization. The investigation therefore holds additional significance since this population lies in an area with a growing rate of infertility, which is known to be exposed to myriads of environmental toxicants. They could trigger protamine-DNA dissociation, adversely influencing sperm function. Literature surveys have indicated that molecular studies on sperm nuclear integrity have not been carried out earlier in populations of this area. It was our endeavour therefore, to identify specific alterations, which later could serve as biomarkers to diagnose such impaired fertility.

2. MATERIALS AND METHODS

The present study included molecular analysis of selected genes *PRM1* and *PRM2* for sperm nucleoproteins and dual staining for toroid integrity in sperm from normal and infertile cases.

2.1 Subjects Selected For Study

Individuals attending the hospital with diagnosis of male unexplained infertility in the reproductive age group of 25 to 40 years, (n=23) were selected as study subjects (Group II). Normal, healthy, age-matched volunteers of proven fertility were selected as the controls (Group I; n=25). The details of each case were recorded on a standard proforma. Patients diagnosed with couple infertility, whose female partners had no discernible cause, were selected for this study. Patients with history of addictions, therapy and occupational exposure to hazardous chemicals were excluded from the study.

2.2 Sample Collection

The blood and semen samples were collected through regular pickups at Vani Hospital, Ahmedabad by written patient consent. This project was approved by the Institutional Human Ethics Committee (IEC; November, 2015). Peripheral blood was collected by EDTA vacutainer under sterile conditions at the Vani Hospital under medical supervision. A volume of 3 ml of blood was collected from each Normal and Infertile subject for isolation of genomic DNA.

2.3 Sperm Toroid Integrity (STI) Assay

The sperm toroid integrity assay described by Chan et al. [13] was employed to determine the whether the DNA-protamine binding was intact or disrupted. The technique employs a dual stain which competes with the basic protamines for DNA binding sites. A high intensity stain indicates penetration of stain and displacement of protamines.

Observations of the stained slides were carried out using a Lawrence and Mayo Lynx 2000 binocular research microscope under 100 X magnification. In accordance with criteria laid down in the technique, observations of intense, dark blue stained sperm heads indicate spermatozoa with loss of compact chromatin packaging since the stain could displace the Protamine and bind effectively to the DNA, reflecting a disrupted toroid assembly. Spermatozoa with heads stained light blue suggested that the stain could not displace the protamines in binding to DNA and hence such nuclei had normal toroid assembly. Spermatozoa which showed intermediate stain intensity were scored as susceptible, vulnerable to dissociation or with partially disrupted assembly (Fig. 1).

2.4 Molecular Analysis

SNPs were analysed among infertile patients and were compared with the established fertile individuals by running Polymerase Chain Reaction (PCR for the two selected nucleoprotein genes namely protamine 1 (*PRM1*) and protamine 2 (*PRM2*).The genomic DNA was isolated from the whole blood sample [14]. The PCR and DNA Sequencing for SNPs were then carried out.

In correlation with this analysis, Sperm Toroid Integrity Test (STI) was [13] for determining the integrity of sperm chromatin. Observations were carried out under 40X and 100 X oil immersion, using a Lawerence and MayoLynx microscope.

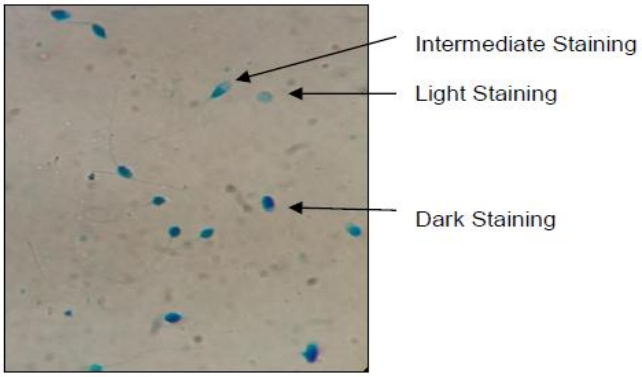


Fig. 1. Staining intensities seen in Sperm Toroid Integrity (STI) test

2.4.1 Quantity and quality check of isolated DNA

Isolated DNA was checked by separation using standard agarose gel electrophoresis and visualized under UV Trans illuminator. The quantitation of isolated DNA was checked by using Implen Nano photometer P300, taking the absorbance at 230 nm, 260 nm, 280 nm, 320 nm and evaluating the ratios of 260/280 and 260/230 to determine the purity of isolated DNA.

2.4.2 Cycle sequencing of PCR amplified products

Two primer sets, the 5'-3'sequence of forward and reverse primers, for each gene were designed and optimized to amplify each of the *PRM1* and *PRM2* coding exons and flanking intronic sequences. These primers were designed specifically according to the notation given in the reviewed literature [15]. The genes were amplified using TopTaq™ Master Mix QIAGEN kit. It was carried out in Thermal Cycler (ABI, Veriti). The PCR protocol designed for 30 cycles for the primers used is given in Table 1. After the genes were amplified, the amplicon (PCR product) was subjected to 1.2% agarose gel electrophoresis in order to check its quality (Fig. 2). The DNA was quantified using gel analysis software against reference DNA ladder.

2.4.3 Cycle sequencing of purified products

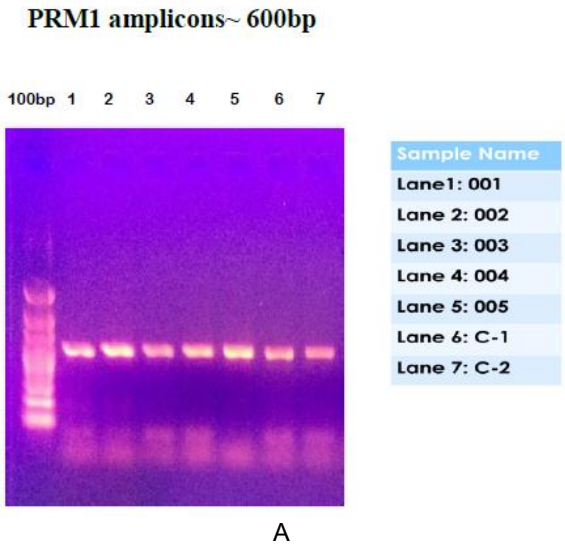
The PCR amplicon was purified with Exosap enzymatic purification in order to remove primer-dimers, unbound primers, etc. as per the manufacturer instruction (ABI). This was followed by reaction in thermal cycler (ABI, Veriti) keeping reaction conditions as shown in Table 2. After the purification, the products were subjected to Sanger sequencing in ABI, 3730XL DNA analyser using BdTv3.1 chemistry. Forward and Reverse DNA sequencing reaction of PCR amplicons of respective samples was carried out with *PRM1* (F/R) and *PRM2* (F/R) primers using BDT v3.1 Cycle sequencing kit and was allowed to undergo reaction in thermal cycler (ABI, veriti) as per the conditions shown in Table 3. After the run, the sequencing product was purified according to the protocol provided in the BDT v3.1 Cycle sequencing kit. The samples were then loaded into the ABI 3730xl Genetic Analyzer and sequence was retrieved.

Table 1. Steps and conditions of thermal cycling for PCR (PRM1 and PRM2)

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	4 min.	1
Denaturation	94°C	30 sec.	30
Annealing	62°C	30 sec.	
Extension	72°C	40 sec.	
Final Extension	72°C	5 min.	1

Table 2. Exosap purification reaction conditions

Stage	Temperature	Time duration
Enzyme activation	37°C	15 minutes
Enzyme inactivation	80°C	15 minutes
Storage	4°C	Infinity



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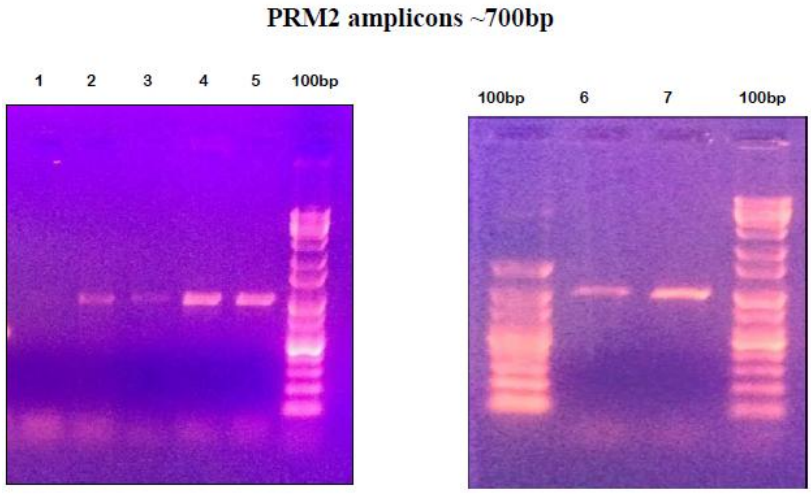

B

Fig. 2. PCR: A prominent band of amplified DNA could be obtained following PCR amplification, a) shows PRM1 amplicon of 600 bp; b) shows PRM2 amplicon of 700 bp

Table 3. Reaction conditions for cycle sequencing

Steps	Temperature	Time	Cycles
Initial denaturation	96°C	1 min.	1
Denaturation	96°C	10 sec.	
Annealing	50°C	0.05 sec.	25
Extension	60°C	4 min.	
Final Extension	04°C	∞	1

2.5 Statistical Analysis

A minimum of six replicates were carried out for staining parameters. Values were expressed as Mean ± S.E. Variation between samples was analyzed taking *P*< .05 level of significance using GraphPad Prism version 6.

3. RESULTS

Preliminary semen analysis was carried out by standard methods [16]. Table 4 represents results of these parameters which were comparable to normal WHO standards and indicate that the sperm counts and motility of the infertile cases were within the normal range.

Table 4. Sperm count and motility in control and infertile cases

Groups	Diagnosis	Sperm count (10^6/ml)	Motility (%)
I	Control (n=25)	76.6 ± 6.3	77.8 ± 9.2
II	Infertile (n=23)	40.0 ±5.4	60.5 ±7.3

3.1 Sperm Toroid Integrity (STI) Assay

The results obtained after staining to evaluate the sperm toroid integrity (STI) from the semen samples of control and infertile males (Groups I and II) are shown in Table 5.

The results obtained after scoring 40 separate non-overlapping fields from each sample indicated that normal control (Group I) samples showed a higher percentage of spermatozoa having intact toroid assembly, with a significantly lower percentage of sperm showing disrupted nuclear chromatin. It was observed that controls showed a high percentage of spermatozoa with normal orientation of toroid. Scoring of the stained slides for sperm toroid assembly in Group II revealed a significant decline in the normal assembly of the sperm toroid as compared to the control. Moreover, these samples portrayed a significantly higher ($P<$ .001) score of susceptible toroid assembly (Table 5; Fig. 3).

3.2 Molecular Analysis

3.2.1 DNA isolation

Genomic DNA from each Control and Patient's samples yielded a ratio of absorbance A260/A280 and A260/A230 within the range 1.7-2.0 as shown in Table 6 which suggested that the isolated DNA was pure without any protein/phenol or RNA contamination and was fit for further analytical purposes. The peak absorbance values at 260nm indicated sufficient DNA yield. The data of 2 Controls(C-1, C-2) and 5 patients (Cases 001-005) have been shown in this paper as representative of the findings.

3.2.2 SNP analysis on gene PRM1 and PRM2

Analysing the sequencing product using the Certified CLC Genomics Workbench software for both the genes, sequence findings of only few representative cases are mentioned here, since the data from all cases is similar with synonymous/no SNPs recorded after analysis.

Table 5. Toroid assembly after STI assay in control and infertile cases

Groups	Diagnosis	Toroid assembly		
		Normal (%)	Susceptible (%)	Disrupted (%)
I	Control (n=25)	56.8±3.7	39.8±2.5	3.35±0.06
II	Infertile (n=23)	37.2±2.9**	58.5±8.3**	4.3±0.07

*Values are Mean ± S.E; **P<.001*

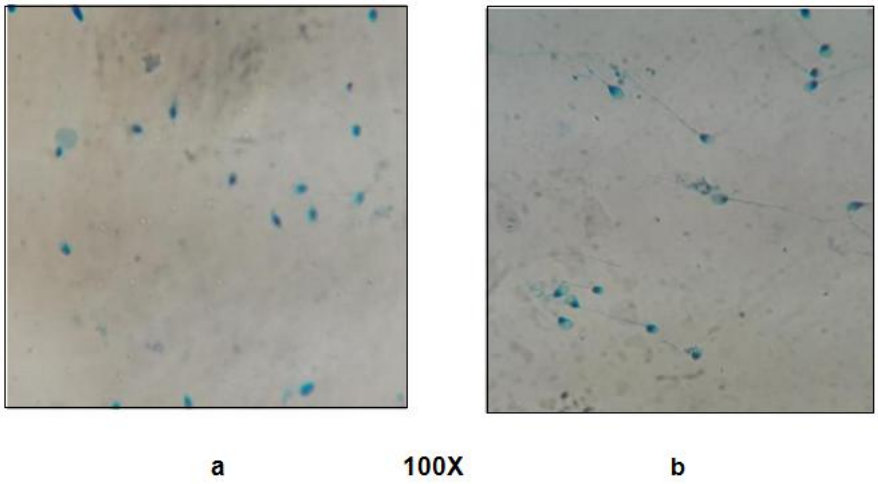

Fig. 3. Sperm toroid integrity test: a) shows increased light stained sperm cells in control depicting normal toroid integrity. b) shows increased intermediately stained sperm cells in infertile cases depicting less alteration in the toroid integrity making DNA prone to damage

Table 6. Absorbance values at different absorbance rates and ratios referring to DNA purity

Groups	Absorbance values				Ratio	Ratio	Inference
	A230	A 260	A280	A320	A260/A280	A260/A230	
I	0.080	0.195	0.106	0.002	1.8	2.4	Pure DNA
II	0.050	0.163	0.088	0.002	1.8	3.3	Pure DNA

A distinct SNP at nucleotide 239 (C>A) (NCBI SNP Cluster ID rs737008) was observed only in forward sequence in gene *PRM1* (ENSG00000175646, NCBI Gene ID 5619). In first control (C-1), a heterozygous A/C peak while in second control (C-2), a clear peak indicating C nucleotide in homozygous condition has been observed. In Cases 001 and 005 of Group II, a clear peak indicating C nucleotide is observed and in Cases 002 and 004, a nucleotide peak in homozygous condition has been observed while in Case 003, a heterozygous A/C peak is seen similar to the first control. However this transition appears to be synonymous and does not affect the amino acid and it codes for arginine itself and the protamine conformation remains unaltered.

Further potential association was established in the 3 common SNPs (rs35576928, rs35262993 and rs11544792) between*PRM1* and male infertility. On analysis, no mutation was observed in our cases.

In gene *PRM2* (ENSG00000122304, NCBI Gene ID 5620), there was no SNPs observed in controls as well as in infertile samples and matched completely with the reference sequence provided in NCBI database. The search included only those SNPs which are already registered in the NCBI database. Hence the analysis does not rule out the presence of new SNPs which may not be registered in the NCBI database.

4. DISCUSSION

Recent reports of the rapid decline in male infertility [1,2] have triggered the need for investigation into the causes for the increased failure of *in vivo* and *in vitro* fertilization. The sperm genome is under duress from a vast array of environmental stressors including environmental toxicants, drugs, addiction, therapy, occupational exposure and infection, and could prove detrimental to both *in-vivo* or *in-vitro* fertilization, culminating in infertility. Apart from an abnormal semen profile, it has been emphasized that abnormal organization of sperm chromatin, poor compaction of its DNA could make the sperm unfit to fertilize the oocyte. In addition, disruption of the toroid assembly would cause the DNA to be increasingly vulnerable to damage, which may be carried forward to the next generation.

Ioannou et al. [17] have pointed out that until recently, little concern has been raised about analysing the genetic quality of sperm prior to ICSI trials or evaluating the impact of genomic aberrations on sperm fertilizing potential and early stage embryonic development. The present investigation therefore holds specific significance in tracking the impact of protamine alteration on sperm nuclear integrity which could have far reaching implications on the establishment and maintenance of a viable pregnancy since the sperm has been recognised to exert subtle epigenetic control over early embryo development.

After specifically staining the spermatozoa, using the STI assay to evaluate the sturdiness of the toroid assembly of sperm DNA, it was found that in 60% of the infertile cases, a significantly higher (P<.001) percentage of susceptible toroids occurred as compared to controls, indicating a loosened protamine DNA binding or greater DNA-Protamine dissociation. Hence, these results point to the fact that the protamines play a key role in keeping the DNA compactly protected and in cases of unexplained infertility where routine diagnostic tests do not reveal any cause, this DNA-Protein binding appeared distorted as compared to that of spermatozoa from samples of normal volunteers.

Analysis of the toroid assemblage [13] has yielded a positive correlation between pregnancy loss, failed implantations and abnormal toroid integrity, which reflects the validity and significance of the method. It is apparent therefore that a sturdy toroid configuration is vital to preserve the sperm DNA and the technique provides an important means for establishing the status of intactness of the nuclear chromatin in this unique cell which reins fertility. In corroboration with our observations, Simon et al. [18] have demonstrated that altered protamines were correlated with decreased sperm fertilizing ability.

The distorted protamine binding observed in the unexplained infertility samples in this study conform to the findings of Carrell et al. [19] who correlated poor toroid assembly with spermatogenic defects. For the complete sperm DNA packaging, nucleoprotamines P1 and P2, encoded by *PRM1* and *PRM2* genes bind firmly to sperm DNA, protecting it from damage. Hence, in the event of any variation such as Single Nucleotide Polymorphisms (SNPs) in these genes i.e. *PRM1* or *PRM2*, amino acid alteration would occur, disrupting the normal protein required for the proper nucleo-protamine structure. Due to an improper conformation, protamines are loosely/unable to bind to DNA and the DNA molecule is vulnerable to damage. Our results (sperm toroid staining) clearly indicated an increase in the percentage of spermatozoa with loosened or partially disrupted protamine-DNA binding in the infertile cases studied, correlated with which single nucleotide polymorphisms in genes *PRM1* and *PRM2* would be expected.

Contrary to our expectation, sequencing of the Protamine gene *PRM1* revealed the presence of a single, apparently synonymous SNP at nucleotide 239 (rs737008) in the *PRM 1* gene in 60% of cases. This would therefore have no bearing on the structure of protein P1, nor would it alter the DNA-Protamine interaction since the variation does not lead to amino acid alteration and results in the characteristic protamine conformation required for normal toroid assembly. Tanaka et al. [9] have also reported the same SNP in *PRM1* gene, which had no impact on the protein expressed. The sequencing data obtained for the gene *PRM 2* also revealed no variations in the gene sequence in the infertile and control cases when compared with the standard protamine reference gene from NCBI database.

In contrast, several researchers have reported SNPs on these nucleoprotein genes that lead to male infertility in different populations. Iguchi et al. [20] found a novel SNP G197T (rs35576928) in *PRM1* gene, which caused amino acid variation from arginine to serine (R34S) in a highly conserved arginine cluster. This SNP creates a new RS sequence, a putative phosphorylation site for the enzyme; SR protein specific kinase 1, known to phosphorylate serines in the RS motifs of *PRM1*.Thus, improper phosphorylation could substantially alter both DNA binding and protamine to protamine interactions in the sperm nucleus. In our study however no such SNP associated alteration was observed. Research by Gazquez et al. [21] revealed a single nucleotide polymorphism -190 C A that could lead to a change in *PRM1* expression resulting in abnormal morphology and infertility. It was also noted by Yang et al. [22] that the $G_{398}C$ polymorphism in PRM2 gene was associated with male infertility in Chinese Han population. Moreover Siasi et al. [12] have elucidated the association of six SNPs in *PRM 1*, *PRM2* and *TNP* genes in Iranian populations.

A meta-analysis carried out by Jiang et al. [23] has confirmed that polymorphisms in *PRM 1* and *PRM 2* could facilitate risk prediction in male infertility and could be appropriate markers for understanding the aetiology of male infertility. They have further asserted that -190C>A (rs2301365) polymorphism is associated with risk for male infertility while rs1646022 polymorphism has a protective effect in Asian populations. This explains the discrepancy in comparing SNPs from population to population.

Moreover, Venkatesh et al. [24] in his study did not observe any nucleotide change with increased risk frequency in infertile cases in the Southern Indian population as compared to control. SNP analysis from samples of this region of Western India also yielded a synonymous SNP; no mutations. Similarly, substantiating our results of relatively few SNPs in the protamine genes, Jodar and Oliva [25] have remarked that the presence of highly penetrant protamine mutations although significant, is a rare occurrence. In a study on the Greek population, Markandona et al. [26] have reported only a single SNP in the MLH3 gene that may be linked to oligozoospermia in Caucasian men.

The outcome of our analysis also indicated no significant variation in the gene or protein sequence in the infertile cases but there was evidence of partial DNA-Protamine dissociation with increased susceptibility of the toroid assembly to disruption.

In contrast,in the normal control samples although no SNPs were detected with no alteration to the protein, the staining indicated that the spermatozoa had a higher percentage of cells with normal compact, intact toroid. This finding suggests that the sperm nuclear chromatin in the infertile cases was possibly inherently labile and despite no nucleotide mutations in the P1 and P2 genes, the toroid assembly was significantly more vulnerable to

any external or internal pressures. A differential sensitivity may therefore exist in the sperm DNA-Protamine assembly of normal and infertile males.

Since most known SNPs were not present in the genes of the studied cases, these SNPs possibly cannot serve as molecular markers for diagnosis of failed fertilization in this population as initially proposed, and hence sequencing should now focus on newer exonic or intronic sites. Identification of such novel SNPs could possibly lead to identification of the new Molecular markers which are now the need of the hour to establish fertility status of a semen sample from the local population for IVF and ART technologies. However, a step in this direction would require further analysis and would make a significant contribution to current infertility strategies, since declining male fertility is now a recognised global threat and requires urgent attention [27].

5. CONCLUSIONS

Our findings after the search for SNPs in the 3' UTR, intron or exon sequences of the genes PRM 1 and PRM 2 uncovers a few remarkable facts:

1. It is known that SNPs in these genes are rare in certain populations as demonstrated by Venkatesh et al. [23] in the Southern population of this country. On the other hand, a higher incidence of SNPs in *PRM1* and *PRM2* have been reported in other populations [10,12,20,22], correlated with infertility. Could there be a population specific differential sensitivity in these genes?
2. The low incidence of polymorphism observed in the *PRM1* and *PRM2* genes in samples of the local population, reflected an unchanged DNA sequence; however the protamine-DNA binding dissociation may be attributed to the impact of environmental pressures or an epigenetic mechanism resulting in altered gene expression. Several authors [24,28] have demonstrated that variations in protamine gene expression resulted in altered proteins and poor sperm function.
3. The current analysis does not rule out the possibility of the presence of novel SNPs which have not yet been registered in the NCBI database. Hence, further sequence alignment is warranted using clustal w software to ascertain mismatch and occurrence of new SNPs which may be unique to the cases under study and may prove to be biomarkers of promise.
4. The key observation of this study was therefore, that while there were no non-synonymous SNPs in both the control and Infertile samples, as per the sequencing data, evidence of a differential susceptibility was remarkable with the chromatin of the infertile sperm nuclei manifesting increased fragility as compared to the control.
5. For the local population, this is possibly the first attempt to identify SNPs in *PRM1* and *PRM2* associated polymorphisms with male infertility and further correlate toroid instability with increased susceptibility of DNA to damage. There could be a differential sensitivity of the genetic material such that spermatozoa in certain individuals may be genetically less susceptible to environmental stressors and hence under adverse influences, do not build up nucleotide changes that could have a detrimental impact.

CONSENT

As per international standard and our university criteria, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per International standard or University standard, written approval of Institutional Ethics Committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Carlsen E, Giwercman A, Keiding N, Skakkebæk NE. Evidence for decreasing quality of semen during past 50 years. Bmj. 1992;305(6854):609-13.
2. Centola GM, Blanchard A, Demick J, Li S, Eisenberg ML. Decline in sperm count and motility in young adult men from 2003 to 2013: Observations from a US sperm bank. Andrology. 2016;4(2):270-6.

3. Barratt CL, Björndahl L, De Jonge CJ, Lamb DJ, Osorio Martini F, McLachlan R, et al. The diagnosis of male infertility: An analysis of the evidence to support the development of global WHO guidance—challenges and future research opportunities. Human Reproduction Update. 2017;1-21.
4. Kumar N, Singh AK. Trends of male factor infertility, an important cause of infertility: A review of literature. Journal of Human Reproductive Sciences. 2015;8(4):191.
5. Barroso G, Valdespin C, Vega E, Kershenovich R, Avila R, Avendaño C, Oehninger S. Developmental sperm contributions: Fertilization and beyond. Fertility and Sterility. 2009;92(3):835-848.
6. Ward WS. Function of sperm chromatin structural elements in fertilization and development. Molecular human reproduction. 2010;16(1):30-36.
7. Singh NP, Muller CH, Berger RE. Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertility and Sterility. 2003;80(6):1420-1430.
8. Zini A, Sigman M. Are tests of sperm DNA damage clinically useful? Pros andcons. Journal of Andrology. 2009;30(3):219-229.
9. Tanaka H, Miyagawa Y, Tsujimura A, Matsumiya K, Okuyama A, Nishimune Y. Single nucleotide polymorphismsin theprotamine-1and-2genes of fertile and infertile human male populations. Molecular Human Reproduction. 2003; 9(2):69-73.
10. He XJ, Ruan J, Du WD, Chen G, Zhou Y, Xu S, et al. *PRM1* variant rs35576928 (Arg>Ser) is associated with defective spermatogenesis in the Chinese Han population. Reproductive Biomedicine Online. 2012;25(6):627-634.
11. Tüttelmann F, Křenková P, Römer S, Nestorovic AR, Ljujic M, Štambergová A, et al. A common haplotype of protamine 1 and 2 genes is associated with higher sperm counts. International Journal of Andrology. 2010;33(1):240-e248.
12. Siasi E, Aleyasin A, Mowla J, Sahebkashaf H. Association study of six SNPs in *PRM1*, *PRM2* and TNP2 genesin Iranian infertile men with idiopathic azoospermia. Iranian Journal of Reproductive Medicine. 2012;10(4):329.
13. Chan PJ, Orzylowska EM, Corselli JU, Jacobson JD, Wei AK. A simple sperm DNA toroid integrity test and risk of miscarriage. BioMed Research International; 2015.
14. John SWM, Weitzner G, Rozen Rand Scriver CR. A rapid procedure for extracting genomic DNA from leukocytes. Nucleic Acids Research.1991;19(2):408.
15. Aoki VW, Christensen GL, Atkins JF, Carrell DT. Identification of novel polymorphisms in the nuclear protein genes and their relationship with human sperm protamine deficiency and severe male infertility. Fertility and Sterility. 2006;86(5):1416-1422.
16. Edition, Fifth. Examination and processing of human semen. World Health Edition, F (10). 2010;286.
17. Ioannou D, Miller D, Griffin DK, Tempest HG. Impact of sperm DNA chromatin in the clinic. Journal of assisted reproduction and genetics. 2016;33(2):157-166.
18. Simon L, Castillo J, Oliva R, Lewis SE. Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. Reproductive Biomedicine Online. 2011;23(6):724-734.
19. Carrell DT, Emery BR, Hammoud S. Altered protamine expression and diminished spermatogenesis: What is the link? Human Reproduction Update. 2007;13(3):313-327.
20. Iguchi N, Yang S, Lamb DJ, Hecht NB. An SNP in protamine 1: A possible genetic cause of male infertility? Journal of Medical Genetics. 2006;43(4):382-384.
21. Gázquez C, Oriola J, Mateo S, Vidal-Taboada JM, Ballescà JL, Oliva R. A common protamine 1 promoter polymorphism (− 190 C→ A) correlates with abnormal sperm morphology and increased protamine P1/P2 ratio in infertile patients. Journal of Andrology. 2008;29(5):540-548.
22. Yang X, Liu F, Li J, Song Y, Tan Y. Association study of protamine 2 (PRM2) gene polymorphism with male infertility in Chinese Han population. Genes & Genomics. 2016;38(3):311-7.
23. Jiang W, Sun H, Zhang J, Zhou Q, Wu Q, Li T, et al. Polymorphisms in Protamine 1 and Protamine 2 predict the risk of male infertility: A meta-analysis. Scientific Reports. 2015;5:15300.
24. Venkatesh S, Singh A, Shamsi MB, Thilagavathi J, Kumar RK, Mitra D, et al. Clinical significance of sperm DNA damage threshold value in the assessment

of male infertility. Reproductive Sciences. 2011;18(10):1005-1013.
25. Jodar M, Oliva R. Protamine alterations in human spermatozoa in genetic damage in human spermatozoa. Springer New York. 2014;83-102.
26. Markandona O, Dafopoulos K, Anifandis G, Messini CI, Dimitraki M, Tsezou A, et al. Single-nucleotide polymorphism rs 175080 in the MLH3 gene and its relation to male infertility. Journal of Assisted Reproduction and Genetics. 2015;32(12):1795-9.
27. Levine H, Jørgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, et al. Temporal trends in sperm count: A systematic review and meta-regression analysis. Human Reproduction Update. 2017;1-4.
28. Zalata AA, Mokhtar N, Atwa A, Khaled M, Shaker OG. The role of protamine 2 gene expression and caspase9 activity in male infertility. The Journal of Urology. 2016;195(3):796-800.

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