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Association between The SIRT1 and SIRT3 Levels and Gene Polymorphisms with Infertility in War Zones of Kermanshah Province, Iran: A Case-Control Study

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Abstract

Objective: War toxin, mustard gas, alkylating agent results in male infertility via inducing reactive oxygen species (ROS) production and DNA mutagenesis. SIRT1 and SIRT3 are multifunctional enzymes that involve in the DNA repair, oxidative stress responses. This study aim is to assess the correlation between serum levels of SIRT1, SIRT3 and both rs3758391T>C and rs185277566C>G gene polymorphisms with infertility in the war zones of Kermanshah province, Iran.

Materials and Methods: In this case-control study based on the semen analysis, samples were divided into two groups infertile (n=100) and fertile (n=100). High-performance liquid chromatography (HPLC) method was used to determine the malondialdehyde level, and also a sperm chromatin dispersion (SCD) test was used to evaluate the DNA fragmentation rate. Using the colorimetric assays, superoxide dismutase (SOD) activity was measured. SIRT1 and SIRT3 protein levels were determined by using ELISA. The genetic variants of *SIRT1* rs3758391T>C, and *SIRT3* rs185277566C>G, were detected by polymerase chain reaction-restriction fragment length (PCR-RFLP) technique.

Results: Malondialdehyde (MDA) level and the percentage of DNA fragmentation were higher in infertile samples, but serum levels of SIRT1 and SIRT3, and SOD activity was lower in infertile compared to fertile samples ($P<0.001$). The TC+CC genotypes and the C allele from SIRT1 rs3758391T>C polymorphism, and CG+GG genotypes and the G allele from *SIRT3* rs185277566C>G polymorphism could increase risk of infertility ($P<0.05$).

Conclusion: The results of this study suggest that war toxins through the impact on genotypes, decreasing levels of SIRT1 and SIRT3 and increasing levels of oxidative stress, lead to defects in the concentration, motility and morphology of sperms and thus, infertility in men.

Keywords: Infertility, Oxidative Stress, SIRT1, SIRT3, War Toxin

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Introduction

About half of the all infertility is related to male factors (1). In comparison with the antioxidants is an increase in levels of reactive oxygen species (ROS) one of the most important causes of male infertility. Evidence showed that ROS participates in the pathology of 30-80% of infertility in infertile men (2). ROS, eventually, result in genetic mutations, reduced sperm volume and count, reduction of sperm motility, abnormal morphology of sperm via lipid peroxidation, DNA damage, oxidation of proteins, and inactivation of enzymes in spermatozoa (3). The ROS production sources are different and generally classified into two groups: endogenous and exogenous (3, 4). Endogenous sources include genital tract infection, metabolic syndromes and varicocele and etc. Also, exogenous sources comprise,

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smoking, ionizing radiation, war chemical toxin and environmental pollution and etc. Sulfur mustard (SM) is one of the most common war chemical agents, that widely used in several wars, such as the Iran-Iraq war (5). Studies have shown that the SM reduces the seminal plasma antioxidants rate via inducing production of ROS. Consequently the ROS level imbalance and antioxidant defense lead to sperm quality reduction and male infertility (6).

Sirtuins (SIRTs) are nicotinamide adenine dinucleotide (NAD)-dependent deacetylase enzymes that involve in various functions such as DNA repair, oxidative stress resistance, apoptosis and control of metabolic enzymes. They play a key role in transcription factors downstream, such as PGC1α, p53, FOXO, and NF-κB, regulating (7, 8). In mammals, seven SIRTs are known (SIRT 1-7), that are different in intracellular position and their role (9). Among them, SIRT1 and SIRT3 play the most important role in oxidative stress resistance and DNA repair (10).

Human, *SIRT1* gene (10q21.3) has 11 exons and displays expression in the cytoplasm and the nucleus of all tissues, especially in the adrenal and testes (11). Also, *SIRT3* gene, with 10 exons, locates on the chromosome 11 (11p15.5) and expresses in the mitochondria testis, heart and brain and other tissues. *SIRT1* polymorphisms, rs3758391 T/C and *SIRT3* polymorphisms rs185277566 C/G, have been located in the promoter region, and may account for differential *SIRT1*, *SIRT3* expression, function and susceptibility to certain disorders (12).

The precise mechanism of war chemical toxic effect on the reproductive systems and male infertility is not entirely understood (5). Previous studies have shown that a decrease in the SIRT1 and SIRT3 levels leads to spermatogenesis defects and general infertility in the men (10, 13, 14), although, the effect mechanism on the reproductive system is unclear. Also, *SIRT1*, *SIRT3* variants polymorphism associated with susceptibility to urinary bladder cancer, acute coronary syndrome and myocardial infarction (15-17), while there are limited studies of the *SIRTs* variants association with infertility. Therefore, we design of this study.

Materials and Methods

Study subjects

This is a case-control study that study population consist of 200 men, aged between 30-55 years, from Kermanshah who had referred to the Motazedi Hospital, Kermanshah, Iran. According to the World Health Organization (WHO) standards (18) of semen analysis results, participants were divided into two groups: i. Infertile, men who participated in the imposed Iran-Iraq war or live in war zones of Kermanshah (n=100) and fertile, men who did not participate in the imposed war and live in non-war zones of Kermanshah (n=100). The study was approved by the Research Ethics Committee of the Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1399.317). Informed consent was obtained from each participant before participation. The exclusion criteria were bacteria or virus infection, history of diseases such as diabetes mellitus, chronic diseases, hypertension and varicocele and smoking.

Semen samples were collected by masturbation into wide-mouthed sterile containers after 48 hours of abstinence. After liquefaction of semen samples in an incubator at 37°C, sample was divided into 3 tests: i. Routine semen parameter analysis, including macroscopic and microscopic parameters, according to WHO (18) and ii. Sperm DNA fragmentation assessment, and iii. Enzymatic measurements. For enzymatic measurements, the semen sample was centrifuged at 600 g for 10 minutes to separate the sperm cell from the seminal plasma, and stored at -20°C for subsequent measurements.

The whole blood samples were collected from each subject, and transferred into tubes containing anticoagulants (EDTA) for DNA extraction and also, anticoagulant-free tubes for serum isolate samples to analysis serum levels of SIRT1 and SIRT3.

Assessment of antioxidants and oxidative stress biomarkers

The level of malondialdehyde (MDA), Lipid peroxidation marker, was determined by the reverse phase high-performance liquid chromatography (HPLC, Agilent Technologies1200 Series). Device specifications included Nucleodur100–5 C18ec column (Macherey-Nagel, Duren, Germany), a fluorescence detector using EC 250/4.6, mobile phase 60:40 V/V of methanol: potassium monobasicphosphate buffer (50 mM) and detection, Ex515-Em555 (10).

Superoxide dismutase (SOD) activity was measured by the Kiazist SOD kit (Kiazist, Iran) that employs the production of superoxide radicals by xanthine oxidase.

Determination of sperm DNA fragmentation

Sperm chromatin dispersion (SCD) test (Halosperm kit INDAS) used to detect sperm DNA fragmentation. In the SCD method, the spermatozoa with non-fragmented DNA shown a large halo dispersed DNA loop, whereas the spermatozoa containing a fragmented DNA displays very small or no halos (19).

Assessment of SIRT1 and SIRT3 assay

For measurement of SIRT1 and SIRT3, the fasting blood serum was detected by enzyme-linked immunosorbent assay (ELISA) kit (Eastbiopharm, Hangzhou, USA).

DNA extraction and genotyping

The standard procedure of the phenol-chloroform method was used to DNA extraction of whole blood leukocytes (20). The genotypes of single nucleotide polymorphisms (SNP) rs3758391 (*SIRT1*) and SNP rs185277566 (*SIRT3*) were determined by the PCR-RFLP technique. Based on the gene sequences in the GeneBank, Primer 3 online website (https://primer3.ut.ee/) was used to design the pair of primer sequences of *SIRT1* rs3758391 and *SIRT3* rs185277566 SNPs.

The primers

F: 5′-TGGCCAGAACCCATACTAGG-3′

R: 5′-AGCCCTTCCACTTTCCTCTC-3′

Were used to determine *SIRT1* rs3758391 SNP. PCR reaction was carried out in a total volume of 25 µL (18 µL of ddH_2O, 0.5 µL of dNTPs at a concentration of 10 mM, 0.75 µL of $MgCl_2$ at a concentration of 50 mM, 2.5 µL of 10X PCR Buffer, 1 µL of each primer (F and R) at a concentration of 10 pmol and 0.2 µL of Taq DNA Polymerase at a concentration of 5 U/µL). The PCR protocol consisted of 1 cycle initial denaturation (at 95°C for 5 minutes), and 40 cycle amplification (at 95°C for 30 seconds, 61.3°C for 30 seconds, 72°C for 30 seconds), with a final extension at 72°C for 10 minutes. Almost 20 µL of PCR product at 37°C overnight was digested by StyI enzyme. The digested products were electrophoresed in 2% agarose gel. The homozygote mutant CC was not cleaved by the StyI enzyme and a fragment with a length of 205 bp was created, heterozygote mutant TC fragments were 205 bp, 120 bp, 85 bp and homozygote wild TT fragments were 120 bp, 85 bp as shown in Figure 1A.

The primers

F: 5′-ACCCCAGAACGCCATGTT-3′

R: 5′-TGAGACACCAGACTAGGGAACTT-3′

Were used to determine *SIRT3* rs185277566 SNP. PCR reaction was carried out in a total volume of 25 µL (16 µL of ddH_2O, 2 µL of dimethyl sulfoxide (DMSO), 0.5 µL of dNTPs at a concentration of 10 mM, 0.75 µL of $MgCl_2$ at a concentration of 50 mM, 2.5 µL of 10X PCR buffer, 1 µL of each primer (F and R) at a concentration of 10 pmol and 0.2 µL of Taq DNA polymerase at a concentration of 5 U/µL. The PCR protocol consisted of 1cycle initial denaturation (at 95°C for 5 minutes), and 40 cycle amplification (at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds), with a final extension at 72°C for 10 minutes. Almost a 20 µL of each PCR products at 37°C overnight was digested by Cfr421 enzyme. The digested products were electrophoresed in 3% agarose gel. The homozygote wild CC was not cleaved by the Cfr421 enzyme and a fragment with a length of 246 bp was created, heterozygote mutant CG fragments were 246 bp, 186 bp, 60 bp and homozygote mutant GG fragments were 186 bp, 60 bp as shown in Figure 1B.

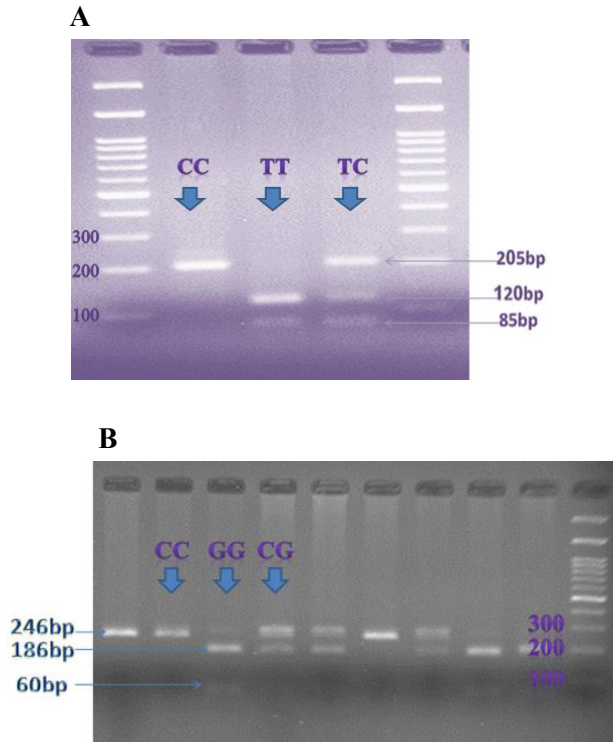


Fig.1: Gel electrophoresis of *SIRT1*, T3758391C digested PCR products with StyI enzyme. **A.** The homozygote mutant CC was not cleaved by the StyI enzyme and a fragment with a length of 205 bp was created, homozygote wild TT fragments were 120 bp, 85 bp and heterozygote mutant TC fragments were 205 bp, 120 bp, 85 bp. **B.** *SIRT3*, C185277566G digested PCR products with Cfr421 enzyme. The homozygote wild CC was not cleaved by the Cfr421 enzyme and a fragment with a length of 246 bp was created, homozygote mutant GG fragments were 186 bp, 60 bp and heterozygote mutant CG fragments were 246 bp, 186 bp, 60 bp. A 1000 bp ladder is used in both SNPs. PCR; Polymerase chain reaction and SNP; Single nucleotide polymorphisms.

Statistical analysis

The normality of distribution of quantitative data was evaluated by Kolmogrov-Smirnov (KS) test. Continuous and comparison data were performed using the Independent t test or Mann-Whitney test. SPSS (version 16, Chicago, IL) was used for analyzing data and Statistical significance was assumed at the $P \leq 0.05$. The frequencies of the genotypes and allele (*SIRT1* and *SIRT3*) in the patients and control groups were compared by the Chi-square test. Odds ratios (OR), 95% confidence intervals (CI) and interaction between the two polymorphisms (*SIRT1* T3758391C and *SIRT3* C185277566G) were determined by the SPSS logistic regression. Pearson correlation was used for Correlation between SIRT1 and SIRT3 levels with evaluated parameters.

Results

Semen analysis

There was no significant difference in the mean age, body mass index (BMI) and semen volume among our groups (Table 1). The mean concentration, motility and morphology of sperm in the infertile group were significantly lower than the fertile group.

Table 1: Demographic and semen parameters data of our groups

Parameters	Groups		P value
	Fertile (n=100)	Infertile (n=100)	
Age (Y)	40.93 ± 3.91	40.73 ± 3.67	0.714
BMI (Kg.M^{-2})	27.85 ± 3.11	27.10 ± 3.20	0.095
Volume (ml)	3.36 ± 0.8	3.60 ± 0.66	0.920
Concentration (10^6 per ml)	40.74 ± 10.19	37.21 ± 13.62	0.041*
Motility (%)	23.45 ± 3.23	16.75 ± 3.51	0.001*
Morphology (%)	6.97 ± 0.95	3.94 ± 1.1	0.001*

Data are presented as mean ± SD. *; P<0.05, significant difference among the groups and BMI; Body mass index.

Levels of oxidative stress biomarkers and sperm DNA fragmentation

The seminal plasma levels of MDA and sperm DNA fragmentation index (DFI) were significantly (P<0.001) higher in the infertile groups in comparison with the fertile group. The SOD activity in the infertile group was remarkable (P<0.001) lower than in the fertile groups (Table 2).

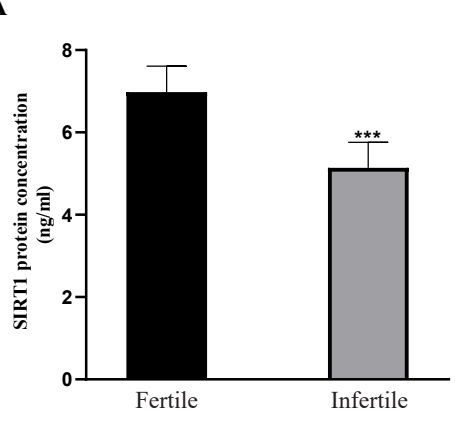
Table 2: MDA concentration, SOD activity and percentage of DNA fragmentation in the fertile and infertile groups

Parameters	Groups		P value
	Fertile (n=100)	Infertile (n=100)	
MDA (µmol/L)	0.91 (0.52-1.12)	1.72 (1.51-3.01)	0.001*
SOD (U/ml)	23.55 (19.83-27.01)	12.17 (8.64-15.01)	0.001*
DFI (%)	20.59 (16-23)	35.94 (33-39.21)	0.001

Data are presented as median and interquartile range (IQR). *; P<0.05, was considered statistically significant, MDA; Malondialdehyde, SOD; Superoxide dismutase, and DFI; DNA fragmentation index.

Quantification of SIRT1 and SIRT3 proteins

A significantly lower (P<0.001) protein level of SIRT1 and SIRT3 was detected in the serum of the infertile groups in comparison with the fertile group. The amount of SIRT1 protein in the fertile was 6.98 ± 0.63 ng/ml and in the infertile was 5.14 ± 0.62 ng/ml (Fig.2A). The amount of SIRT3 protein in the fertile was 4.20 ± 0.34 ng/ml and in the infertile was 3.46 ± 0.66 ng/ml (Fig.2B).



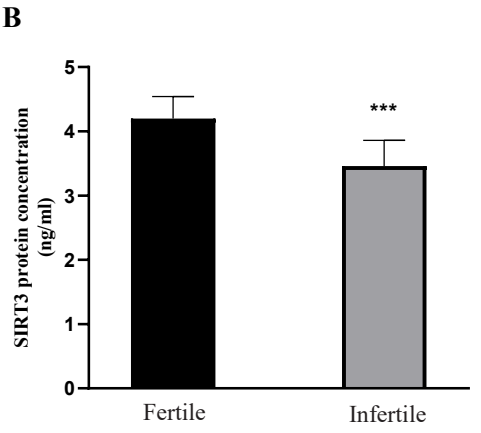


Fig.2: ELISA quantitation of SIRT1 and SIRT3. **A.** SIRT1 and **B.** SIRT3 proteins (ng/ml) in the serum from fertile (n=100) and infertile men (n=100). ***; Significantly different (P<0.001).

Genotypes

The frequency of *SIRT1* rs3758391T>C and *SIRT3* 185277566C>G genotypes and alleles of our group is demonstrated in Tables 3, 4. The distribution of *SIRT1* rs3758391T>C genotypes (χ^2=14.03, df=2, P=0.001) and its alleles (χ^2=15.22, df =1, P=0.014) in infertile men was significantly different in comparison with the fertile men (Table 3). Interestingly, the distribution of *SIRT3* 185277566C>G genotypes (χ^2=6.34, df=2, P=0.04) and its alleles (χ^2 =8.23, df =1, P=0.004) showed a significant difference in the infertile group in comparison with the fertile group (Table 4).

Table 3: Genotype distribution and alleles frequency *SIRT1* (rs3758391) gene polymorphisms in the studied subjects

Genotype	Fertile group	Infertile group	OR (95% CI, P value)
TT	38 (38)	19 (19)	
TC	46 (46)	45 (45)	
CC	16 (38)	36 (38)	
	χ^2=14.03, df =2, P=0.001		
TT	38 (38)	19 (19)	2.61 (1.37–4.96, 0.003)
CT+CC	62 (62)	81 (19)	
	χ^2=8.85, df =1, P=0.003		
Alleles			
T	122 (61)	83 (41.5)	2.20 (1.47–3.28, 0.014)
C	78 (39)	117 (58.5)	
	χ^2=15.22, df =1, P=0.014		

Data are presented as n (%). Distributing alleles and genotype frequency of *SIRT1* in the infertile group in comparison to the fertile group are done by χ^2 test analysis. Odd ratio (OR) was calculated 95% confidence interval (CI) was achieved via χ2 regression binary logistic analysis. P<0.05 was considered significant.

Table 4: Genotype distribution and alleles frequency *SIRT3* (rs185277566) gene polymorphisms in the studied subjects

Genotype	Fertile group	Infertile group	OR (95% CI, P value)
CC	29 (29)	16 (16)	
CG	34 (34)	32 (32)	
GG	37 (37)	52 (52)	
	$\chi2$=6.34, df =2, P=0.04		
CC	29 (29)	16 (16)	2.14 (1.07–4.24, 0.02)
CG+GG	71 (71)	84 (84)	
	$\chi2$=4.84, df =1, P=0.02		
Alleles			
C	92 (46)	64 (32)	1.81 (1.20–2.71, 0.004)
G	108 (52)	136 (68)	
	$\chi2$=8.23, df =1, P=0.004		

Data are presented as n (%). Distributing alleles and genotype frequency of *SIRT3* in the infertile group in comparison to the fertile group are done by χ^2 test analysis. Odd ratio (OR) was calculated 95% confidence interval (CI) was achieved via χ^2 regression binary logistic analysis. P<0.05 was considered significant.

We observed that the presence of TT and TC+CC genotype of *SIRT1* in the infertile men was strongly associated with lower of motility, morphology, SOD, serum level of SIRT1 and SIRT3 and also the higher rate of MDA in comparison with the fertile men. Similar results were observed for *SIRT3* genotypes rs185277566 CC and CG+GG.

Discussion

In this study, we observed a higher rate of MDA and DNA fragmentation in the infertile men. Also, these affected showed a lower rate of SIRT1 and SIRT3 levels, and SOD activity in comparison with fertile man. We observed that the distribution of rs3758391T>C and rs185277566C>G genotypes and alleles in infertile men were significantly different compared to fertile men.

Some studies have focused on the war effects, short-term and long-term, on the fertility (5, 21). These studies reported that patients who have been exposed to war chemical toxins such as SM have reduced the count and motility of sperm and increased abnormal morphology and lead to oligozospermia, asthenozospermia and teretozospermia in comparison with no exposed individuals (5, 22). These results are consistent with our data. Studies have shown, war chemical toxins, especially SM, increase inflammatory reactions, that lead to induce the ROS production rate in the testicular tissue and reducing seminal plasma antioxidants. It seems, this imbalance in the ROS level and antioxidant rate may reduce sperm quality and result in male infertility (1, 23). An increased level of MDA in the seminal plasma inconsistent with the oxidation of sperm membrane lipids, resulted in abnormal morphology and reduced sperm motility. Also, SOD plays an important role that improves the sperm quality (24). Although, no significant correlation among MDA level and SOD activity rate with semen quality have reported by Suleiman et al. (25). Here, we observed that a decrease SOD activity in seminal plasma is associated with poor semen quality, morphology and motility aspects of sperm.

The increased DNA fragmentation rate of sperms can affect negatively the sperm viability and consequences, fertility. In our study, there was a direct relationship between increased DNA fragmentation with decrease concentration, motility and increase abnormal morphology of sperm in infertile samples. The connection between DNA fragmentation and war chemical toxin was investigated. Safarinejad (6) showed the direct correlation between the DNA fragmentation rate and the amount of mustard gas exposure. The frequency of DNA fragmentation was higher in the group with severe mustard gas injury. These data are consistent with our results.

In our previous study, we found that protein levels of SIRT1 and SIRT3, have an important role in sperm quality and it is a major regulator of ROS (10). Our findings suggested that the low serum levels of SIRT1 and SIRT3 protein correlated with high ROS and a DFI of sperm of infertile affected. Loganathan et al. (26) have shown the possible role of SIRT1 and SIRT3 in the spermatogenesis and stated that SIRT1 and SIRT3 play an important role in spermatids differentiation and oxidative stress reduction and absence of this SIRTs leads to DNA damage. In *SIRT1* knockout mice, germ cell differentiation was suppressed (13) and also, a defective acrosome formation (14), an increase of ROS production (10), DNA fragmentation, poor sperm genomic integrity (27) were reported in this animal model that and consequently has led to infertility. SIRT3 is a mitochondrial enzyme that plays a role in the oxygen radical neutralization in the reproductive system. Studies have shown that SIRT3 increases the quality of sperm parameters via an increase of antioxidant enzyme activity (8), and ROS production reduction (26). However, inconsistent with our findings, it seems that SIRT1 and SIRT3 level decrease occurs, follow of war chemical toxin exposure.

Our data detected a significant difference between the genotypes and alleles of SIRT1 rs3758391T>C and SIRT3 rs185277566C>G polymorphism, between the two groups of infertile men and fertile men. The C allele from SIRT1 rs3758391T>C and The G allele from SIRT3 rs185277566C>G polymorphism could increase 2.20 and 1.80 times the risk of infertility in comparison with T and C alleles, respectively. The presence of TC+CC genotype compared to TT genotype of SIRT1 rs3758391T>C 2.61 times and the presence of CG+GG genotype compared to CC genotype of SIRT3 rs185277566C>G 2.14 times increases the risk of infertility.

Sarumaru et al. (28) reported that among Japanese population the distribution of rs3758391T>C polymorphism genotypes had a significant correlation

between autoimmune thyroid patients and control group and the C allele increased the risk of autoimmune thyroid disease. Shafieian et al. (15) stated that the frequency distribution of TT genotype of SIRT1 rs3758391 was significantly different among urinary bladder cancer and the control group. In their study, the wild TT genotype increases the risk of urinary bladder cancer. While in our study the mutant CC genotype is associated with an increased risk of infertility. Inconsistent results between the SIRT1 rs3758391 polymorphism and the incidence of a disease may be due to differences in the type of damaged tissue, lifestyle, environmental factors and ethnicity. Consistent with our results, there is a significant relationship between *SIRT1* gene genotypes with the serum levels of this protein. Hu et al. (29) evaluated the correlation the C allele of *SIRT1* gene genotypes with the SIRT1 serum level reduction in acute coronary syndrome patients. Consistently, in the Peng et al. (30) study, the distribution of rs3758391T>C polymorphism genotypes associated with a decrease in SIRT1 serum level that reduces the susceptibility to diabetic foot ulcers.

Few studies have examined the genotype of SIRT3 rs185277566C>G with the incidence of a disease. Yin et al. (17) examined the correlation of SIRT3 rs185277566C>G genotypes with myocardial infarction in the Chinese population. Their results showed a significant correlation between the G allele and increase risk of myocardial infarction.

In the study of Mostafa et al. (31) a negative correlation was observed between SIRT1 level with concentration, motility and morphology of sperm in varicocoele affected men. Nasiri et al. (10) stated that there is a positive relationship between SIRT1, SIRT3 with antioxidants and a negative relationship between these proteins and oxidative stress in plasma seminal. Loganathan et al. (26) showed that a negative correlation among SIRT1 and SIRT3 level with DNA fragmentation and infertility. According to these results, it can be guessed that lower serum levels of SIRT1 and SIRT3 proteins may lead to an increase of ROS level and DNA fragmentation that results in male infertility.

Conclusion

The results of know study demonstrated a correlation between SIRT1 rs3758391 and SIRT3 rs185277566 polymorphisms with infertility in the war zones of Kermanshah province, Iran. The genetic variants of SIRT1 rs3758391 and SIRT3 rs185277566 were correlated with diminish serum levels of SIRT1 and SIRT3 in infertile men. The levels of ROS and DNA fragmentation in people exposed to war chemical toxins are high. A significant positive correlation between serums SIRT1, SIRT3 levels with sperm motility, sperm morphology and SOD as well as a negative relationship between MDA, DFI with the level of these proteins. In summary, the results of the present study showed that war chemical toxin through the impact on genotypes, decreasing levels of SIRT1 and SIRT3 and increasing levels of oxidative stress, lead to defects in the concentration, motility and morphology of sperms and thus, infertility in men.

Acknowledgments

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Authors' Contributions

A.N., S.M.H.; Study design, data collection, statistical analyses, and manuscript writing. H.M., MR.; PCR-RFLP performance and data interpretation. All authors have read and approved the final manuscript.

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