

Analysis of *PRM1* and *PRM2* Polymorphisms in Iranian Infertile Men with Idiopathic Teratozoospermia

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Abstract

Single nucleotide polymorphisms (SNPs) in a number of genes involved in sperm maturation are considered as one of the main factors for male infertility. The aim of the present case-control study was to examine the association of SNPs in protamine1 (*PRM1*) and protamine2 (*PRM2*) genes with idiopathic teratozoospermia. In this case-control study, some SNPs in *PRM1* (c.49 C>T, c.102 G>T and c.230A>C) and *PRM2* (rs545828790, rs115686767, rs201933708, rs2070923 and rs1646022) were investigated in 30 idiopathic infertile men with teratozoospermia (case group) in comparison with 35 fertile men (controls). Genotyping of SNPs was undertaken using polymerase chain reaction (PCR)-direct sequencing. For *PRM1*, c.230A>C, as a synonymous polymorphism, was detected in both teratozoospermic men (heterozygous n=26, homozygous minor n=1) allele frequency C(48) A(52) and controls (heterozygous n=15, homozygous minor n=4). All cases and controls were genotyped for rs545828790 in *PRM2*, a missense polymorphism, as well as rs115686767 and rs201933708, both of which synonymous variants. The findings showed an intronic variant in *PRM2* (rs2070923) was also present in both groups. Also, rs1646022, a missense polymorphism, occurred in teratozoospermic men (heterozygous n=10, homozygous minor n=5) and controls (heterozygous n=13, homozygous minor n=2). However, there were no significant differences in SNPs of *PRM1* and *PRM2* between the two groups, however, for c.230A>C, the frequency of the CA genotype was significantly higher in infertile men with teratozoospermia (P=0.001). We demonstrate that *PRM2* G398C and A473C polymorphisms were associated with the teratozoospermia and its genetic variation was in relation to semen quality, sperm apoptosis, and morphology in the Iranian population. This study is a preliminary study and presenting data as part of a future comprehensive study to clinically establish whether these gene polymorphisms are biomarkers for susceptibility to teratozoospermia.

Keywords: Single Nucleotide Polymorphisms, Sperm, Teratozoospermia

Citation: Dehghanpour F, Fesahat F, Miresmaeili SM, Zare Mehrjardi E, Honarju A, Talebi AR. Analysis of *PRM1* and *PRM2* polymorphisms in Iranian infertile men with idiopathic teratozoospermia. *Int J Fertil Steril.* 2019; 13(1): 77-82. doi: 10.22074/ijfs.2019.5650.

Genetic factors are responsible for 50% or more of male infertility etiology and nearly 7% of men suffer from infertility worldwide (1, 2). It is generally accepted that abnormalities in sperm chromatin and DNA are one of the main factors affecting pregnancy rates. Sperm DNA packaging, which occurs during spermiogenesis, is a unique process because of histone-protamine replacement.

Two types of protamines are known to exist in humans, namely protamine 1 (*PRM1*) and protamine 2 (*PRM2*). The expression of this two proteins in the sperm nucleus is almost equal (3). The protamine proteins are characterized by an arginine-rich core and cysteine residues (4). Protamine has specialized in sperm for several reasons including more chromatin condensation, faster spermatozoa, effective oocyte fertilization, protecting the maternal genome from nuclease and toxins, permitting oocyte to reprogramme the paternal genome, and for having an im-

printing pattern and reactivation upon fertilization (5).

Therefore, due to the importance of protamines in male fertility, many studies have shown altered expression of protamines in several groups of infertile men (3, 6-8). *PRM1* and *PRM2* are located on chromosome 16 and both genes contain a single intron (9). A number of reports have verified different variations in *PRM1* and *PRM2* sequences (NM_002761.2 and NM_002762.3) in humans with various associations with male infertility (10-13). Infertile men with high levels of abnormal sperm morphology are considered to teratozoospermia (14). There is some evidence that protamine mutations or polymorphisms may induce conformational changes the protein level, altering their incorporation into sperm chromatin thus leading to sperm defects. *PRM* deficiency causes sperm morphology defects, motility reduction, and infertility as a result of haploinsufficiency in mice (15, 16). *PRM1* variant rs35576928 is a single nucle-

Received: 18/April/2018, Accepted: 14/July/2018

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Royan Institute
International Journal of Fertility and Sterility
Vol 13, No 1, April-June 2019, Pages: 77-82

tide polymorphism (SNP) that is present at a significantly higher frequency in infertile patients with non-obstructive azoospermia and altered morphology of the spermatozoa (17). Also, variants of *PRM1* and *PRM2* have been shown to be associated with male infertility and abnormal sperm morphology. A polymorphism in the *PRM1* promoter (-190 C→A) is known to increase *PRM1* to *PRM2* ratio (18-20). The aim of our study was to examine the association of SNPs in *PRM1* and *PRM2* with idiopathic teratozoospermia.

In this case-control study, semen samples were collected from 35 fertile men (control group) and 30 infertile men (case group) referred to our Andrology Lab at the Research and Clinical Center for Infertility of Yazd. Sperm samples were obtained after informed consent from the participants. A comprehensive evaluation was undertaken to identify the etiology of infertility including physical examination, smoking history, and reproductive hormonal assays. The infertile man was defined as a man who had no child after a period of unprotected intercourse for more than one year. The control group included fertile donors with one naturally conceived child during the past 12 months who also had normal semen parameters according to the recommendations of the World Health Organization (WHO, 2010). Heavy smokers (more than one pack of cigarettes per day during the past year), drug addicts, alcohol consumers, men with a history of varicocele and those aged more than 45 years were excluded from the study. Theliquefiedsemen of each man was evaluated for sperm parameters according to WHO 2010 (14). Sperm morphology was evaluated using the strict criteria of Kruger et al. (21) and at least 200 cells were examined per slide. To determine the genetic status of protamine genes, the DNA sample was extracted using the salting out method from the peripheral blood of each individual (22). This study was approved by the Ethics Committee of the Yazd Research and Clinical Center for Infertility.

Genomic DNA isolation from peripheral blood samples was performed using the protease and phenol purification protocol (23). Each polymerase chain reaction (PCR) reaction consisted of 1-2 µl (100 ng) of DNA, 0.5-1 µl (0.2 µM) of each specified primer (Pishgam co., Iran) (Table 1) (11) and 12.5 µL of the 2X mastermix (amplicon) in a total volume 25 µL. The cycling conditions for PCR were an initial denaturation of DNA at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 66°C for *PRM1* and 69°C for *PRM2* for 45 seconds, 72°C for 30 seconds, and a final extension of 10 minutes at 72°C. To verify fragment lengths, 2 µl of each PCR fragment was electrophoresed on a 1.5% agarose gel stained with SYBR DNA Safe Stain (Invitrogen, USA).

Table 1: Two primer pairs for amplification of the *PRM1* and *PRM2* genes

Gene	Sequence primer (5'-3')	Product size (bp)
<i>PRM1</i>	F: CCCCTGGCATCTATAACAGGCCGC R: TCAAGAACAAGGAGAGAAGAGTGG	558
<i>PRM2</i>	F: CTCCAGGGCCCCACTGCAGCCTCAG R: GAATTGCTATGGCCTCACTTGGTG	599

After PCR, all of the PCR products were purified and sequenced on an Applied Biosystems 3730 XL DNA analyzer according to the manufacturer's instructions. Using designed primers (forward and reverse), the amplified products with sizes of 557 nucleotides (from -42 to 515) for *PRM1* and 599 nucleotides (from 49 to 648) for *PRM2* were sequenced. Chromatograms were 3 analyzed using Chromas 2 (Technelysium Pty. Ltd., South Brisbane, QLD, Australia).

In this study, we used SPSS 20 (SPSS Inc., Chicago, IL, USA) for all statistical analyses. The frequency of SNPs in *PRM1* and *PRM2* in case and control groups were compared using logistic regression. Differences between groups were examined using one-way ANOVA (followed by Turkey test) for sperm characteristics.

A total of 65 semen samples were examined in two groups. The mean age of participants was 35.21 ± 5.5 vs. 33.71 ± 4.5 years in the case and control groups respectively. In the case and control group, we observed three SNPs in *PRM1* and five SNPs in *PRM2*, namely C230A, G102T, and C49T in *PRM1*, and C288T, C401T, C248T, G398C, A473C, and G271C in *PRM2*. The PCR products were verified on agarose gels (Fig. 1).

The frequency of these SNPs differed in groups of fertile and infertile men. The three SNPs G102T and C49T in *PRM1*, and C248T in *PRM2* were not observed in either group. Other SNPs were found in non-coding regions (Table 2).

Table 3 shows the association of the most frequent genotypes (three SNPs) with seminal characteristics of participants (Table 3).

Abnormal morphology, as well as sperm apoptosis (TUNEL+), was significantly elevated in the GG genotype compared with other genotypes in rs1646022 and rs2070923 in *PRM2*. However, in *PRM1* rs737008, the highest percentage of abnormal morphology, apoptotic sperms, and abnormal motility belonged to the AA genotype. There was no difference between genotypes regarding sperm protamine deficiency and sperm concentrations for all SNPs in *PRM1* and *PRM2* (Table 4).

In this study, infertile men with a history of defects at sperm head morphology and stretch of this region (tapered head) were analyzed for *PRM1* and *PRM2* polymorphisms and compared with fertile men. The findings showed an intronic variant in *PRM2* (rs2070923) which was also present in both groups. Also, rs1646022, a missense polymorphism, occurred in teratozoospermic men (heterozygous n=10, homozygous minor n=5) and controls (heterozygous n=13, homozygous minor n=2). However, there were no significant differences in SNPs of *PRM1* and *PRM2* between the two groups for c.230A>C, the frequency of the CA genotype was significantly higher in infertile men with teratozoospermia.

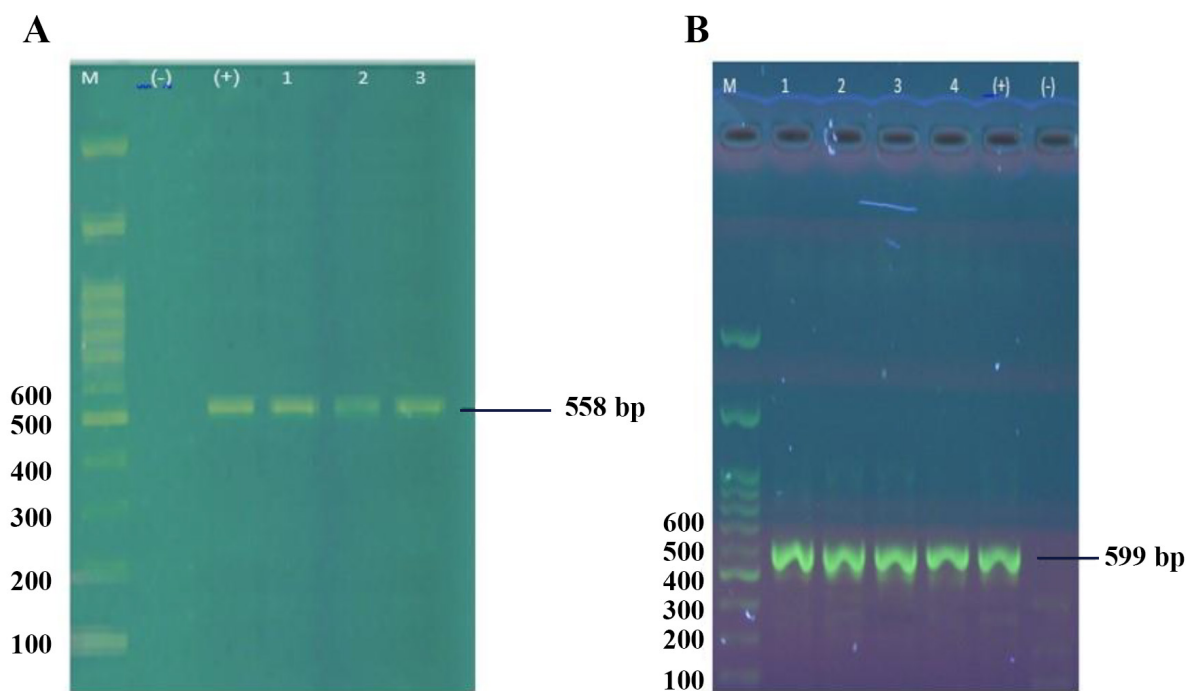


Fig.1: Polymerase chain reaction (PCR) product of genomic DNA using specific primers. **A.** *PRM1* with 557 bp and **B.** *PRM2* with 599 bp length was detected. M; Molecular marker (100 bp), (+); Positive control, and (-); Negative control.

Table 2: The frequency of single nucleotide polymorphisms (SNPs) in *PRM1* and *PRM2* in case and control groups

Gene	Nucleotide change	Region	AA change	NCBI ID	Infertile		Fertile		P value
					Genotype frequency (%)	Allele frequency	Genotype frequency (%)	Allele frequency	
<i>P1</i>	C230A	Exon	None	rs737008	CC: 0.0 CA: 96 AA: 4	C(48) A(52)	CC: 26 CA: 57 AA: 17	C(54.5) A(45.5)	0.000 0.002
	G102T	Exon	R→S	-	GG: 100 GT: 0.0 TT: 0.0	G(100) T(0)	GG: 100 GT: 0.0 TT: 0.0	G(100) T(0)	NS NS
	C49T	Exon	R→C	-	CC: 100 CT: 0.0 TT: 0.0	C(100) T(0)	CC: 100 CT: 0.0 TT: 0.0	C(100) T(0)	NS NS
<i>P2</i>	C288T	Intron	None Coding	rs115686767	CC: 100 CT: 0.0 TT: 0.0	C(100) T(0)	CC: 94.11 CT: 5.89 TT: 0.0	C(97.05) T(2.95)	NS NS
	C401T	Intron	None Coding	rs545828790	CC: 100 CT: 0.0 TT: 0.0	C(100) T(0)	CC: 100 CT: 0.0 TT: 0.0	C(100) T(0)	NS NS
	C248T	Exon	E-Q	-	CC: 100 CT: 0.0 TT: 0.0	C(100) T(0)	CC: 100 CT: 0.0 TT: 0.0	C(100) T(0)	NS NS
	G398C	Intron	None Coding	rs1646022	GG: 44.44 GC: 38.86 CC: 16.7	G(63.87) C(36.13)	GG: 31.25 GC: 62.5 CC: 6.25	G(62.5) C(37.5)	0.004 0.012
	A473C	Intron	None Coding	rs2070923	AA: 33.33 AC: 33.34 CC: 33.33	A(50) C(50)	AA: 43.75 AC: 39.59 CC: 16.66	A(63.55) C(36.45)	0.073 0.007
	G 271C	Intron	None Coding	rs201933708	GG: 100 GC: 0.0 CC: 0.0	G(100) C(0)	GG: 93.75 GC: 6.25 CC: 0.0	G(96.87) C(3.13)	NS NS

Logistic Regression Modeling was used for statistical analysis. All P values were two-sided and considered significant at the 0.05 level and showed the comparisons between the allele frequencies in case and control groups. *P1*; *PRM1*, *P2*; *PRM2*, AA; Amino acid R; Arginine, S; Serine, C; Cysteine, Q; Termination codon, E; Glutamic acid, and NS; No significant.

Table 3: Association of C230A polymorphism in *PRM1* with sperm characteristics

Genotype	Apoptosis	Protamine deficiency	Abnormal motility	Concentration	Abnormal morphology
CC (n=6)	45.66 ± 16.23	35.6 ± 12.34	41.85 ± 6.89	64.83 ± 58.67	5.26 ± 3.49
CA (n=55)	30.8 ± 12.45	27.33 ± 9.67	45.33 ± 7.34	77.47 ± 60.5	48.48 ± 29.78
AA (n=4)	56 ± 18.96	29.66 ± 10.56	23.5 ± 3.45	49.25 ± 32.75	1.4 ± 0.32
P value	<0.001*	0.157	<0.001*	0.602	<0.001*

Values are presented as a mean ± standard deviation. Tukey's test was used for statistical analysis. *; The P<0.05 were considered to indicate statistical significance. Evaluating the sperm parameters was according to the World Health Organization (WHO, 2010).

Table 4: Association of *PRM2* G398C and A473C polymorphisms with human sperm characteristics

Genotype	Apoptosis	Protamine deficiency	Abnormal motility	Concentration	Abnormal morphology
rs1646022					
GG (n=6)	43.4 ± 18.3	28.75 ± 9.25	47 ± 8.45	74.4 ± 34.5	2.33 ± 2.13
GC (n=55)	29.9 ± 9.94	30.9 ± 15.45	57.54 ± 12.36	52.54 ± 28.75	28.7 ± 18.5
CC (n=4)	12.25 ± 3.88	37.75 ± 17.72	60 ± 14.87	74.5 ± 38.65	27.75
P value	<0.001*	0.63	0.12	0.11	0.003*
rs2070923					
AA (n=16)	24.83 ± 8.25	27.33 ± 6.85	55.66 ± 10.76	84.83 ± 64.23	33.33 ± 23.32
AC (n=34)	13.5 ± 3.97	26 ± 11.83	63.33 ± 8.69	102.66 ± 85.43	37 ± 30.54
CC (n=15)	41 ± 14.28	26.66 ± 12.35	44.66 ± 10.73	54.66 ± 47.23	5 ± 4.55
P value	<0.001*	0.92	0.02*	0.12	<0.001*

*; The P<0.05 were considered to indicate statistical significance. Data are presented as mean ± SD. Post-hoc test to ANOVA was used for statistics evaluating the sperm parameters was according to the World Health Organization (WHO, 2010).

Protection and support of the sperm genome are the main functions of protamines. It is shown that incomplete protamination of sperm DNA causes high susceptibility of the genome to nucleases, endogenous and exogenous free radicals and mutagens (24). Studies have also DNA damage (25, 26). Consistent with our results, Aoki et al. (27) analyzed 15 SNPs such as G102T and C203A in *PRM1* and observed similar frequencies between an infertile population and normal controls. But, in contrast to Aoki et al. (27), C203A(rs737008) was different between two groups in the present study. The frequency of C49T, located in the exonic region, was the same between groups whereas these results were different in study by Jodar et al. (28). They found that infertile men with normal sperm count but with both abnormal motility and morphology had a nonsynonymous substitution at position C49T (R17C). A recent review article demonstrated that C230A variant had a higher frequency in infertile men compared with controls (19) which were also in agreement with our results. In addition, Tanaka et al. (11) reported 5 gene polymorphisms in *PRM1* and 3 polymorphisms in *PRM2* in infertile men. Cho et al. (15) found one stop-gained variant, which converted glutamic acid to a stop codon (known as C248T), in one individual from an infertile azoospermic group. We identified G398C (rs1646022) and A473C (rs2070923) in our samples, however, Tanaka et al. (11) did not show the above-mentioned SNPs in azoospermic and oligospermic patients. Another study conducted by Jiang al. (19) showed that G398C is present in infertile men, which was in line with our findings.

Although Aoki et al. (27) identified 15 SNPs, however, given that their frequencies in cases were almost identical to controls, they did not consider them as the underlying genetic cause of abnormalities in the expression of *PRMs* in infertile couples. In this study, the C281T substitution was selected and evaluated among other SNPs and as in Aoki et al. (27) results, we did not observe C281T in either group. It is likely that the variation in results of these studies is due to differences in study populations, which in fact shows that most of these SNPs were at variable frequencies in different populations, indicating that the distribution of genotypes related to different polymorphisms of *PRM1* and *PRM2* genes have ethnic variation. We also detected significant associations between the frequencies of GG and CC genotypes of *PRM2* rs1646022 and rs2070923 respectively with apoptosis, morphology and total motility. Interestingly, these genotypes were the most frequent genotypes in infertile men with taper head spermatozoa. In contrast, there was no association between genotype AA at rs737008 with male infertility. In our previous study, we reported sperm protamine deficiency, lower rates of normal sperm parameters, and apoptosis in infertile men with idiopathic teratozoospermia compared to the controls.

We saw that the concentration of sperm cells was lower in the case group than controls. Also, total motility and sperm morphology were significantly lower in the case group than in the control group. Furthermore, we showed significantly higher rates of protamine deficiency as well as sperm apoptosis in patients with tapered sperm compared with the fertile group using CMA3 fluorescent stain-

ing and TUNEL assay respectively (29). The findings of the present survey are in agreement with the mentioned previous study, demonstrating the probable relationship between male infertility in patients with tapered head sperms and *PRM2* polymorphisms as well as sperm protamine deficiency, apoptosis rate, and morphology. These damages may affect the quality of the ejaculated spermatozoa and decrease their fertility potential in natural conception or ART cycles. Furthermore, a recent study reported that *PRM2* G398C is associated with the pathogenesis of male infertility in idiopathic infertile men from Chinese Han population, which was in line with our results despite having studied different populations (18). Also, another recent study reported that the c.-190 C>A transversion may be involved in the susceptibility for oligozoospermia and could be used as a non-invasive molecular marker for genetic diagnosis of idiopathic oligozoospermia (30). Finally, a more recent study showed that c.-9C>T and c.368A>G polymorphisms of *H2BFWT* may be genetic risk factors for male infertility so we suggested these polymorphisms investigated in the teratozoospermia group (31).

We show that *PRM2* G398C and A473C polymorphisms are associated with male infertility in men with teratozoospermia and sperm parameters including semen quality, sperm apoptosis, and morphology in the Iranian population. This study is a preliminary study of a larger comprehensive research program aiming to identify clinically relevant polymorphisms as biomarkers for susceptibility to teratozoospermia.

Acknowledgements

The authors especially thank Jalal Ghasemzadeh for his technical assistance during this study. No financial support was provided by an external funding agency. The authors declare that they have no conflict of interests.

Authors' Contributions

A.R.T., F.D.; Participated in study design and contributed extensively in the interpretation of the data and the conclusion. F.F.; Performed semen specimen analysis, contributed extensively in the interpretation of the data and the conclusion, and conducted molecular experiments and PCR analysis. E.Z.M., S.M.M. A.H.; Contributed to conception and design, all experimental work, data and statistical analysis, and interpretation of data, drafted the manuscript. All authors performed editing and approved the final version of this paper for submission.

References

1. Yatsenko AN, O'neil DS, Roy A, Arias-Mendoza PA, Chen R, Murthy LJ, et al. Association of mutations in the zona pellucida binding protein 1 (ZPBP1) gene with abnormal sperm head morphology in infertile men. *Mol Hum Reprod.* 2012; 18(1): 14-21.
2. Behre HM, Nieschlag E. Testosterone substitution in the aging man. *Urologe A.* 2000; 39(5): 421-424.
3. Carrell DT, Emery BR, Hammoud S. Altered protamine expression and diminished spermatogenesis: what is the link? *Hum Reprod Update.* 2007; 13(3): 313-327.

4. Balhorn R. The protamine family of sperm nuclear proteins. *Genome Biol.* 2007; 8(9): 227.
5. Oliva R. Protamines and male infertility. *Hum Reprod Update.* 2006; 12(4): 417-435.
6. Aoki VW, Emery BR, Liu L, Carrell DT. Protamine levels vary between individual sperm cells of infertile human males and correlate with viability and DNA integrity. *J Androl.* 2006; 27(6): 890-898.
7. Park KW. Protamine and protamine reactions. *Int Anesthesiol Clin.* 2004; 42(3): 135-145.
8. Carrell DT, Liu L. Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. *J Androl.* 2001; 22(4): 604-610.
9. Krawetz SA, Herfort MH, Hamerton JL, Pon RT, Dixon GH. Chromosomal localization and structure of the human P1 protamine gene. *Genomics.* 1989; 5(3): 639-645.
10. Iguchi N, Yang S, Lamb DJ, Hecht NB. An SNP in protamine 1: a possible genetic cause of male infertility? *J Med Genet.* 2006; 43(4): 382-384.
11. Tanaka H, Miyagawa Y, Tsujimura A, Matsumiya K, Okuyama A, Nishimune Y. Single nucleotide polymorphisms in the protamine-1 and-2 genes of fertile and infertile human male populations. *Mol Hum Reprod.* 2003; 9(2): 69-73.
12. Ravel C, Chantot-Bastaraud S, El Houate B, Berthaut I, Verstraete L, De Larouziere V, et al. Mutations in the protamine 1 gene associated with male infertility. *Mol Hum Reprod.* 2007; 13(7): 461-464.
13. Tüttelmann F, Krenková P, Römer S, Nestorovic AR, Lujic M, Stambergová A, et al. A common haplotype of protamine 1 and 2 genes is associated with higher sperm counts. *Int J Androl.* 2010; 33(1): e240-e248.
14. World Health Organization. WHO laboratory manual for the examination and processing of human semen. Geneva: World Health Organization; 2010.
15. Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, Xu Z, et al. Protamine 2 deficiency leads to sperm dna damage and embryo death in mice 1. *Biol Reprod.* 2003; 69(1): 211-217.
16. Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi Y-C, Hecht NB, et al. Haploinsufficiency of protamine-1 or-2 causes infertility in mice. *Nat Genet.* 2001; 28(1): 82-86.
17. 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. *Reprod Biomed Online.* 2012; 25(6): 627-634.
18. 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): 311-317.
19. 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. *Sci Rep.* 2015; 5: 15300.
20. Gázquez C, Oriola J, Mateo S, Vidal-Taboada JM, Balleascá 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. *J Androl.* 2008; 29(5): 540-548.
21. Kruger TF, Ackerman SB, Simmons KF, Swanson RJ, Brugo SS, Acosta AA. A quick, reliable staining technique for human sperm morphology. *Arch Androl.* 1987; 18(3): 275-277.
22. Sambrook J, Russell DW. *Molecular Cloning.* Hum Mutat. 2001; 18: 1-2231.
23. Sambrook J, Russell DW. Purification of nucleic acids by extraction with phenol: chloroform. *CSH Protoc.* 2006; 2006(1): pii: pdb.prot4455.
24. de Yebra L, Balleascá JL, Vanrell JA, Bassas L, Oliva R. Complete selective absence of protamine P2 in humans. *J Biol Chem.* 1993; 268(14): 10553-10557.
25. Dym M, Kokkinaki M, He Z. Spermatogonial stem cells: mouse and human comparisons. *Birth Defects Res C Embryo Today.* 2009; 87(1): 27-34.
26. Cheng CY. *Molecular mechanisms in spermatogenesis.* Springer Science & Business Media; 2009.
27. 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. *Fertil Steril.* 2006; 86(5): 1416-1422.
28. Jodar M, Oriola J, Mestre G, Castillo J, Giwercman A, Vidal-Taboada JM, et al. Polymorphisms, haplotypes and mutations in the protamine 1 and 2 genes. *Int J Androl.* 2011; 34 (5 Pt 1): 470-485.
29. Dehghanpour F, Tabibnejad N, Fesahat F, Yazdinejad F, Talebi AR. Evaluation of sperm protamine deficiency and apoptosis in infertile

- men with idiopathic teratozoospermia. *Clin Exp Reprod Med.* 2017; 44(2): 73-78.
30. Jamali S, Karimian M, Nikzad H, Aftabi Y. The c.- 190 C> A transversion in promoter region of protamine1 gene as a genetic risk factor for idiopathic oligozoospermia. *Mol Biol Rep.* 2016; 43(8): 795-802.
31. Rafatmanesh A, Nikzad H, Ebrahimi A, Karimian M, Zamani T. Association of the c.-9C> T and c. 368A> G transitions in H2BFWT gene with male infertility in an Iranian population. *Andrologia.* 2018; 50(1).
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