Invited Article

Impact of sperm protamine on semen quality and fertility

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ABSTRACT

Protamines are the nuclear proteins essential for chromatin compaction during spermatogenesis. During chromatin compaction, histones are replaced by transition proteins, which are then replaced by protamines. This process is essential for DNA stability. Protamines are rapidly evolved proteins with high evolutionary variation and encompass positively charged amino acids, especially 48% of arginine. Cysteines present in their sequence allow the formation of disulfide bonds between adjacent protamine molecules. Protamine 1 (PRM1), Protamine 2 (PRM2), and Protamine 3 (PRM3) are reported in mammals. Among these, PRM1 and PRM2 were extensively studied. The normal PRM1 and PRM2 ratios in men, stallions, and mice are 1:1, 3:1, and 1:2, respectively. However, in infertile males, the PRM1: PRM2 ratio is altered due to decreased PRM2 expression, which, in turn, is due to incomplete PRM2 precursor processing and zinc deficiency. In bull, ram, and buck, PRM2 mRNA is present but not PRM2 protein. In mice, rats, bulls, and men, the protamine cluster contains an open reading frame called protamine 3 (gene-4 or protamine-3). The proportion of protamine deficient sperm in the sample is indicative of problems in protamination. Recently, *omics* technologies, RT-qPCR, and gene knockout-based studies also reported the presence of protamine in sperm. All these semen quality and knockout studies envisage that protamines are indispensable for fertility. Henceforth, protamine-like biomolecules also may be evaluated for fertility prediction or markers in addition to the existing structural and functional attributes of sperm.

Keywords: Protamines, Chromatin compaction, Chromomycin A3, Infertility, Histone

INTRODUCTION

Commonly, the semen quality was evaluated merely by assessing sperm phenotypic characteristics and sperm function. However, nowadays, many techniques have emerged to evaluate the molecular details of the sperm. The sperm proteins, especially nuclear proteins like protamine, were studied in the near past concerning male fertility and semen quality. Friedrich Miescher was the first to report protamine in the semen of salmon fish in 1874.[1] Protamine sulfate neutralizes the anticlotting activity of heparin; therefore, it was used as an antidote for heparin toxicity.[2] It was used in gene therapy because it condenses plasmid Deoxyribonucleic acid (DNA). In addition, it can be used to prevent obesity as it deters low-density lipoprotein. Commonly, protamine is extracted from milt (i.e., fish semen) and used along with insulin to increase the half-life of isophane insulin to treat diabetes. Protamine is also used as a carrier for DNA or Ribonucleic acid (RNA) in drug delivery system in nanopharmaceuticals. The protamine-based RNA vaccines were developed for cancer treatment and also against infectious diseases.[3] On the other hand, from semen biology point of view, protamine is essential during

spermatogenesis for chromatin compaction. Protamines are rapidly evolved proteins with a high evolutionary variation. Protamine contains positively charged amino acids, especially rich in arginine (48%). Cysteines present in their sequence allow the formation of disulfide bonds between adjacent protamine molecules.[4,5] Protamine 1 (PRM1), Protamine 2 (PRM2), and Protamine 3 (PRM3) are reported in mammals.[6] Among these, PRM1 and PRM2 have been studied extensively. In bull, ram, and buck spermatozoa, PRM2 mRNA is present but not PRM2 protein. In mice, rats, bulls, and men, the protamine cluster contains an open reading frame called PRM3 (gene-4 or protamine-3). PRM3 is misleading, as it lacks arginine clusters instead of being rich in glutamic acid. Gene4/PRM3 will not bind to DNA and should not be called protamine.[4,7]

The elongating spermatids have soluble polyribosomes in their cytoplasm that synthesizes the protamines and they can pack the genome of sperm. In primates, 10–15% of the sperm genome is packed with histones.[8] The γ-globin, ε-globin, and telomeric DNA were identified as packed in the mature sperm by histones.[9] Protamines begin to appear in elongating spermatids, during the final process of chromatin condensation. In specific stages of spermatids, the transcription and translation of protamines occur. However, the mRNA of protamines was not reported in Leydig or Sertoli cells. Few studies reported the existence of protamines in the fetal brain, placenta, and kidney, but it is not sure whether this protamine expression is due to artifact or biological expression.[10]

The PRM1 is located in the major groove with every DNA helix turn having one PRM1 molecule.[11] PRM2 is larger than PRM1 and shares around 60% sequence similarity with PRM1. PRM1 and PRM2 can bind up to 11 and 15 base pairs of DNA, respectively.[12] The sperm of horse, tapir, zebra, elephant, hare, and rabbit encompasses two variants of PRM2 (i.e., precursor and processed form). Two additional PRM2 variants were reported in stallion – one containing 62 amino acids (St2a) and the other containing 58 amino acids (St2b).[13] However, the PRM2 content was never reported to be more than 80%. Some species expressed only PRM1, but no species expresses only PRM2. This indicates that PRM2 alone cannot condense the DNA properly. As like two molecules of each H2A, H2B, H3, and H4 create histone octamers to form nucleosomes along with 147 bp of DNA, the PRM1 and PRM2 also interrelate among them and attach to DNA. Protamines can make the molecules of DNA pack together as it counteracts the negative charge of phosphodiester of DNA. The continuation of this process during epididymal transport of spermatozoa can form a disulfide bridge between PRM1 and PRM2 as well as between two molecules of PRM1 that can culminate in condensation of sperm nucleus up to 20 times more than that of the nucleus of somatic cell.[14] This chromatin hypercondensation contributes to the shape of the

sperm head. The cleaved PRM2 rather than mature PRM2 was reported to be associated with elongated sperm head in rodents. The arginine content of PRM1 has a role in head shape as it was negatively related to the shape of sperm head in eutherian mammals.[4,10]

FUNCTIONS OF PROTAMINES

The major functions of the PRMs can be enlisted as follows:
1. These PRMs aid in maintaining the compact and hydrodynamic nucleus with a condensed genome; this is vital because sperm with a hydrodynamic nucleus moves faster
2. Protects paternal genetic information from free radicals, nucleases, and other mutagens
3. Removes transcription factors and other proteins, resulting in a blank paternal genetic message, devoid of epigenetic information, therefore allowing its reprogramming by the oocyte
4. Imprinting of the paternal genome during spermatogenesis
5. Confer an epigenetic mark on some regions of the sperm genome, affecting its reactivation on fertilization
6. Activation of oocyte creatine kinase II.[6,10]

ROLE OF PROTAMINE IN CHROMATIN COMPACTION

Spermatogenesis involves numerous intricate processes. One of those is chromatin compaction, which happens during spermiogenesis. The process of chromatin compaction is depicted in [Figure 1]. During this process, histones are replaced by transition proteins, which are later replaced by protamines. This process is essential for DNA stability.[10,15] Protamines are the major nuclear proteins in the male germ cells of many species. During spermatogenesis, nucleohistones are replaced by nucleoprotamines in the following steps:
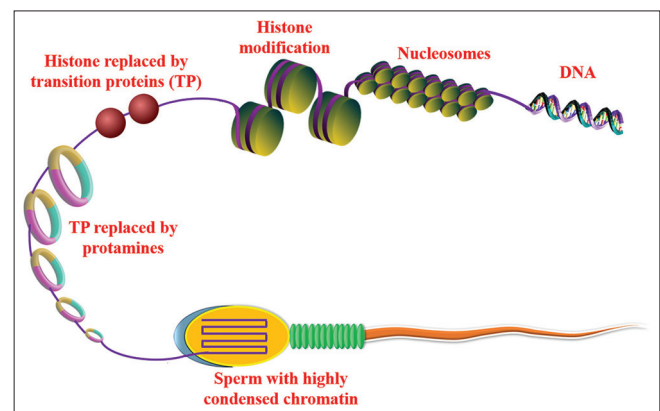

Figure 1: Histone to protamine transition and chromatin compaction.

1. Testis-specific histone variants (TH2B) are incorporated into the sperm DNA
2. Hyperacetylation reduces the DNA binding affinity of histones and results in DNA relaxation through topoisomerase activation
3. Replacement of histones with transition proteins 1 and 2 (TNP1 and TNP2)
4. TNPs are then replaced by protamines to form compact chromatin.[10,14]

The histone acetylation (mainly H4 acetylation), phosphorylation, and ubiquitination are responsible for the histone displacement. The chaperons are also required for chromatin remodeling as they displace the histones that were modified post-translationally. The neutralization of the basic proteins, i.e., transition proteins and protamine is vital for chromatin compaction that is achieved by phosphorylation of these proteins.[16]

One or more phosphorylation sites were typically found on the amino-terminal peptide sequence flanking the DNA binding domain and the DNA binding domain of PRM1 in humans, bulls, and stallions. These sites in PRM1 are phosphorylated instantly after its synthesis and also during the entrance of sperm into the oocyte. The major phosphorylation sites are threonine, serine, and tyrosine.[10,17] Another post-translational modification in protamine is the formation of disulfide bonds during the final stages of sperm maturation and epididymal transport, but these disulfide bonds are reduced when sperm enters the oocyte.[14]

METHODS TO EVALUATE CHROMATIN CONDENSATION/PROTAMINE DEFICIENCY IN SPERMATOZOA

To detect the presence and quantify the protamine, currently, the following methods are being adopted:
1. Toluidine blue staining
2. Feulgen reaction
3. Aniline blue staining
4. Real-time PCR (qPCR)
5. Transcriptomics (Microarray or next-generation sequencing)
6. Western blotting
7. Proteomics
8. Chromomycin A3 staining
9. Sperm Chromatin Structure Assay (SCSA).

The toluidine blue staining is performed usually after acid treatment, which stains normal spermatozoa with different shades of green while the sperm with abnormal chromatin stain dark blue to violet.[18] The Schiff's reagent is used in Feulgen staining, the normal sperm stain uniform magenta to deep purple color, whereas the sperm with finely granular, coarse clumped, total clearing, and apical clearing appearance are considered to have chromatin alteration.[19] The aniline blue stains sperm with immature nuclei, whereas the sperm that does not stain, is considered mature sperm with well-condensed chromatin.[20] These three methods are indirectly indicating protamine deficiency as abnormal chromatin condensation is usually due to problems in protamination. The qPCR and transcriptomics can be used to estimate protamine mRNA, whereas western blotting and proteomics are for protamine protein estimation, but these techniques are expensive, time-consuming, and require more skill. Therefore, among all these techniques, CMA3 and SCSA are commonly used as they are not consuming much time. CMA3 is a fluorophore that competes with the binding sites of protamines in DNA.[21] The CMA3 binding in sperm indicates protamine deficiency. The sperm stained with CMA3 (protamine deficient sperm) glows with brighter yellow fluorescence, compared to the remaining sperm. The fluorescent microscopic image of CMA3 staining is shown in [Figure 2]. The presence of a higher proportion of CMA3-stained sperm is indicative of a higher proportion of immature sperm in the given ejaculate that can hamper fertility. Protamine reversibly binds with G-C base pairs in the minor groove of the DNA strand. It needs the presence of Mg^{2+} to bind to the DNA, so the samples are usually treated with a CMA3 solution containing $MgCl_2$ (McIlvaine's buffer).[22] In SCSA, and Acridine Orange (AO) stain is used to detect damaged DNA by staining intact DNA (dsDNA) as green and denatured (ssDNA) as red. However, SCSA can also be used for detecting protamine deficiency by assessing high DNA stainability (HDS%).[23]

ROLE OF PROTAMINE IN SEMEN QUALITY

The protamine content in the fertile patients was equal to protamine in an infertile patient with normal seminal parameters. However, protamine in the fertile patient is not equal to protamine in an infertile patient with abnormal
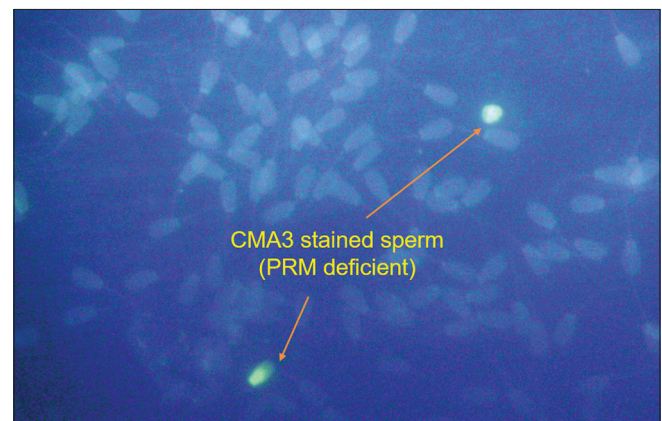

Figure 2: Fluorescent microscopic representation of chromomycin A3 staining.

seminal parameters.[24] Sperm with a round head contains more histone and less protamine.[25] The primary and secondary sperm defects positively correlated with chromatin alteration.[19] Protamine levels correlated with viability and DNA integrity.[26]

Protamine ratio

Normal human sperm encompasses PRM1 and PRM2 in equal quantities, but in infertile patients, they are present in altered amounts. Therefore, infertile patients have aberrant PRM1 and PRM2 ratios.[27,70-73] The proportion of PRM1 and PRM2 changes between different species due to variations in PRM2 content. The normal PRM1 and PRM2 ratios in men, stallions, and mice were 1:1, 3:1, and 1:2, respectively. Altered PRM1 and PRM2 ratios indicate DNA fragmentation and infertility. The protamine ratio in fertile and infertile conditions reported in previous studies is depicted in [Table 1]. The altered ratio is due to decreased PRM2 expression in infertile patients, which is due to incomplete PRM2 precursor processing and Zinc deficiency. However, the PRM3:PRM1 ratio remained unaltered in infertile patients.[28,29] An increased proportion of H2B to protamine was reported in infertile men.[30] Khara *et al.*, in 1997,[31] reported a negative correlation (-0.6119) between PRM1/PRM2 ratio and the IVF rate.

Relation of sperm protamine with semen quality and male fertility

Various authors have correlated the deficiency in protamines with fertility [Figure 3]. Protamine deficient (chromomycin positive) spermatozoa of buffalo bull (-0.37[32]) have a negative correlation with fertility. Moreover, a higher proportion of protamine deficient sperm was seen in medium and low fertile bulls. Protamine-deficient sperm of human semen ($-0.641--0.40$[33-36]) was reported to have a negative correlation with fertility. Likewise, spermatozoa having excessive histones are also negatively correlated (Bull = -0.9[37]) with fertility. Dogan *et al.* (2015)[38] evaluated the PRM1 content by western blotting, which has a correlation of 0.254 with fertility [Table 2]. These studies collectively indicate that sperm with deficient protamine or excessive histones has less fertilizing potential. In addition, sperm protamine concentrations were negatively correlated with DNA fragmentation.[26] This indicates the role of protamines in protecting sperm DNA from damage. Therefore, sperm with protamine deficiency is prone to DNA damage and subsequent reduction in fertility. Cutoff values of HDS% detected from SCSA range from 10% to 15% in humans and 3.5% in bulls [Table 3].[74-79] If it increases more than these values, it could compromise fertility. Therefore, SCSA will be the best tool for simultaneous assessment of intactness of both DNA and nuclear protein in the chromatin.

Table 1: Protamine ratio in relation to fertility (Mean±SE).

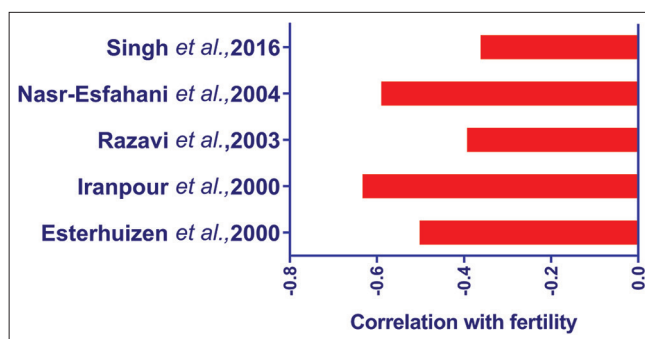| Fertile men	Infertile men
0.98±0.12	1.58±0.24[70]
0.99±0.06	1.50±0.05[71]
1.10±0.08	3.00±2.84[72]
0.55 to 1.29 range	Outside 0.55–1.29 range[31]
1.01±0.15	1.51±0.48 (Oligozoospermia)[73]
	1.23±0.65 (Asthenozoospermia)[73]
1.06±0.01	<0.8 (13.6%)[15]
	0.8–1.2 (46.7%)[15]
	>1.2 (39.7%)[15]
1.00±0.01	<0.8 (61 patients)[26]
	0.8–1.2 (71 patients)[26]
	>1.2 (34 patients)[26]

Figure 3: Proportion of protamine deficient (CMA3 stained) sperm assessed by different authors and its correlation with fertility.

PRM1 is the most abundant transcript present in the bovine spermatozoa as per the RNA sequencing results of two studies.[39,40] Protamine mRNA is reported in several species such as human,[41-43] bovine,[32,39,44,45] equine,[46] and porcine[47,48] using various techniques such as RNA-*Seq*,[32,39,40,46] microarray,[44] and RT-PCR.[39,48] PRM1 is a nuclear protein formed during the condensation of sperm chromatin[4,49] and involved in the process of cell differentiation, chromosome condensation, multicellular organism development, and spermatogenesis. It also has functions such as packaging and stabilizing sperm DNA[44] and the formation of sperm chromatin structure.[50] PRM1 is reported to be abundant in spermatozoa and delivered to the oocyte.[51]

PRM1 is indicative of sperm fertility and is studied based on different fertility indices [Table 4].[80-83] Its concentration was found to be higher in high fertile bulls than in low fertile bulls ($P < 0.05$) by microarray and RT-PCR analysis.[44] Similarly, bulls in a good quality semen-producing group have shown a higher level of PRM1 expression compared to the poor quality semen-producing group ($P < 0.05$).[52] In boar, PRM1 was significantly ($P < 0.05$) found to be higher in the high embryo cleavage group semen samples ($n = 10$) than in the

Table 2: Correlation between protamine and fertility.

Parameters	Correlation with fertility	Fertility calculated as	Species
Chromatin maturity (Reduced protamination) Toluidine blue%	−0.61 (*P*<0.001)	SD of CR	Bull[38]
PRM1 content (Western blotting)	0.254 (*P*=0.050)	SD of CR	Bull[38]
PRM2/PRM1 mRNA ratio (qPCR)	0.274 (*P*<0.05)	28NRR	Stallion[28]
PRM1/PRM2 ratio (Protamine1/Protamine2)	−0.6119 (*P*<0.05)	FR	Human[31]
Chromomycin (CMA$_3$) (Protamine deficiency)	−0.37 (*P*=0.056)	CR	Buffalo bull[32]
	−0.641 (*P*<0.001)	FR	Human[33]
	−0.401 (*P*=0.002)	FO-ICSI	Human[35]
	−0.598 (*P*=0.000)	FO-ICSI	Human[36]
	−0.51 (*P*=0.0001)	FR	Human[34]
Aniline blue (Excess Histones)	0.186 (*P*=0.174)	FO-ICSI	Human[35]
	−0.90 (*P*<0.001)	SD of CR	Bull[37]

Table 3: Cut off values of HDS % (High DNA stainability).

HDS%	Fertility calculated as	Species
15	PL after IVF ICSI	Human[74]
15	CP PL after IVF	Human[75]
15	BP CP IR after IVF ICSI	Human[76]
10	BP CP after IVF ICSI	Human[77]
15	CP after IVF ICSI	Human[78]
3.5	Pregnancy rate 90 d after AI	Bull[79]

BP: Biochemical pregnancy, CP: Clinical pregnancy, PL: Pregnancy loss, SCD: Sperm chromatin dispersion test, ICSI: Intracytoplasmic sperm injection, HDS: High DNA stainability

Table 4: Abundance of Protamine mRNA and protein in relation to fertility.

Protamine	Inference	Species
PRM1 mRNA	Low in low fertile	Bull[44]
PRM1 mRNA	High in good motile	Bull[52]
PRM2 mRNA	Not differ based on motility	
PRM1 mRNA	High in low motile sperm	Men[80]
PRM1, PRM2 mRNA	Upregulated in bulls with poor motility	Bull[81]
PRM1, PRM2 protein	Low in DFI	Men[15]
PRM1, PRM2 mRNA	P1, P2 mRNA retention in infertile	Men[15]
PRM1, PRM2 protein	Vary in every individual cell, correlate with viability and DNA integrity	Men[26]
PRM1, PRM2 mRNA	Decreased in asthenozoospermia	Men[42,82]
PRM2 mRNA	Not differ between normal and abnormal spermatogenesis	Men[83]
PRM1 mRNA	High in high embryo cleavage group	Boar[53]
PRM1 mRNA	No difference in fresh and frozen epididymal sperm	Mithun[55]

low embryo cleavage group semen samples (*n* = 10) by RT-PCR.[53] On validating the 27 transcripts observed in RNA-*Seq* on nine bulls with conception rate scores of −6.7 to 3.6, PRM1 was found to be the only transcript present in all the sires by RT-PCR.[39] Furthermore, on validation of spermatozoal transcripts, PRM1 showed significant (*P* < 0.05) relation to good quality semen and poor-quality semen of crossbred bulls by RT-PCR.[54] It is said that during cryopreservation, the PRM1 will be affected invariably; however, Mondal *et al.* (2013)[55] reported that there is no difference in the relative expression of PRM1 between fresh and frozen-thawed epididymal semen from *Bos Frontalis* by RT-PCR. Using RT-PCR, a down-regulated expression of PRM1 was observed in the asthenozoospermic patients.[42] The sperm transcript of the PRM1 gene is present in all livestock species, and its abundance ratio is high irrespective of the semen condition (fresh and frozen) and techniques (RNA-Seq and microarray analysis). It is observed as a full-length coverage transcript having the possibility of protein-coding, and thus, PRM1 could be used as a non-invasive tool for fertility markers.

Effect of protamine knockout

Disruption of the coding sequence of one allele in either PRM1 or PRM2 in the embryonic stem cell by homologous recombination and the injection of those embryonic stem cells into blastocyst produced the chimeric mice, which produced the sperm with disturbed PRM1 or PRM2 allele. These sperm had an abnormal nuclear formation, problems in protamine-2 processing, and abnormal sperm function. This shows that both the protamine is indispensable for male fertility and the haploinsufficiency of either PRM1 or PRM2 has resulted in male infertility in mice.[56] Takeda *et al.*[57] generated the PRM1 lacking female chimeric mice having the oocytes lacking PRM1. These mice produced the male PRM1 (+, −) offspring. The sperm from these male

mice were used for *in vitro* fertilization to produce viable healthy offspring. This study revealed that the sperm from PRM1 (+,−) males can produce healthy offspring, despite having abnormal sperm with high DNA fragmentation, decreased mitochondrial membrane potential, and slight irrationality in expression profile. Schneider *et al.*[58] used the CRISPR/Cas9 for gene editing in oocytes to produce PRM2 deficient mice. Interestingly, the sperm of heterozygous male mice was having normal motility and head morphology. However, problems in chromatin condensation were noticed in the sperm lacking PRM2 due to the perturbed histone to protamine transition. In addition, these sperm had problems in acrosome formation and membrane abnormalities. This study indicated that in PRM2 deficient mice, heterozygous males were fertile and homozygous males were sterile. TNP1 and TNP2 knockout mice spermatids showed the normal shape of the nucleus, translation suppression, histone disappearance, and protamine deposition but irregular chromatin condensation, PRM2 processing, and DNA breaks. However, the integrity of the genome was preserved in mature spermatids as the intracytoplasmic sperm injection resulted in successful fertilization and produced offspring. Nevertheless, the retention of spermatids in the testis was observed, which resulted in the abnormal and reduced number of sperm in epididymis.[59]

PROTAMINE AND ASSISTED REPRODUCTIVE TECHNOLOGIES (ART) OUTCOME

The interventions in reproduction through ART are emerging continuously. Intracytoplasmic sperm injection (ICSI) is an ART, in which a single spermatozoon is inserted into the oocyte for fertilization. The fertilization capacity was higher in both ICSI and IVF when the sperm with normal levels of protamine mRNA was used.[60] The chromatin maturity measured by Toluidine blue and CMA3 staining was deranged in the patients with poor ART outcomes in terms of fertilization rate.[61] The rate of fertilization and the embryo quality was less in the sperm with high protamination.[62] The histone to protamine ratio (HPR) was negatively correlated with the blastocyst formation rate and delivery rate regardless of the type of ART used.[63] The histone retention and protamine ratio were significantly associated with blastocyst quality after ART.[64]

Mutations in protamine gene and male fertility

The screening for mutation in PRM1 revealed the c.102G > T transversion and c.119G > A, p. Cys40Tyr novel missense mutation in oligospermic individuals. Variants such as c.−107G > C in −15 bp from the transcription initiation site and a c.*51G > C in the 3′UTR of PRM1 were detected in the PRM1 of oligospermic individuals.[65] A total of five SNPs were identified in the Swedish population and seven SNPs were identified in the Spanish population in PRM1 gene. A total of 15 variants were reported in the PRM2 of the Spanish population.[66] The c.65G > A mutation in PRM1 gene and −67C > T mutation in PRM2 promotor region was reported to be associated with spermatogenic failure.[67] The SNP in the PRM2 gene that terminates the translation can cause haploinsufficiency of PRM2 and hence male infertility.[68] A heterozygous SNP in PRM1 that can impair the highly conserved arginine clusters vital for normal DNA binding was also reported in the infertile male patients.[69] All these reports are indicating that the protamine gene without any mutation is essential for male fertility.

CONCLUSION

Protamine is the highly abundant transcript and protein found in spermatozoa, as reported by many studies. Many semen quality-related studies and knockout studies showed that protamine is indispensable for fertility. However, many studies are conducted on humans only. Therefore, the role of protamine, its correlation with fertility, and its cutoff value in domestic animals need to be elucidated by studies in the future. Like protamine, other biomolecules associated with fertility also may be ascertained in the future. In addition to the structural and functional attributes of sperm, protamine-like biomolecules also can be evaluated for fertility prediction and may be used as potential markers for fertility.

Authors' contributions

KE, AK, and TRT conceptualized the review. All authors were involved in the literature review and development of the manuscript and approved the manuscript for publication.

Declaration of patient consent

Patient's consent not required as there are no patients in this study.

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Conflicts of interest

There are no conflicts of interest.

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