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Association Between Semen Paraoxonase1- Activity Level and L55M Gene Variants with Risk of Male Infertility

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ABSTRACT

Today evaluation of polymorphisms of the antioxidant enzyme-encoding genes, which affect the activity of antioxidant enzymes, could be used as risk prediction models for male infertility. This study aims to evaluate the coloration of serum paraoxonase (PON1) activity levels in the semen and its L55M gene variants with the risk of male infertility. In a case-control study, semen samples were collected from 80 healthy controls and 128 infertile men at Fatemeh Al-Zahra IVF and Pastor Laboratory (Babol, Mazandaran, Iran). PON1 activity of semen samples was measured by spectrophotometric methods. Genotyping of all individuals based on *PON*1-L55M loci performed by PCR-RFLP and PCR-sequencing and molecular effects of leucine (L) to methionine (M) substitution were investigated by bioinformatics tools. Results showed a significant difference in genotype frequencies of PON1-L55M polymorphism between patient and control groups, and c.163T>A transition effect on the structure and function of PON1 protein. Also, TA genotype (OR=1.754, 95%CI=0.971 to 3.166, *P*= 0.062) and AA genotype (OR=5.067, 95%CI=1.366 to 18.789, *P*= 0.015) were associated with male infertility. Men with the mutant allele (AA+TA) are exposed to be at risk of male infertility (OR= 1.990, 95%CI= 1.118 to 3.54, P= 0.019). Also, the allelic analysis showed that the A allele was associated with the increased risk of idiopathic male infertility (OR= 1.749, 95%CI= 1.143 to 2.676, P= 0.010). Additionally, PON1 activity was higher in the TT (LL) individuals compared to the TA (LM) and AA (MM) men in both groups (LL> LM> MM). Since the PON1-L55M gene variants are related to PON1 activity levels in the semen and serum paraoxonase is known as an important antioxidant calcium-dependent enzyme, and it could be implicated in male infertility. Based on these findings, the presence of mutant allele (A) and or decreasing semen's PON1 level may be an indicator/prediction factor for male infertility.

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INTRODUCTION

According to World Health Organization (WHO) guidelines, male infertility as a global health issue, refers to a man's inability to cause pregnancy in a fertile female after 12 months of regular, unprotected sexual intercourse (1). Approximately one in 20 adult men in the reproductive age group suffer from male factor infertility (2). Nearly 25% of these cases of male infertility are unknown and defined as idiopathic male infertility (3). Many factors can impact male infertility including epigenetic, genetic, environmental, and lifestyle-related factors (4). One of the main causes of male infertility is oxidative stress (OS). Reactive oxygen species (ROS) are strongly related to impaired spermatogenesis and male infertility (5, 6). ROS is essential for signal transduction pathways, modulation

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of activities of redox-sensitive transcription factors, and regulation of mitochondrial enzyme activities in all living aerobic cells (7). ROS includes free radical agents such as superoxide anions (O_2•), hydrogen peroxide(H_2O_2), peroxyl (ROO•), and hydroxyl (OH•) radicals that are highly reactive (8, 9). ROS in low levels is essential for sperm capacitation, acrosomal reaction, hyperactivation, and fertilization but overproduction of ROS deactivates antioxidants in the seminal plasma and causes OS. Many studies have reported that infertile men have high levels of seminal ROS and low levels of total antioxidant capacity (10). Seminal plasma leukocytes and the mitochondria in the spermatozoa are the primary cellular sources of ROS production (1). According to many studies, high levels of seminal oxidative stress have been associated with sperm dysfunction such as imperfect metabolism, morphology, motility and fertilization due to lipid peroxidation in sperm cellular membranes and sperm DNA fragmentation, eventually leading to cell death (11, 12). ROS causes reproductive problems in men by damaging the balance of sex hormones (13). Detoxification of ROS concentrations is conducted by a well-organized antioxidative system that includes non-enzymatic and enzymatic antioxidants. The enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and paraoxonase (PON). Most of these enzymes are encoded by SOD, glutathione peroxidase (GPX), CAT and PON genes (10, 14). Many studies demonstrated that genetic mutations and genetic polymorphisms in these genes including the single nucleotide polymorphisms (SNPs), can reduce the rate of fertilization (15, 16). A wide range of genetic, epigenetic and lifestyle-related factors can break the balance between the antioxidant defense system and ROS and lead to infertility (17). Serum paraoxonase1 (PON1) is a 43 kDa extracellular HDL-associated antioxidant enzyme that is dependent on Ca^{2+} and prevents LDL and HDL oxidation to protect cells against OS (18, 19). Infertility may be caused by hyperlipidemia; therefore, PON1 prevents the occurrence of this disease by its key role in preventing lipid peroxidation (20, 21). Sperm parameters such as concentration, motility, and morphology can be influenced by paraoxonase levels and its activity in spermatozoa (22). The long arm of chromosome 7 between q21.3 and q22.1 belongs to a multigene cluster that consists of PON1, PON2 and PON3 (23). Liver is the predominant source of PON1 and PON3 expression, which can be detected in the blood. However, PON2 is extensively expressed in numerous tissues such as the testis, brain, kidney and liver but is not secreted into the blood (24). All three PON proteins are localized in spermatozoa; however, only PON1 and PON3 were found in Sertoli and Leydig cells (25, 26). PON1 mRNA has been detected in some tissues such as the kidney. It has been documented that the ability of HDL to prevent both the oxidation of LDL and the interaction between macrophages and endothelium by inactivation of PON1 increases (27, 28). Several researchers have reported that PON1 is present at various stages of spermatogenesis; however, the exact role of PON1 in the male reproductive system is unknown (21, 25). More than seven polymorphisms in the coding region and five in the promoter region have been identified in the PON1 gene. It seems that the substitution of glutamine (Q) by arginine (R) at position 192 (Q192R) and leucine (L) by methionine (M) at position 55 (L55M) of coding region are important functional genetic polymorphisms of PON1 protein (29, 30). Genetic analysis demonstrated that the replacement of leucine by methionine at position 55 influences the paraoxonase and aryl esterase activities and the stability of the protein, which can reduce sperm motility (26, 31). Some reports showed that L55M (as known p.L55M, p.Leu55Met or rs854560, c.163T>A) SNP could be a risk factor in male infertility and influence the stability of the protein (19). The L55M polymorphism shows three phenotypes (LL, LM and MM) in which paraxonase activity in the MM form is lower compared with LL and LM (26). As of this date, little research has been conducted on the PON1-L55M gene polymorphism and male infertility. The cytogenetic location (gene position), preferred name, rs number, and Human Genome Variation Society (HGVS) of PON1-L55M are shown in figure 1A. In this present study, we determined the association of L55M polymorphism with male infertility. Additionally, PONase activities, the level of malondialdehyde (MDA) and total antioxidant capacity (TAC) in infertile men of the Mazandaran population (North of Iran), were assessed.

METHODS AND MATERIALS

Subjects and sample collection

In this case-control study, semen samples were collected from 128 individuals with idiopathic male infertility and 80 fertile men without any history of infertility in their first-degree family, forming a healthy control group from January 2020 to October 2022. All semen samples were collected at Fatemeh Al-Zahra IVF and Pastor Laboratory (Babol, Mazandaran, Iran), and stored at -20°C for further use. The infertile men who were referred to these centers had no history of cryptorchidism, orchitis, infectious disease, diabetes mellitus, drug abuse, obstruction of the vas deferens, varicocele, abnormal profiles of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone, as well as abnormal karyotype Y-chromosome microdeletion. Additionally, the women of infertile men women had no reproductive system problems. Moreover,

there were no signs of coronary heart disease (CHD), atherosclerosis, liver diseases, hypertension, diabetes and cancer in both fertile and subfertile cases, which are diseases affecting paraoxonase. The semen analysis was conducted following the guidelines outlined by the World Health Organization (32). guidelines concerning sperm motility (normal ≥ 25%) concentration (normal ≥ 20× 10^6 spermatozoa/ml), and normal morphology (normal ≥ 14%).

This study was conducted following the principles outlined in the Declaration of Helsinki and received approval from the ethics committees of the University of Mazandaran (#IR.UMZ.REC.1399.033).

DNA extraction and PON1-L55M genotyping

Genomic DNA was extracted from semen samples using the conventional salting-out method described by Mwer et al (33). and then stored at -20°C until use. The PON1-L55M gene polymorphisms were genotyped using a polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP).

One pair of primers was designed using oligo primer analysis software ver. 7.0 and listed as follows: forward primer:

5´-GAAGAGTGATGTATAGCCCCAGTT, reverse primer: 5´-AGTGGGCATGGGTATACAGAAA. The amplification reactions were carried out in a total volume of 25 µl, consisting of 100 ng (2 µl) genomic DNA, 10 pmol (1 µl) of each primer, 2.5 µl of 10X PCR buffer, 0.5 µl of four mixed dNTPs (10 mM, Cinnagen Inc, Iran), 1µl of $MgCl_2$ (50 mM, Cinnagen Inc, Iran), and 0.25 µl of 5U/µl *Taq* DNA polymerase (Cinnagene, Co., Iran). The PCR program used for amplification was as follows: 5 min at 94°C, 32 cycles of 30s at 94°C, 30s at 56°C, 30s at 72°C and finally extension step 5 min at 72°C. PON1 gene rs854560 polymorphism was amplified by PCR and then genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. For the PCR RFLP analysis, PCR products were digested with the *Hin1*II restriction enzyme (Thermo Fisher Scientific, USA). The restriction reactions were carried out in a total volume of 10 µl containing: 5µl PCR product (~100ng); 3µl DNAase free H_2O; 1.5 µl 10X fast digest green buffer and 0.5µl (~3 Units) of the restriction enzymes. These components were incubated for 5min at 37°C. Restriction fragments were separated by 1.5% agarose gel electrophoresis and visualized by UV-transilluminators after staining with 1 µg/ml ethidium bromide (34).

TAC, MDA and PON1 activities

The total antioxidant capacity (TAC) level was measured by its ability to reduce Fe^{3+} to Fe^{2+} using the ferric-reducing ability of plasma (FRAP) method (35). Malondialdehyde (MDA) level was determined by its reaction with thiobarbituric acid (TBA) at 100 °C (36). The procedure for these methods in semen fluid was described by Fallah et al (37).

The PONase activity was assessed spectrophotometrically using paraoxon (Sigma Chem, USA) as a substrate, and the absorbance was recorded at 412 nm due to 4 -4-nitrophenol formation (38). Briefly, the paraoxonase activity was measured at 25°C for 3 min after adding 10 µl of seminal plasma to each well containing 150 µl of Tris-Cl (100 mM, pH 8.5) buffer, including 2 mM $CaCl_2$ and 6 mM paraoxon. All results are expressed in U/ml, defined as 1 nmol of 4- nitrophenol formed per minute. The PON1 enzymatic activity was calculated using the molar extinction coefficient 18 053 M^{-1} cm^{-1}.

Statistical analysis

Statistical analysis of the difference in allele and genotype frequencies between controls and infertility groups was performed using SPSS ver. 26.0 (SPSS, Inc., Chicago, IL, USA) software. After assessing the normality of the constant variables, using the Shapiro-Wilk test, quantitative data were presented as mean± SD for normally distributed data, while qualitative variables were represented as a number or percentage. The Hardy–Weinberg equilibrium (HWE) test was used to estimate genotype frequencies. Results were reported by odds ratios (ORs) and 95% confidence intervals (CI). Both clinical and laboratory data were checked for their correlation with PON1 polymorphism. A p-value less than 0.05 (typically ≤ 0.05) was considered statistically significant.

In silico analysis

L55M is one of the most studied polymorphisms associated with PON1 levels and activity. In this study, several bioinformatics tools were used to investigate the molecular effects of this substitution. To predict the effects of L55M substitution on the structure and function of the PON1 protein, the PolyPhen-2 in silico prediction server (http://genetics.bwh.harvard.edu/pph2/index.shtml) was utilized. The SIFT server is an online tool to predict if an amino acid substitution affects protein function (https://sift.bii.a-star.edu.sg/). The MutPred server, based on SIFT, was developed to classify amino acid substitutions (AAS) as benign or disease-associated (http://mutpred.mutdb.org/).To determine the effect of mutation on protein stability and structure, based on the free energy change value (ΔΔG), the I-Mutant 2.0 online server was employed (https://folding.biofold.org/i-mutant/i-mutant2.0.html). SNP & GO is an online server predicting human disease-related mutations in proteins by determining of Reliability Index (RI) (https://snps-and-go.biocomp.unibo.it/snps-and-go/). To assess the

effect of this polymorphism on m-RNA secondary structure, the RNAsnp database was used. For finding PON1 expression in different tissues and establishing the relationship between rs854560 polymorphism and PON1 expression levels in testis, the GTEx portal was consulted. The Kyte-Doolittle scale was employed to detect protein hydrophobic and hydrophilic tendencies before and after the mutation.

RESULTS

A total of 208 individuals participated in this study, including 128 patients with idiopathic male infertility and 80 fertile men as the control group. The average age of the healthy donors was 35.25±7.19. The case group comprised 128 infertile men with an average age of 37.62±9.24. Seminal factors such as motility, sperm count, morphology and volume in both infertile and fertile groups are summarized in Table 1.

The levels of TAC and MDA (as biochemical parameters) in the seminal fluid were assayed by the FRAP and TBA methods, respectively, with results reported in Table 1. The concentration of MDA in the seminal fluid of infertile men (9.65±5.49) and fertile subjects (7.61±2.53) was evaluated. Statistically, the plasma levels of MDA significantly increased in infertile men compared with fertile subjects (p<0.001). In contrast, seminal TAC levels were significantly higher in fertile donors than in men with idiopathic infertility (1949.33± 229.56 vs 1513.43±412.65, p<0.001). In general, seminal MDA levels were significantly higher, but seminal TAC levels were considerably lower in men with idiopathic infertility than in fertile individuals (Table 1).

Genotyping results

The amplified 259 bp fragment of PON1-L55M, which flanked the PON1 L55M (c.163T>A) loci, was used for SNP genotyping. After digestion of PCR products by *Hin1 II* and subjecting them to electrophoresis on a 1.5 % agarose gel, gene variants were displayed in Fig. 1B-C. In this figure, an undigested single band of 259 bp was detected in LL homozygotes, digested fragments of 125 and 134 bp were identified in MM genotypes, and three different fragments (134, 125 and 259 bp) were obtained for heterozygous genotypes. The direct DNA sequencing revealed that PCR-RFLP results for the three mentioned SNPs are reliable Fig. 1D.

Distribution of genotypic and allelic frequency

The analysis of c.163T>A polymorphism correlation with sperm abnormality and sperm motility showed that these clinical data of infertile patients were associated with c.163T>A gene polymorphism. Statistical analysis showed that the PON1 genotype distribution for the c.163T>A polymorphism did not deviate from the Hardy-Weinberg equilibrium in the fertile ($\chi 2$= 3.38, p = 0.065) and infertile ($\chi 2$= 3.52, p = 0.060) subjects (all p>0.05).

The distribution of genotypic and allelic frequencies of the L55M polymorphism in infertile and fertile men are shown in Table 2. As shown in Table 2, the TA heterozygous genotype (OR=1.754, 95%CI=0.971 to 3.166, *P*= 0.062) and AA genotype (OR=5.067, 95%CI=1.366 to 18.789, *P*= 0.015) were associated with idiopathic male infertility. Also, A allele carriers (AA+TA) were exposed to the risk of male infertility (OR= 1.990, 95%CI= 1.118 to 3.54, P= 0.019). Also, the allelic analysis showed that the A allele of the c.163T>A polymorphism was associated with the increased risk of idiopathic male infertility (OR= 1.749, 95%CI= 1.143 to 2.676, P= 0.010). In general, the investigation of allele and genotype frequencies

Table 1. Sperm parameters and biochemical parameters of fertile and infertile men.

Drug	DrugMechanism of Effectiveness	Additional Information
Remdesivir	Inhibits RNA-dependent RNA polymerase (RdRp), disrupting viral replication	Used primarily for treating COVID-19; works by incorporating into viral RNA, causing premature termination
Chloroquine/Hydroxychloroquine	Increases endosomal pH, inhibiting viral/cell fusion and glycosylation of cell receptors.	Initially used for malaria; investigated for COVID-19 treatment; potential immunomodulatory effects.
Methylprednisolone	Reduces inflammation by suppressing the immune response.	Corticosteroids used for various inflammatory and autoimmune conditions; can reduce cytokine production.
Combination of Ritonavir and Lopinavir	Ritonavir inhibits the breakdown of lopinavir, enhancing its antiviral activity against HIV.	Used in HIV treatment; ritonavir acts as a pharmacokinetic enhancer.
Favipiravir	Inhibits viral RNA-dependent RNA polymerase, preventing viral replication.	Broad-spectrum antiviral; used for influenza and investigated for COVID-19.
Fingolimod	Modulates sphingosine-1-phosphate receptors, reducing immune cell migration to the central nervous system.	Used for multiple sclerosis; prevents lymphocyte egress from lymph nodes.
Bevacizumab	Inhibits vascular endothelial growth factor (VEGF), reducing tumor blood vessel formation.	Monoclonal antibody used in cancer therapy; targets angiogenesis.

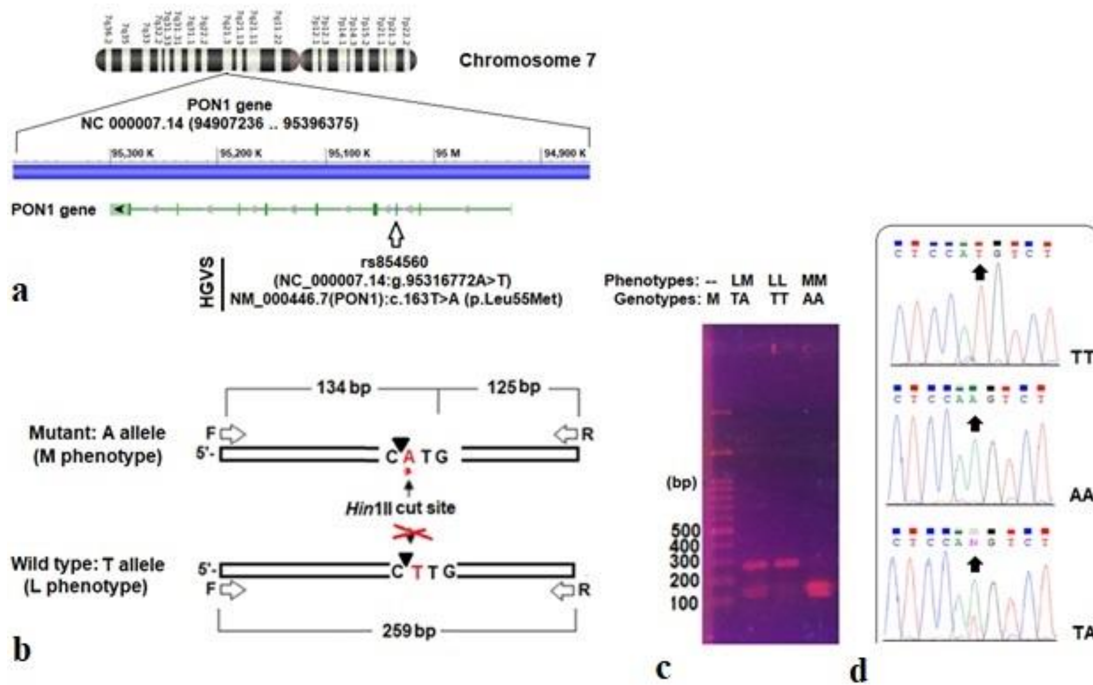

Fig 1. PON1 gene map, RFLP map and DNA sequencing results of PON1-L55M (c.163T>A) loci. a Human PON1 gene map retrieved from the NCBI database, L55M polymorphism located in exon 7. b Schematic of RFLP map, PCR product sizes, and Hin1 II restriction map. c Restriction digest pattern on the 1.5% agarose gel electrophorese, which was stained by ethidium bromide. d Results of PCR-directed sequencing which showed TT, TA, and AA genotypes. M= 100 bp DNA Marker (Fermentas Co., Germany).

Table 2. Analysis of PON1 L55M (c.163T>A) gene variants with the risk of male infertility.

Genotype/ Allele	No. and Percentage		OR (95% CI)	p-value
	Control (80)	Case (n=128)		
Genotype (Phenotype)				
TT (LL)	38 (47.50%)	40 (31.25%)	-	-
TA (LM)	39 (48.75%)	72 (56.25%)	1.754(0.971-3.166)	**0.062***
AA (MM)	3 (3.75%)	16 (12.5%)	5.067(1.366-18.789)	**0.015***
AA(MM) + TA (LM)	42 (54.54%)	88 (68.75%)	1.990(1.118-3.542)	**0.019***
Allele				
T	115(71.87%)	152(59.37)	-	-
A	45(28.22 %)	104(40.62)	1.749(1.143-2.676)	**0.010***

OR = odds ratio; CI = confidence interval; **P value < 0.05***

for the PON1-L55M polymorphism showed that the frequency of the 163A allele in the infertile was higher than the control group (40.62% and 28.22%, respectively). Finally, subjects who receive the A allele have an increased risk of idiopathic male infertility.

Analysis of paraoxonase activity in seminal plasma

Our findings revealed a significant difference in plasma PON activity between the infertile and control groups ($p<0.001$). Infertile patients had considerably lower PON activity (0.046±0.002) in semen plasma compared with healthy men (0.063±0.002) (Fig. 2A). Collectively, PON activities in the LL genotype were the highest followed by LM and MM genotypes, respectively (MM< LM< LL) in both patients and controls. (Fig. 2B).

***In silico* results**

According to the PolyPhen-2 online tool, L55M was classified as benign. The L55M polymorphism, submitted to PolyPhen, was also assessed by the SIFT server, revealing a strong correlation between the results obtained from the PolyPhen and SIFT server. There was a notable relationship between the results obtained from PolyPhen and the MutPred servers. As per the I-Mutant 2.0 online server, a negative ΔΔG value suggests that the mutated protein has lower stability, supporting our findings that the L55M substitution can reduce the protein stability (ΔΔG=-0.64). The SNP& GO server indicated that rs 854560 with a Reliability Index value greater than 5 (RI=7) may have disease-causing potential while the cell continues to survive despite it. Each amino acids have numeric values

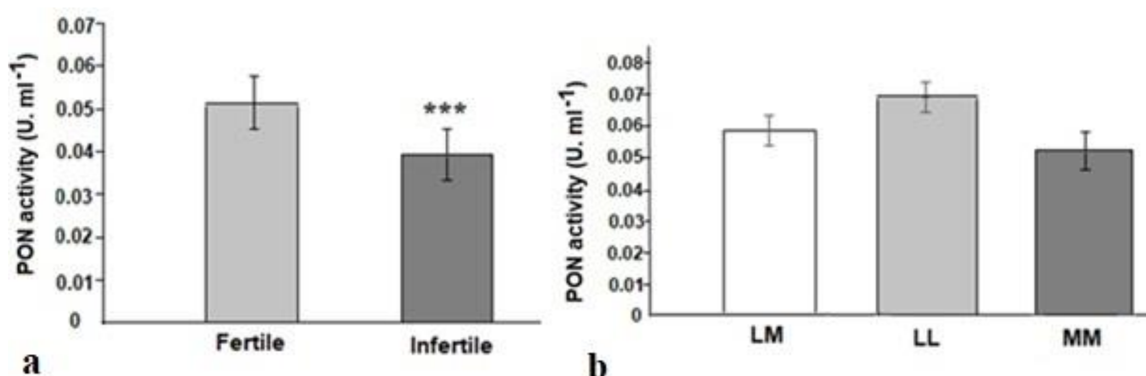

Fig 2. Paraoxonase activity level in the different groups. a PON activity in the infertile and fertile groups, which is a significant difference between the PON activity levels between infertile and fertile groups (***$p < 0.001$). b Paraoxonase activity in different genotypes, in which PON levels were the highest in the LL genotype followed by LM and then MM genotype (MM<LM<LL) in both infertile and fertile groups.

indicating its hydrophobic or hydrophilic nature, and a change in an amino acid can affect protein hydrophilicity and hydrophobicity. The Kyte scale analysis of PON1 (Kyte-Doolittle Hydropathy Plot) revealed that the peak of hydrophobic pattern in the wild type at position 55 for leucine is -0.44 (Fig. 3B) higher than that for methionine in the mutant variant (-0.82) (Fig. 3A). Consequently, the leucine-to-methionine substitution can decrease the tendency of PON1 protein hydrophobicity (Fig. 3). Using GTEx, PON1 gene expression in different tissues such as adrenal glands, breast, fallopian Tube, liver, nerve, ovary, prostate, testis, thyroid and vagina is illustrated in Fig. 4. According to GTEx portal outcomes, the rs854560 variant is remarkably related to different levels of PON1 expression in the liver, testis and ovary (Fig. 4). The data in the RNAsnp database revealed that leucine substitution by methionine in this polymorphism didn't bring about a significant change in m-RNA secondary structure $p = 0.8427$; the p-value < 0.2 is a fundamental structural change, (Fig. 5).

DISCUSSION

Infertility is one of the most significant social problems, known as a multifactorial disease. Epigenetic modifications, genetic variants and environmental factors play a key role in the risk of male infertility (2, 39). The male reproductive system may be affected by environmental factors and pollution. Cigarette smoking is one of the main risk factors for male infertility. Acrosome reaction and capacitation, two necessary processes for fertilization, can be impaired by smoking, and smoking can cause sperm ultrastructural abnormalities. According to the American Society of Reproductive Medicine, research shows that cigarette smoking is associated with poorer semen parameters in smokers than in non-smokers (40). Sperm creatine kinase activity can be reduced by using cigarettes (Smoking can lower sperm creatine kinase activity). Cigarette smoking modifies the PON1 thiol groups and then inhibits the hydrolytic activity of this enzyme (41). Pregnant women exposed to smoking during the first trimester of pregnancy showed reduced PON1 activity (42).

Stress oxidative (OS) and reactive oxygen species (ROS) are strongly related to impaired spermatogenesis and male infertility (5). ROS has both favorable and disadvantageous effects on spermatozoa, as low levels of ROS are necessary for sperm fertilization, motility, capacitation and acrosome reaction. On the other hand, high levels of ROS may cause male infertility by inducing sperm morphology, membrane and DNA damage (11, 12). The human body has several antioxidant enzymes to protect itself against ROS damage (10, 43). Antioxidant enzymes play a key role in maintaining the oxidant and antioxidant balance (22). PON1 is an antioxidant calcium-dependent enzyme that appears to play an essential role in the development of a large variety of diseases. This enzyme possesses three enzymatic activities: lactonase, aryl esterase, and paraoxonase activity. PON1 is mainly synthesized in the liver, then transported from the liver to several tissues and released into the blood circulation where it binds to cell membranes and protects lipids against peroxidation (44). PON1 received its name from its ability to hydrolyze paraoxon (diethyl p-nitrophenyl phosphate). Several studies have reported that PON1 is present at various stages of spermatogenesis (21, 25). The sperm plasma membrane has a high level of polyunsaturated fatty acids. The PON1 gene encodes an enzyme that protects the sperm plasma membrane against lipid peroxidation (22). There is enough evidence showing that genetic risk factors play an important role in increasing the risk of male infertility (45, 46). L55M is one of the most common types of genetic variation in the coding region identified in the PON1 gene, and it affects PON1 serum concentration

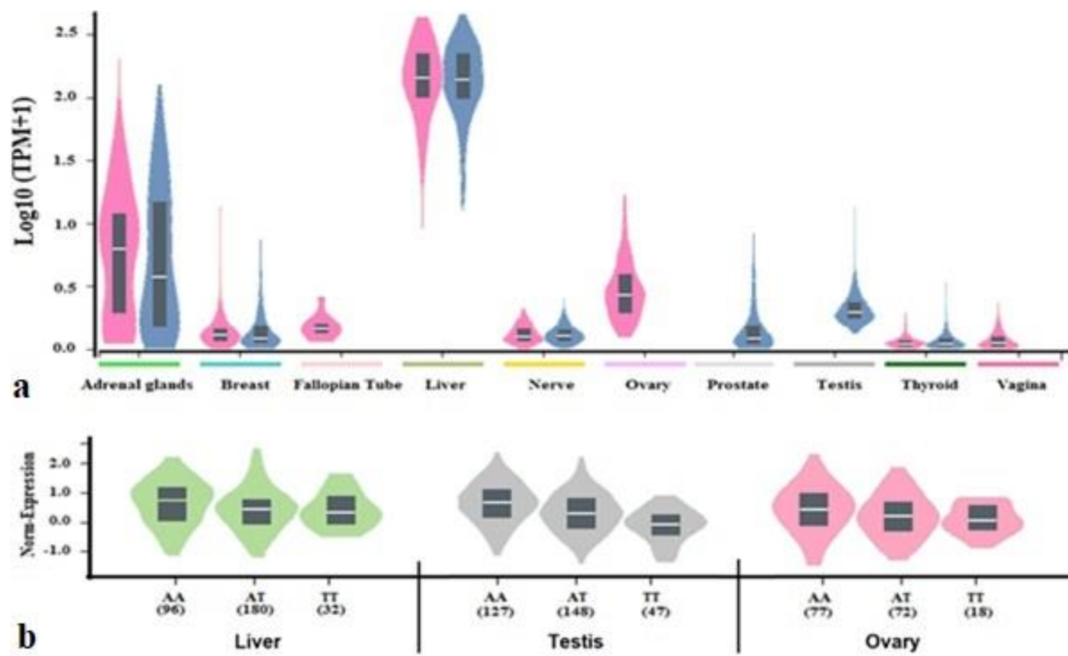

Fig 4. The data deduced from the GTEx server showed. a The expression of PON1 in different tissues such as adrenal glands, breast, fallopian tube, liver, nerve, ovary, prostate, testis, thyroid and vagina in female (pink) and male (blue). b Result of the GTEx portal revealed that leucine substitution by methionine is appreciably related to different levels of PON1 expression in the liver, testis, and ovary.

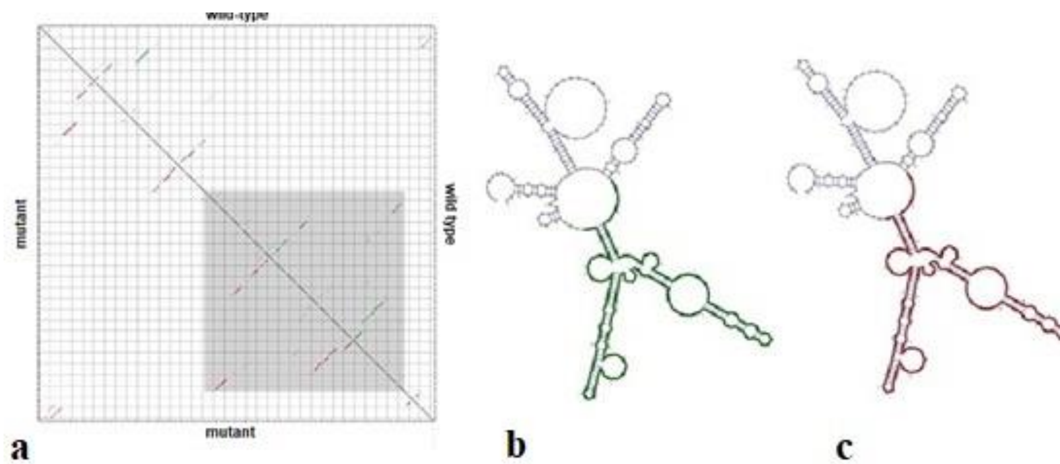

Fig5. The effect of L55M polymorphism on PON1 mRNA secondary structure. An RNA structure alignment plot for the mutant and normal variety. b The wild type secondary structure is depicted in green vs. c The mutant variant secondary structure is depicted in red.

and activity (47). PON1 L55M polymorphism has been reported in various diseases, including cardiovascular diseases (48), rheumatoid arthritis (49), Breast cancer and Parkinson's (28). In this present study, the TA heterozygous genotype (OR=1.754, 95%CI=0.971 to 3.166, *P*= 0.062) and AA genotype (OR=5.067, 95%CI=1.366 to 18.789, *P*= 0.015) were associated with idiopathic male infertility. Also, A allele carriers (AA+TA) were exposed to the risk of male infertility (OR= 1.990, 95%CI= 1.118 to 3.54, P= 0.019). Also, the allelic analysis showed that the A allele of the c.163T>A polymorphism was associated with the increased risk of idiopathic male infertility (OR= 1.749, 95%CI= 1.143 to 2.676, P= 0.010). We found a significant association between the substitution of leucine (TTG) at position 55 by methionine (ATG) at the third exon (L55M) and the risk of male infertility. Previous studies show conflicting results. For example, in 2018, Behrouzi et al investigated the association between PON1 192 Q/R polymorphism and the risk of idiopathic male infertility in Northern Iran. Their result indicated that the PON1 192 Q/R polymorphism is associated with a decreased risk of idiopathic male infertility. In 2020, Alizadeh et al investigated the association between PON1 L55M and Q192R polymorphisms and recurrent pregnancy

loss risk. In their study, statistical analysis of the L55M polymorphism for the MM genotype in the case group compared with the control group showed a significant difference, but none for the LM and LL genotypes (50). The contradictory results of different studies can be caused by the differences in sample sizes, and geographical, racial, and environmental factors.

The L55M polymorphism (rs854560, c.163T>A) displays three phenotypes (LL, LM and MM) in which paraxonase phosphodiesterase activity in the MM form is lower than LL and LM (26). Although serum PON1 aryl esterase activity wasn't linked to this polymorphism, L55M can regulate PON1 aryl esterase activity in the blood (42). M allele carriers have significantly lower PON1 mRNA and protein levels. Some research has suggested that men with MM genotype might be predisposed to infertility. On the other hand, it was shown that the c.163T>A genotype is associated with less sperm motility. As a keynote, L55M could be a risk factor in male infertility and influence the stability of the PON1 protein (19).

In Conclusion, the present study establishes an association; L55M polymorphism can be a genetic marker for male infertility in the Iranian population. One limitation of this study was the sample size. We suggested that future studies focused on some factors such as environmental and epigenetic factors in a larger sample size to the significance of these findings.

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Authorship Contribution Statement

All authors contributed to the writing of this article: **AHC** was involved in the conceptualization, methodology, and software sections. **HM** and **FF** contributed to the material preparation, data curation and analysis, and preparation of the original draft. **AT** and **GJ** contributed to the data analysis and editing of the manuscript.

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Availability of Data and Materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

Informed consent was obtained from all subjects, and the study protocol was approved by the ethics committee of the University of Mazandaran and conducted in accordance with Iran National Committee for Ethics in Biomedical Researches (#IR.UMZ.REC.1399.033).

Declarations

Consent to Participate

As corresponding author, I confirm that the manuscript has been read and approved for submission by all the named authors.

Consent to Publish

All authors would like to submit this manuscript, to be considered for publication as a Research Article, in the Personalized Medicine Journal journal. We declare that it is original, has not been published before and is not currently being considered for publication elsewhere.

Conflict of Interest

There is no conflict of interest to declare.

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