



PROTAMINE GENES POLYMORPHISMS AND THEIR RELATIONSHIP WITH THE INCIDENCE OF OLIGOZOOSPERMIA AND ASTHENOZOOSPERMIA IN IRAQI PATIENTS

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Abstract

Single nucleotide polymorphisms are considered as one of causes of male infertility. The replacement of histones with protamines led to proper sperm chromatin packaging which associated with male fertility. The defects of protamine genes have been reported to cause sperm DNA damage and male infertility. In this study, the relationship between protamines genes SNPs that include PRM1 (C321A) and PRM2 (C248T) were studied in 30 oligozoospermic, 30 asthenozoospermic patients and 30 apparently healthy subjects. Analysis of these SNPs was performed using restriction fragment length polymorphism (PCR-RFLP) and PCR sequencing. As related with PRM1 (C321A), CA genotype frequency was significantly ($p < 0.05$) higher in severe oligozoospermia group than apparently healthy control group, higher in control group than in asthenozoospermia group and higher in severe oligozoospermia group than in asthenozoospermia group. In contrast, AA genotype frequency was significantly ($p < 0.05$) higher in control group than severe oligozoospermia group and in asthenozoospermia group than in severe oligozoospermia group. As related with PRM2 (C248T) only CT genotypes (100%) were found in all samples studied. In PRM1 gene, CA genotype of C321A SNP was associated with severe oligozoospermia rather than asthenozoospermia whereas there is no association between C248T SNP of PRM2 gene and the incidence of both oligozoospermia and asthenozoospermia in Iraqi patients.

Key words: polymorphisms, oligozoospermia, asthenozoospermia.

Introduction

Infertility is a major health problem worldwide, affecting at least one in every eight couples and affecting people both medically and psychosocially (Fisher and Hammarberg, 2012). Five to 10% of normal fertile couples take more than a year or 2 to conceive. Some couples, therefore present with a delay in conceiving purely by chance, having normal low fertility rather than subfertility. Many of these supposedly “infertile” couples will eventually conceive, even without treatment. About 6% of men between the ages of 15 and 50 years are infertile. According to the World Health Organization (WHO), 60-80 million couples suffer from infertility worldwide (Rutstein *et al.*, 2004). A male partner factor contributes to 40% of cases of infertility (Alam, 2009).

In Iraq Infertility is a problem among men and women which considered as an important public health and clinical problem in Iraqis. In a study conducted in

Iraq, Razzak and Wais, (2002) reported incidence of primary infertility as 77.2% and secondary infertility as 22.8% these percentages give a strong indication of infertility among Iraqi couples. Oligozoospermia is low sperm count which mean the men who have Sperm concentration of less than 20 million/ml is classified as oligozoospermic (WHO Laboratory Manual, 1999), is a cause of human male infertility. Asthenozoospermia, a disorder of sperm motility, is a cause of human male infertility and is implicated in 19% of infertile cases (Curi *et al.*, 2003).

The methods for evaluation of male infertility have typically been limited to a semen analysis measuring count, motility and morphology of the sperm. Up to 8% of infertile men have been shown to have high levels of sperm DNA fragmentation despite a normal semen analysis (Sakkas *et al.*, 2010). Since sperm DNA has little capacity for repair, protection against damage is

particularly important (Simon *et al.*, 2011). Simon *et al.*, (2011) indicated that sperm DNA is subjected to major restructuring to achieve a tighter packaging at the final stage of sperm differentiation (spermiogenesis). This is performed via replacement of approximately 85% of histones by the more basic protamines (Barone *et al.*, 1994). Sperm DNA fragmentation is an indicator of male infertility as demonstrated by several studies (Agarwal *et al.*, 2009; Barratt *et al.*, 2010; Lewis and Aitken, 2005; Lewis *et al.*, 2008; Sakkas *et al.*, 2002; Zini and Sigman, 2009).

During spermatogenesis, spermatogonia differentiate into spermatocytes then spermatocyte undergo meiosis producing four spermatids then spermatid develop into sperm and in this time the acrosome, tail and highly condensed nucleus are produced. In sperm nuclei the DNA-protamine complex compacts, stabilizes and protects the haploid genome.

Protamines are basic proteins and all mammals have protamine 1 (PRM1) whereas some species including human have protamine 2 (PRM2) (Naoko *et al.*, 2005). Both PRM1 and PRM2 are rich in amino acids, required for DNA-binding and disulfide bridge formation, arginine and cysteine (Hecht, 1989; Oliva and Dixon, 1991). These genes are located within a 25-kb fragment of chromosome 16p13.3 (Engel *et al.*, 1992). In mice, Cho *et al.*, (2001) and Cho *et al.*, (2003) found that the disruption of either the PRM1 or PRM2 gene leads to haplo insufficiency, abnormal chromatin compaction, sperm DNA damage and male infertility due to the important roles the protamines play in spermatid differentiation, aberrations in protamine expression or changes in protein structure. Three major functions of protamines have been postulated: (i) condensation of the sperm nucleus (Balhorn, 1982); (ii) protection of the paternal genome from nucleases (Sotolongo *et al.*, 2003) and free radicals (Alvarez *et al.*, 2002) and (iii) imprinting of the paternal genome (Oliva, 2006; Balhorn, 2007).

During spermiogenesis, transition proteins (TNP) and protamines (PRM) are needed for spermatid chromatin compaction (Sassone-Corsi, 2002). During this process, somatic histones are replaced with the transition proteins, TNP1 and TNP2. Then, in elongating spermatids, the protamine proteins, PRM1 and PRM2, replace TNP1 and TNP2 (Ravel *et al.*, 2007).

There is a considerable interest in the impact of mutations or variants in the protamine genes on male fertility. Al-Ramahi *et al.*, (2012) indicated that the splice site mutation might generate a total elimination of one exon generating frame reading mutation which produce a truncated protein. Lee *et al.*, (1995) observed that premature translation of the PRM1 gene in mice caused

precocious nuclear condensation leading to the arrest of spermatid differentiation. In addition, a number of reports have noted abnormal PRM1/PRM2 ratios in the sperm of infertile human males, suggesting that the relative amounts of each protamine is important for proper spermatid differentiation (Steger *et al.*, 2003; Aoki *et al.*, 2005, 2006; Carrell *et al.*, 2007).

The aims of this study was to identification of PRM1 and PRM2 genes polymorphism and compare the frequency of genotypes and alleles in these genes among oligozoospermic, asthenozoospermic patients and control subjects.

Materials and Methods

Subjects and sampling

Patients (30 oligozoospermic and 30 asthenozoospermic) and 30 apparently healthy subjects (Control) were recruited from Kamal Al-Samraee hospital, Baghdad, Iraq. All infertile patients in this study were selected on the basis of clinical and laboratory examination. Blood samples were collected in EDTA tubes from all infertile and fertile males in this study. The laboratory study was conducted in Genetic Engineering and Biotechnology Institute (GEBI), university of Baghdad, Iraq during a period from 15 November, 2014 to 1 May, 2015.

Analysis of genes

Genomic DNA was extracted from whole blood of infertile and fertile males using Wizard genomic DNA purification kit (Promega). PCR amplifications were performed using two primer pairs (Table 1).

Two fragments were amplified as a 558 bp fragment (PRM1 gene) and 599 bp fragment (PRM2 gene). The PCR condition for amplification of PRM1 fragment was as, 30 cycles of denaturation at 94°C for one minute, annealing at 60°C for one minute and extension at 72°C for one minute. PCR condition for amplification of PRM2 fragment was as, 38 cycles of denaturation at 98°C for ten seconds, annealing at 64°C for 45 seconds and extension at 72°C for 45 seconds. The PCR products were electrophoresed using 2% agarose gel.

Genotyping

For analysis of PRM1 C321A (g.82994C>A, rs737008, AC009121.8) polymorphism RFLP assay, *Bst*uI restriction endonuclease enzyme was used to recognized CGCG sequence. The enzyme digestion was carried out in 2 hours at 37°C that cut 558 bp PCR products contained wild type C allele into 363 and 195 bp fragments. For RFLP assay of PRM2 C248T (g.87825C>T, AC009121.8) polymorphism, *Bsr*I restriction endonuclease enzyme was

Table 1: Primers used for amplification of protamine genes in this study.

Gene	Primers	Sequence	Band size (bp)
PRM1	Forward	5'-CCCCTGGCATCTATAACAGGCCGC-3'	558
	Reverse	5'-TCAAGAACAAGGAGAGAAGAGTGG-3'	
PRM2	Forward	5'-CTCCAGGGCCCACTGCAGCCTCAG-3'	599
	Reverse	5'-GAATTGCTATGGCCTCACTTGGTG-3'	

used to recognized CCAGT sequence. The enzyme digestion was performed for 2 hours at 65°C that digest 599 bp PCR products contained wild type C allele into 400 and 199 bp fragments. All DNA fragments were separated using 2% agarose gel electrophoresis and visualized on UV transilluminator by ethidium bromide staining.

Sequencing

PCR products of PRM1 gene were sent for sequencing to MacroGen Company (USA). The results of sequencing were analyzed by BLAST_n in NCBI (AC009121.8).

Statistical analysis

Comparisons of genotype and allele frequencies among study groups were determined using *chi* square test by SAS user guide version 7th (2004).

Results and Discussion

Apparently healthy samples were frequency-matched with patient groups according to three age groups, smoking status, mean marriage period, sperm concentration and mean weight (Table 2).

All criteria mentioned above were obtained from the questioner forma that used in this study. As related with age results, there are equal numbers in each age group within and among control and patients groups. Seventy percent of apparently healthy individuals were smokers, while, this percentage was 63.3 and 60% in severe

oligozoospermic and asthenozoospermic patients, respectively. Also, mean marriage periods were 5, 7 and 8 years apparently healthy subjects, severe oligozoospermic and asthenozoospermic patients, respectively. Sperm concentration of apparently healthy individuals was more than 55 million sperm per ml, while, sperm concentrations were less than 5 million sperm per ml and nil in those with severe oligozoospermia and asthenozoospermia, respectively. Mean weights were 71, 81 and 79 kg for the groups of apparently healthy, severe oligozoospermia and asthenozoospermia, respectively.

The fragment size amplified that flanking the C321A SNP (g.82994C>A) was 558 bp in PRM1 gene as shown in fig. 1. By using specific primers, the targeted fragment that flanking C321A SNP was determined according to GenBank, AC009121.8 between 82631-83188 in chromosome 16 (clone RP11 – 485G7).

The PRM1 gene polymorphism at position 82994 (GenBank: AC009121.8) was defined using PCR-RFLP with *Bst*uI restriction enzyme. The targeted fragment contains one restriction site for *Bst*uI enzyme and the studied SNP (C321A) found within enzyme sequence (CGCG). PCR fragments with cytosine (C) at position 82994(C321A,rs737008) were cut into two fragments (195 and 363 bp), whereas in fragments with adenine (A) at the same position there is no restriction site for this enzyme (558 bp), therefore, the genotypes CC, CA and AA are of wild-type homozygous, heterozygous mutant and homozygous mutant, respectively (Fig. 1).

The distribution of genotype and allele frequency at 82994 site (C321A) of PRM1 gene presented in table 3.

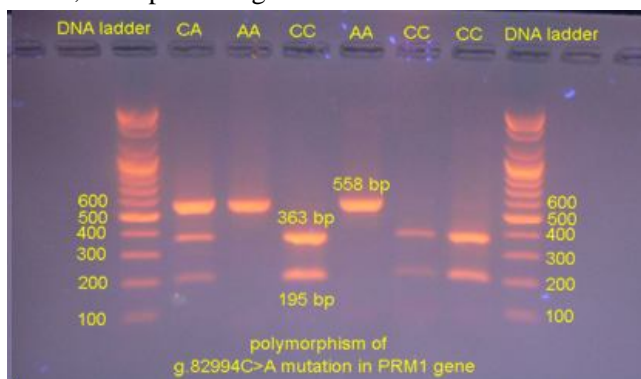


Fig. 1: PCR-RFLP analysis of *Bst*uI digest of the PCR product that include C321A SNP (rs737008) at position 82994 (AC009121.8) of the PRM1 gene separated on a 2% agarose gel. DNA ladder = 100 bp, CC = wild-type homozygous; CA = heterozygous mutant; AA = homozygous mutant.

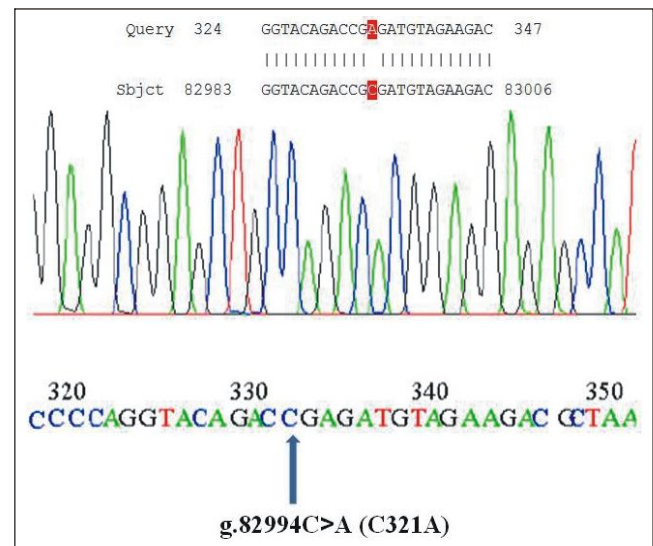


Fig. 2: Electropherogram depicting the g.82994C>A (C321A) position and its flanks.

Table 2: Characteristics of severe oligozoospermic and asthenozoospermic patients and apparently healthy control subjects.

Parameters	Control Apparently healthy	Patients	
		Severe Oligozoospermic	Asthenozoospermic
Age (year)			
21-30	10 (33.3%)	10 (33.3%)	10 (33.3%)
31-40	10 (33.3%)	10 (33.3%)	10 (33.3%)
41-50	10 (33.3%)	10 (33.3%)	10 (33.3%)
Smoking			
Yes	15 (50%)	19 (63.3%)	18 (60%)
No	15 (50%)	11 (36.7%)	12 (40%)
Mean marriage period (year)	5	7	8
Sperm concentration (10 ⁶ /ml)	55<	5>	nil
Mean weight (kg)	71	81	79

As related with CC genotype, there are no significant differences between control group and severe oligozoospermia group, while, CA genotype frequency was significantly ($p < 0.05$) higher in severe oligozoospermia group than apparently healthy control group (60 versus 50%, respectively; $X^2 = 4.61$). In contrast, AA genotype frequency was significantly ($p < 0.05$) higher in control group than severe oligozoospermia group (23 versus 13%, respectively; $X^2 = 4.61$). As related with CC and AA genotypes, there were no significant differences between control and asthenozoospermia groups, while, CA genotype frequency was significantly ($p < 0.05$) higher in control group than in asthenozoospermia group (50 versus 37%,

Table 3: The allele and genotype frequencies of g.82994 C>A (C321A) mutation in *PRM1* gene.

Asthenozoospermic patients (G3)	Oligozoospermic patients (G2)	Apparently healthy Control (G1)	
Genotype frequency, n (%)			
10(33%)	8(27%)	8(27%)	CC
11(37%)	18(60%)	15(50%)	CA
9(30%)	4(13%)	7(23%)	AA
CC, $X^2 = 0.00$ NS			G1
CA, $X^2 = 4.61$ *			versus
AA, $X^2 = 4.61$ *			G2
CC, $X^2 = 1.29$ NS			G1
CA, $X^2 = 5.02$ *			versus
AA, $X^2 = 2.17$ NS			G3
CC, $X^2 = 1.29$ NS			G2
CA, $X^2 = 7.94$ **			versus
AA, $X^2 = 6.22$ **			G3
Allele frequency (%)			
0.52	0.57	0.52	C
0.48	0.43	0.48	A

* = $p < 0.05$, ** = $p < 0.01$, NS = No-significant.

respectively; $X^2 = 5.02$). There were no significant differences between severe oligozoospermia and asthenozoospermia groups as related with CC genotype. CA genotype frequency was significantly ($p < 0.01$) higher in severe oligozoospermia group than in asthenozoospermia group (60 versus 37%, respectively; $X^2 = 7.94$). AA genotype frequency was significantly ($p < 0.01$) higher in asthenozoospermia group than in severe oligozoospermia group (30 versus 13%, respectively; $X^2 = 6.22$). The frequencies of C and A alleles were 0.52 and 0.48 in apparently healthy control group, 0.57 and 0.43 in severe oligozoospermia group and 0.52 and 0.48 in asthenozoospermia group, respectively.

Tanaka *et al.*, (2003) found, as related with C321A SNP(rs737008) at amino acid 47, that 55.3% and 47.8% were homozygous CC genotype, 38.1% and 43.3% were heterozygous CA genotype and 6.6% and 8.9% were homozygous AA genotype in the infertile and fertile Japanese populations, respectively.

These results are agreed with the results of the present study as related with severe oligozoospermia versus control in CA and AA genotypes. While, Tanaka *et al.*, (2003) indicated similar prevalence for infertile and fertile group in Japanese population as related with C321A SNP. In addition, Lee *et al.*, (2005) found in C321A SNP of *PRM1* gene that genotype frequencies

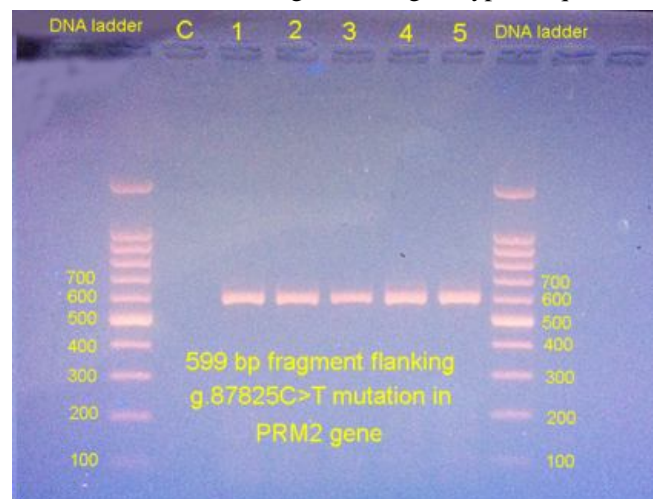


Fig. 3: PCR product (599 bp) of C248T SNP (g.87825C>T, GenBank: AC009121.8) visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 5 volt / cm for 2 hours. DNA ladder = 100 bp, C = negative control.

Table 4: The allele and genotype frequencies of g.87825 C>T (C248T) mutation in *PRM2* gene.

	Apparently healthy control	Severe oligozoospermic patients	Asthenozoospermic patients
Genotype frequency, n (%)			
CC	0(0%)	0(0%)	0(0%)
CT	30(100%)	30(100%)	30(100%)
TT	0(0%)	0(0%)	0(0%)
Allele frequency, (%)			
C	0.50	0.50	0.50
T	0.50	0.50	0.50

of CC, CA and AA were 56.9, 35.4 and 7.7%, respectively in Korean infertile men. In Iranian population, Elham *et al.*, (2012) found in C321A SNP that genotype frequencies of CC, CA and AA were 22.5, 29.9 and 43.6% in the idiopathic azoospermia group and 24, 29 and 47% in the control group, respectively, while in this study were 33, 37 and 30% in the asthenozoospermia group; 27, 60 and 13% in the severe oligozoospermia group and were 27, 50 and 23% in the apparently healthy control group.

PCR products of 10 samples from each group that include all samples with homozygous and heterozygous mutants (C321A) and some homozygous normal were sent for sequence analysis by AB13730XL APPLIED BIOSYSTEMS machine in NICM Co., USA. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program (BLAST_n) which is available at the National Centre of Biotechnology Information (NCBI) online (<http://www.ncbi.nlm.nih.gov/nucleotide>). The sequence analyses of *PRM1* gene fragment of studied samples were compared with *PRM1* gene reference sequence (GenBank: *AC009121.8*). From the information of sequencing, fig. 2, presented the electropherogram depicting the 82994 position (C321A) and its flanks.

PCR was used to amplify the targeted fragment which specified by using specific primers. The fragment size amplified that flanking the C248T position (87825: GenBank: *AC009121.8*) in *PRM2* gene was 599 bp as shown in fig. 3.

The *PRM2* gene polymorphism at position of C248T SNP was defined using PCR-RFLP with *BsrI* restriction enzyme. The targeted fragment contain one sequence of ACCGG in which the cytosine in the position of SNP, when this cytosine is replaced with thymine as a result of C248T this sequence will convert to ACTGG which represent the site sequence of *BsrI* restriction enzyme, therefore, CC genotype carriers (homozygous normal) had one fragment (599 bp), CT genotype carriers

(homozygous mutant) had three fragments (599, 400 and 199 bp) and TT genotype carriers (homozygous mutant) had two fragments (400 and 199 bp) because two site sequences of *BsrI* were available in the DNA of target fragment due to single nucleotide transition in the two strands of DNA (Fig. 4).

The distribution of genotype and allele frequency at 87825 site (C248T) of *PRM2* gene presented in table 4. In this study, only CT genotypes (100%) were found in all samples studied. In addition, the frequencies of C and T alleles were equal in all studied groups.

The C248T SNP which changes glutamine (CAG) to a stop codon (TAG) was found in the middle of the *PRM2* coding region. This SNP produces severe changes in the *PRM2* protein.

Elham *et al.*, (2012) found, as related with C248T SNP of *PRM2* gene, that the frequency of CC genotype was 100% while were 0 and 0% for CT and TT genotypes, respectively, in both fertile and infertile Iranian mens. In contrast, in this study, the frequency of CT genotype was 100% while were 0 and 0% for CC and TT genotypes, respectively, in apparently healthy subjects, severe oligozoospermic and asthenozoospermic patients. Therefore, CC and TT genotypes are completely absent and are not associated with male infertility in Iraqi patients with severe oligozoospermia and asthenozoospermia under this study condition and therefore this SNP may not represent a molecular marker for genetic diagnosis of male infertility. Tanaka *et al.*, (2003) identified presence of C248T SNP among of 266 azoospermic patients and they contributed these results to the

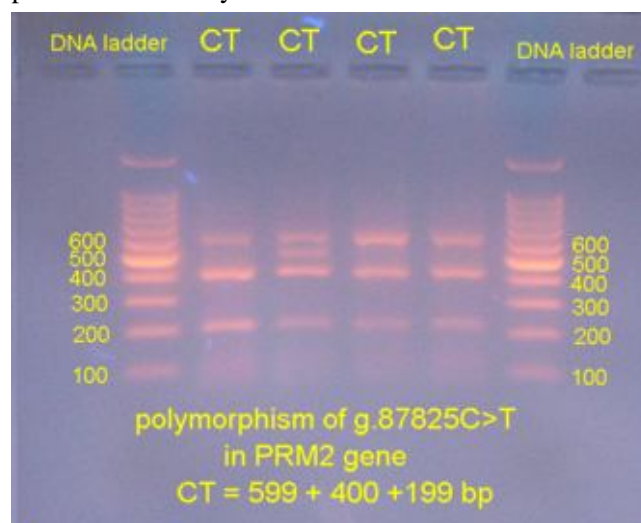


Fig. 4: PCR-RFLP analysis of *BsrI* digest of the PCR product that include C248T SNP at position 87825 (*AC009121.8*) of the *PRM2* gene separated on a 2% agarose gel. DNA ladder = 100 bp, CT = heterozygous mutant.

appearance of a stop codon and premature termination of the protamine 2 mRNA leading to azoospermia.

Conclusion

In PRM1 gene, CA genotype of C321A SNP was associated with severe oligozoospermia rather than asthenozoospermia whereas there is no association between C248T SNP of PRM2 gene and the incidence of both oligozoospermia and asthenozoospermia in Iraqi patients.

References

- Agarwal, A., A.C. Varghese and R.K. Sharma (2009). Markers of oxidative stress and sperm chromatin integrity. *Methods Mol. Biol.*, **590**: 377-402.
- Alam, N. (2009). Male factor infertility basics revisited. *J. Coll. Physicians Surg. Pakistan.*, **19**: 205-206.
- Al-Ramahi, I.J., A.H.M. Al-Faisal, I.A. Abdul-Hassan, R. Al-Atar and S. Barusrux (2012). Screening of g.IVS5+1G to a mutation of TG gene and thyroid hormone level among Iraqi thyroid disorders. *Journal of Medical Genetics and Genomics.*, **4(1)**:1-5.
- Alvarez, J.G., R.K. Sharma, M. Ollero, R.A. Saleh, M.C. Lopez and A.J. Thomas *et al.*, (2002). Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. *Fertil. Steril.*, **78**: 319-329.
- Aoki, V.W., B.R. Emery, L. Liu and D.T. Carrell (2006). Protamine levels vary between individual sperm cells of infertile human males and correlate with viability and DNA integrity. *J. Androl.*, **27**: 890-898.
- Balhorn, R. (2007). The protamine family of sperm nuclear proteins. *Genome Biol.*, **8**: 227.
- Balhorn, R. (1982). A model for the structure of chromatin in mammalian sperm. *J. Cell Biol.*, **93**: 298-305.
- Barone, J.G., J. De Lara, K.B. Cummings and W.S. Ward (1994). DNA.
- Barratt, C.L., R.J. Aitken, L. Bjo`rndahl, D.T. Carrell, P. de Boer, U. Kvist and S.E. Lewis *et al.*, (2010). Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications-a position report. *Hum. Reprod.*, **5(4)**: 824-838.
- Carrell, D.T., B.R. Emery and S. Hammoud (2007). Altered protamine expression and diminished spermatogenesis: what is the link. *Human Reproduction Update.*, **13(3)**: 313-327.
- Cho, C., H. Jung-Ha, W.D. Willis, E.H. Goulding, P. Stein, Z. Xu, R.M. Schultz, N.B. Hecht and E.M. Eddy (2003). Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. *Biol. Reprod.*, **69**: 211-217.
- Cho, C., W.D. Willis, E.H. Goulding, H. Jung-Ha, Y.C. Choi, N.B. Hecht and E.M. Eddy (2010). Haploinsufficiency of protamine -1 or -2 causes infertility in mice. *Nat. Genet.*, **28**: 82-86.
- Curi, S.M., J.I. Ariagno, P.H. Chenlo, G.R. Mendeluk, M.N. Pugliese, L.M. Sardi, H.E. Repetto and A.M. Blanco (2003). Asthenozoospermia: analysis of a large population. *Arch Androl.*, **49**: 343-49.
- Elham, S., A. Ahmad, M. Javad and S. Hamid (2012). Association study of six SNPs in PRM1, PRM2 and TNP2 genes in Iranian infertile men with idiopathic azoospermia. *Iran. J. Reprod. Med.*, **10(4)**: 329-336.
- Engel, W., S. Keime, H. Kremling, H. Hameister and G. Schluter (1992). The genes for protamine 1 and 2 (PRM1 and PRM2) and transition rotein 2 (TNP2) are closely linked in the mammalian genome. *Cytogenet. Cell Genet.*, **61**: 158-159.
- Fisher, J.R. and K. Hammarberg (2012). Psychological and social aspects of infertility in men: an overview of the evidence and implications for psychologically informed clinical care and future research. *Asian J. Androl.*, **14**: 121-129.
- Hecht, N.B. (1989). Molecular biology of structural proteins of the mammalian testis. In: Adolph KW, eds. Molecular biology of chromosome function. New York, NY: Springer. 396-420.
- Lee, H., H. Choi, Y. Park, J. Seo, M. Koong and J. Jun (2005). Screening of the Single Nucleotide Polymorphisms in the Protamine 1 and 2 Genes of Korean Infertile Men. *Kor. J. Fertil. Steril.*, **32(3)**.
- Lee, K., H.S. Haugen, C.H. Clegg and R.E. Braun (1995). Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. *Dev. Biol.*, **92**: 12451-12455.
- Lewis, S.E. and R.J. Aitken (2005). DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell Tissue Res.*, **22**: 33-41.
- Lewis, S.E.M., I. Agbaje and J. Alvarez (2008). Sperm DNA tests as useful adjuncts to semen analysis. *Syst. Biol. Reprod. Med.*, **54(3)**: 111-125.
- Naoko, I., Y. Shicheng, J.L. Dolores and B.H. Norman (2005). A SNP in protamine 1: a possible genetic cause of male infertility. *JMG*, 10.1136/jmg.2005.037168.
- Oliva, R. (2006). Protamines and male infertility. *Hum. Reprod. Update.*, **12**: 417-435.
- Oliva, R. and G.H. Dixon (1991). Vertebrate protamine genes and the histone-toprotamine replacement reaction. *Prog. Nucleic Acid Res. Mol. Biol.*, **40**: 25-94.
- Organization in human spermatozoa. *J. Androl.*, **15**: 139-144.
- Ravel, C., S. Chantot-Bastaraud, B. El Houate, I. Berthaut, L. Verstraete, V. De Larouziere, D. Lourenco, A. Dumaine, J.M. Antoine and J. Mandelbaum *et al.*, (2007). Mutations in the protamine 1 gene associated with male infertility. *Mol. Hum. Reprod.*, **13**: 461-464.
- Razzak, A.H. and S.A. Wais (2002). The infertile couple: A Cohort study in Duhok, Iraq. *East Mediterr Health J.*, **8(2-3)**: 234-238.
- Rutstein, S.O. and I.H. Shah (2004). Infecundity, Infertility and Childlessness in Developing Countries. ORC Macro.

- MEASURE DHS + (Programme) and World Health Organization. Calverton, MD, Geneva, Switzerland: ORC Macro World Health Organization.
- Sakkas, D. and J.G Alvarez (2010). Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome and analysis. *Fertil. Steril.*, **93**: 1027-1036.
- Sakkas, D., O. Moffatt, G.C. Manicardi, E. Mariethoz, N. Tarozzi and D. Bizzaro (2002). Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol. Reprod.*, **66**: 1061-1067.
- SAS (2004). Statistical analysis system, User's guide. Statistical Ver. 7th edition SAS. Inst. Inc. Cary USA.
- Sassone-Corsi, P. (2002). Unique chromatin remodeling and transcriptional regulation in spermatogenesis. *Science.*, **296**: 2176-2178.
- Simon, L., D. Lutton and J. McManus *et al.*, (2011). Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and IVF success. *Fertil. Steril.*, **95**: 652-657.
- Sotolongo, B., E. Lino and W.S. Ward (2003). Ability of hamster spermatozoa to digest their own DNA. *Biol. Reprod.*, **69**: 2029-2035.
- Steger, K., L. Fink and K. Failing (2003). Decreased protamine-1 transcript levels in testes from infertile men. *Mol. Hum. Reprod.*, **9**: 331-336.
- Tanaka, H., Y. Miyagawa, A. Tsujimura, K. Matsumiya, A. Okuyama and Y. Nishimune (2003). Single nucleotide polymorphisms in the protamine-1 and -2 genes of fertile and infertile human male populations. *Mol. Hum. Reprod.*, **9**: 69-73.
- WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical mucus Interaction (1999). 4th ed. (Cambridge University Press).
- Zini, A. and M. Sigman (2009). Are tests of sperm DNA damage clinically useful? pros and cons. *J. Androl.*, **30(3)**: 219-229.