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Original article

The interaction between a genetic variant in the *NQO1* gene and environmental influences involving coenzyme Q10 has a significant impact on sperm motility

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Abstract. *Background and aim:* Asthenozoospermia is a condition characterized by reduced sperm motility. Oxidative stress is known to impact sperm parameters, but Coenzyme Q10 (CoQ10), as an antioxidant, protects sperm from such damage. CoQ10 antioxidant activity requires reduction by the NAD(P) oxidoreductase 1(NQO1) enzyme. This study investigated the association between CoQ10 levels in seminal plasma, the *NQO1*^{P187S} variant of the *NQO1* gene, and asthenozoospermia risk. *Research Design and Methods:* A case-control study that included 127 asthenozoospermic patients and 55 normozoospermic controls was employed. Levels of CoQ10 in seminal plasma were measured using high-performance liquid chromatography (HPLC). The amplification refractory mutation system (ARMS)-PCR was used to detect the *NQO1*^{P187S} polymorphism. *Results:* Asthenozoospemic patients had significantly lower levels of CoQ10 compared to normozoospermic controls (P=0.02). Additionally, asthenozoospermic patients with the CT/TT genotypes had lower levels of CoQ10 than controls carrying the same genotypes of the *NQO1*^{P187S} variant. Regression analysis showed that CT/TT genotype was associated with an 8-fold increase in asthenozoospermia risk (OR=8.57, 95%CI: 2.09-3517, P=.003). Furthermore, a 1-unit increase in CoQ10 levels in participants with the CT/TT genotype was associated with a 3% reduction in asthenozoospermia risk. *Conclusion:* This study is the first in Jordan to provide evidence that an association between asthenozoospermia and CoQ10 levels in seminal plasma is influenced by the genetic background. CoQ10 protective role is affected by genetic variations of the *NQO1*^{P187S}. This finding highlights an interaction between genetic and environmental factors in determining the risk of asthenozoospermia. (www.actabiomedica.it)

Key words: asthenozoospermia, *NQO1* ^{P187S}, CoQ10 levels, HPLC, ARMS-PCR

Introduction

Infertility is a medical condition defined as the inability to conceive a child after one year or more of regular unprotected sexual intercourse (1,2). Infertility affects around 15% of couples worldwide, with the male factor accounting for approximately 50% of these cases (3, 4). Several factors can contribute to male infertility, including genetic and environmental factors (5-7). A reduction of sperm's motility below 40%, also defined as asthenozoospermia, negatively impacts the sperm's fertilization capacity (8).

It is widely documented that oxidative stress (OS) can lead to male infertility with a reported 30-80% of

infertile males demonstrating an elevation in the levels of reactive oxygen species (ROS) (9). The elevation could be explained by the presence of an imbalance between the generation of ROS with the antioxidant defense mechanisms of the body (10-12).

ROS are generated as a by-product of cellular metabolism, particularly during the electron transport chain in the mitochondria (13,14). Spermatozoa are particularly vulnerable to ROS. One mechanism attributes the above vulnerability to the high percentage of polyunsaturated fatty acids (PUFAs) in the plasma membrane of spermatozoa (15,16). ROS targets PUFAs within the sperm cell membrane. This leads to lipid peroxidation and a consequent change in fluidity and structural integrity of the membrane(17,18). This ultimately leads to impaired sperm function via a decrease in sperm motility and viability.

Coenzyme Q10 (CoQ10), also referred to as ubiquinone, is a lipophilic molecule found naturally in every cell of the human body and is present in two different states: oxidized (ubiquinone) and reduced (ubiquinol) (19,20). It plays a fundamental role in producing energy in the electron transport chain, where it transfers electrons between complexes I and II to complex III during the oxidative phosphorylation cascade (21,22).

Additionally, CoQ_{10} has other various vital functions within the cell. It is a powerful lipophilic antioxidant in its reduced form (ubiquinol) that protects cellular membranes from ROS-induced oxidative stress (23-25). It is interesting to note that pharmacological interventions that involved CoQ10 supplementation demonstrated an improvement in progressive sperm motility in individuals receiving the supplementation (26).

The *NQO1* gene encodes the NAD(P)H: quinone oxidoreductase 1 (NQO1) enzyme which plays a key role in cellular defense mechanisms and detoxification processes by directly reducing quinone to hydroquinone (27,28). The above detoxification process relies on a direct two-electron-mediated reduction that uses NADH as a substrate (29,30). This mechanism provides cellular protection against oxidative damage by limiting the generation of ROS through a one-electron reduction of quinones, as well as limiting a subsequent formation of superoxide radicals through the formation of semiquinone (31,32).

NQO1 also helps to maintain the reduced form of CoQ10 and protects membrane components from free radicals and lipid peroxidation (33). The genetic variant $NQO1^{P187S}$ (C>T (Pro187Ser)) (rs1800566) has been studied extensively (28,34). Previous studies have shown that the substitution of C with T results in a reduction in NQO1 enzymatic activity (35). It is thus not surprising that in contrast to individuals with the wild-type CC genotype, individuals with the homozygous TT genotype exhibit a significantly lower quinone reductase activity (28,36).

This study investigated the association of CoQ_{10} levels in the seminal plasma and $NQO1^{P187S}$ with asthenozoospermia. We also tested whether CoQ10 levels modify the association between the $NQO1^{P187S}$ polymorphism with asthenozoospermia.

Patients and Methods

Patients and sample collection

One hundred and twenty-seven patients were collected, including 84 asthenozoospermic, 22 terato asthenozoospermic (with lower morphology and motility), 14 oligo-terato-asthenozoospermic (with lower count, morphology, and motility) and seven oligo-asthenozoospermic patients (with lower count and motility). The age of the cases ranged between 23 – 57 years, while the age of the controls ranged between 24 – 50 years. Controls were 55 normozoospermic men with high sperm motility (a percentage of <32%) recruited from the IVF clinic at Prince Rashid Ben Al-Hasan Military Hospital in Irbid, Jordan. Ethical approval was obtained from the Institutional Review Board (IRB) at King Abdullah University Hospital/ Jordan University of Science and Technology in July 2022 (ID: 2022/149/44). Sample collection and experimental procedures were explained to all recruited participants. Written informed consent was obtained from each participant before sample collection.

Semen was obtained through masturbation from all participants after three to five days of sexual restraint. It was collected in a non-toxic, sterile container. Samples were then incubated at 37°C for 30 minutes to allow liquefication. A senior clinical embryologist

Table 1. ARMS primer sequence and PCR conditions used to genotype the *NQO1*^{P187S} variant.

Primers		Sequences (5'→ 3')	Product size base pair (bp)	PCR program
Outer primers	Forward	CACCTGAGAAGGCTAAAATTGGTAACGGC	381bp	30 cycles of 94oC for 30 seconds 60.3oC for 30 seconds 72oC for 5 minutes
	Reverse	TGCCTGGAAGTTTAGGTCAAAGAGGCTG		
Inner primers	Forward C allele	GCATTTCTGTGGCTTCCAAGTCTTAGCAC	175bp 261bp	
	Reverse T allele	GTGCCCAATGCTATATGTCAGTTGCGA		

performed seminal analysis according to the World Health Organization laboratory manual for examining and processing human seminal fluid (8).

Semen samples were centrifuged at 14000 rpm for 10 minutes to separate the seminal plasma from the pellet. The seminal plasma was stored in brown tubes at -20°C to avoid degradation, and the sperm pellet was collected in a microcentrifuge tube and stored at -20°C for DNA extraction.

DNA extraction

Genomic DNA was extracted from the sperm pellet that contains diploid cells (epithelial, leukocytes, and some immature germ cells) in addition to the haploid spermatozoa using the QIAamp DNA Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. The DNA quantity and purity were measured using a Nanodrop spectrophotometer (Thermofisher, USA) with an optimal density ratio of 260/280 of 1.8 or more. Following spectrophotometric evaluation, the DNA was run on a 1% agarose gel containing nucleic acid gel stain (ethidium bromide) and was visualized under UV illumination. This step served to confirm DNA quality.

Tetra-primer Amplification Refractory Mutation System (ARMS)-Polymerase chain reaction (Tetra-ARMS-PCR)

Tetra-ARMS PCR was used to genotype the C>T variant (rs1800566) of the *NQO1* gene. The amplification was performed using two forward and two reverse primers as outlined in Table 1. The PCR mixture was prepared in a total volume of 15 µl, including 0.5 µl of the outer primers and 1 µl of the inner primers, 8 µl of nuclease-free water, and f 1 µl of the extracted DNA. PCR conditions are listed in Table 1. The PCR product was loaded on a 2% agarose gel. The gel was run at 120 V for 45 minutes and was submerged in 1X Tris-borate-EDTA buffer, then visualized under UV light, with a 100 bp ladder used as a reference.

High-Performance Liquid Chromatography (HPLC)

The CoQ10 levels in seminal plasma were measured by HPLC as described by Boto et al. (37), with ultraviolent detection at 275 nm. Coenzyme Q9 was used as an internal standard.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (Version 27) and GraphPad Prism Version 9 (GraphPad Software, San Diego, CA, USA). To examine the differences between cases and controls in the CoQ10 median levels with either Interquartile Range (IQR) or 95 % Confidence Interval (CI), as well as among the different genotypes, the Mann-Whitney-U test and the Kruskal-Wallis H test with Dunn's multiple comparison non-parametric tests were employed.

The Chi-square test was used to compare genotypic and allelic frequencies between the study groups. Binary logistic regression analysis was used to assess the effect of CoQ10 and rs1800566 on the risk of asthenozoospermia after adjusting for age and BMI. An interaction term was included in the model to test the

interaction effect of CoQ10 and rs1800566 in addition to their main effects. The significance level was set at $P<.05$

Results

Participant demographics and semen parameters

Table 2 summarizes the baseline characteristics of the study participants. Upon the comparison of semen parameters between the study groups, significant differences were found in the percentage of motile sperm as well as the percentage of sperm with normal morphology. Patients with asthenozoospermia exhibited a lower percentage of motile sperm with a median of 10% (IQR=11) compared to 62% (IQR=13%) in the controls. Additionally, the percentage of sperm with normal morphology was significantly lower in the patients compared to controls (5% Vs. 16%) (P<.001). No significant difference in sperm concentration was observed between the two groups.

Genotype distribution

The PCR/gel electrophoresis results for the *NQO1*^{P187S} variant show three major bands: a 381 bp band for the outer primers, 175 bp for the homozygous wild-type allele, and 261 bp for the homozygous variant allele (Figure 1).

Table 2. Baseline characteristics of study participants**.**

Variables	**Controls** ***(n=55)***	**Cases** ***(n=127)***	***P* value**
Age (years)	30.00 (6.00)	33.50 (8.00)	0.004
BMI (kg/m^2)	28.06 (3.88)	28.45 (3.50)	0.29
Concentration (million/mL)	44.00 (37.00)	40.00 (44.00)	0.27
Motility (%)	62.00 (13.00)	10.00 (11.00)	<0.001
Normal morphology (%)	16.00 (9.00)	5.00 (5.00)	<0.001
***NQO1*P187S (rs1800566)** **CC** **CT/TT**	 35 (63.64%) 20 (36.36%)	 71 (55.91%) 56 (44.09)	0.33

Values are presented as median (IQR). The P-value was calculated using the Mann-Whitney U test.

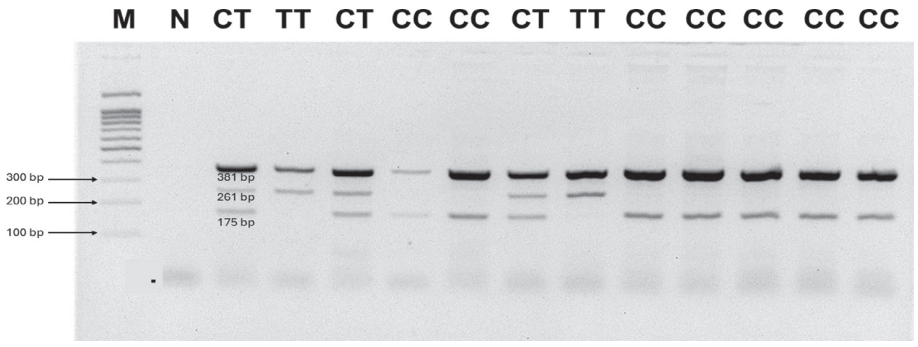

Figure 1. ARMS-PCR agarose gel electrophoresis image for *NQO1*^{P187S} polymorphism. Lane M is the molecular marker (100 bp ladder), and lane N is the negative control, *: primer dimer. The 381 bp band represents the outer primers, 175 bp for the homozygous wild type of CC, (175,261 bp) for heterozygous CT, and 261 bp for homozygous TT.

Table 2 also demonstrates the distribution of *NQO1* (rs1800566) genotypes among the cases and controls under a dominant model of inheritance. Our findings showed no statistically significant differences between the two groups (*P*=.33).

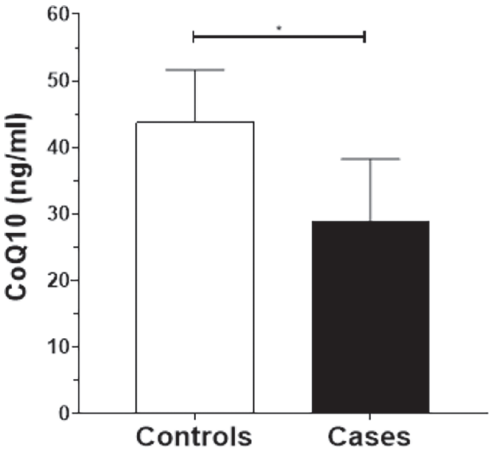

Figure 2. Comparison of Coenzyme Q10 levels (ng/ml) in seminal plasma between cases and controls. The bar represents the median (95% CI). Mann-Whitney U test was used to assess statistically significant differences. $n_{controls}$=55, n_{cases}=127. CoQ10: Coenzyme Q10. *P<0.05.

CoQ10 levels

Notably, as shown in Figure 2 asthenozoospermic patients exhibited significantly lower CoQ10 levels in their semen plasma compared to the control group (*P*=0.02). This result underscores the significant association between CoQ10 levels and asthenozoospermia.

Association between $NQO1^{P187S}$ genotypes and CoQ10 levels

Next, we wanted to determine whether significant differences in CoQ10 semen plasma levels existed among participants following stratification based on their $NQO1^{P187S}$ genotypes. Participants were divided into two groups; participants who carried the CC genotype of $NQO1^{P187S}$ represented one group while participants with the CT or TT genotypes represented the other group. As illustrated in Figure 3A, there was no significant association between CoQ10 levels and the $NQO1^{P187S}$ variant if the analysis was performed on the entire population. Noteworthy, CoQ10 levels were higher in participants carrying the CT/TT genotypes compared to participants carrying the

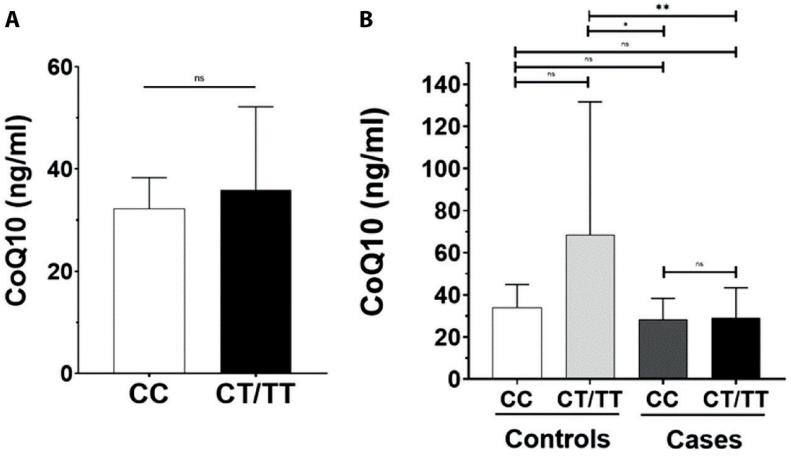

Figure 3. The association between CoQ10 levels and the genotype of the $NQO1^{P187S}$ polymorphism. Subjects were classified according to their genotype: CC or TT/CT. Levels of CoQ10 were compared separately between the different genotypes in A) total population and in B) cases and controls. n=181, n_{CC}= 106, $n_{CT/TT}$=75. The bar represents the median (95%CI). Mann-Whitney-U test and Kruskal-Wallis H test with Dunn's multiple comparison test were used to assess statistically significant differences. *P <0.05, **P<0.01, ns: not significant.

CC genotype (Figure 3A). This difference, however, was not statistically significant.

The above analysis was repeated following further stratification of the population according to their disease status (i.e. cases vs. controls). The results of this analysis are shown in Figure 3B. Analogous to the analysis performed on the entire population, it was demonstrated that a non-significant difference existed in CoQ10 levels among control participants carrying the CC genotype compared to controls carrying either CT or TT genotypes (CoQ10 levels were lower in CC genotype carriers).

Interestingly, patients (i.e. cases) carrying the CT or TT genotypes had significantly lower CoQ10 levels compared to CT/TT genotype carriers of the control group (Figure 3B), The findings above suggested that carrying the CT/TT genotypes of *NQO1*^{P187S} may modify the association of CoQ10 with asthenozoospermia or vice versa. The presence of such a relationship would suggest a gene–environment interaction.

To further test the above hypothesis and investigate the combined effect of both CoQ10 and *NQO1*^{P187S} polymorphism on the risk of asthenozoospermia, a logistic regression analysis was performed (Table 3). In this analysis, CoQ10 and the *NQO1*^{P187S} polymorphism were included as separate predictors of asthenozoospermia (i.e. outcome variable). Additionally, an interaction term including CoQ10 by the CT/TT genotype of *NQO1*^{P187S} was included as a variable in the model.

Using the above regression model with the described interaction terms, it was found that the risk of asthenozoospermia was about 8.5 folds higher in individuals carrying the *NQO1*^{P187S} CT/TT genotypes compared to the CC genotype (OR = 8.57,95% CI: 2.09-35.17, *P*=.003). According to the regression model, CoQ10 levels did not affect disease risk. However, as indicated by the interaction term in the model, a single unit increase (1 ng/ml) in CoQ10 levels in participants carrying the CT/TT genotype was associated with a 3% reduction in the risk of asthezoospermia (95% CI: 1-6%, *P*=.004). These results indicate that higher CoQ10 levels protect individuals who carry the CT/TT genotypes from asthenozoospermia.

Discussion

The present study shows that the seminal plasma CoQ10 levels are associated with asthenozoospermia. Asthenozoospemic patients had significantly lower CoQ10 levels than controls. Using a statistical multivariate model, this report also determined that individuals who carry the CT/TT genotypes of *NQO1*^{P187S} had a higher risk of asthenozoospermia. The same model showed that higher CoQ10 levels reduced the risk of asthenozoospermia in individuals carrying the high-risk CT/TT genotypes of *NQO1*^{P187S}. The above result indicated a tentative gene-environment interaction where higher CoQ10 levels in the semen mitigate the effects of carrying CT/TT genotypes of *NQO1*^{P187S}.

To our knowledge, this is the first study to investigate the association between the *NQO1*^{P187S} genotypes, seminal plasma CoQ10, and asthenozoospermia in a Jordanian cohort.

Previous studies have shown that 30-80% of male infertility and subfertility cases are attributed to

Table 3. Risk factors for asthenozoospermia.

Variable	OR (95%CI)	*P*-value[1]
Age (years)	1.10 (1.02-1.19)	0.01
BMI (kg/m^2)	1.04 (0.90-1.21)	0.60
CoQ10 concentration (ng/mL)	1.01 (0.99-1.02)	0.30
NQO1P187S genetic variant CC CT/TT	 Ref 8.57 (2.09-35.17)	 - 0.003
CoQ10 by CT/TT	0.97 (0.94-0.99)	0.004

P-value was calculated using binary logistic regression analysis. Outcome variable: having asthenozoospermia (Yes vs no). BMI, body mass index; CoQ10, Coenzyme Q10; CI, confidence interval; OR odds ratio.

oxidative stress (38). Sperms are particularly susceptible to oxidative stress due to the high PUFA content in their membranes and susceptibility to environmental factors. Peroxidation damage to sperms' plasma membranes affects their motility (2), leading to asthenozoospermia.

COQ10 is a crucial antioxidant in the seminal plasma and is negatively associated with sperm motility (39). Therefore, by scavenging free radicals, regenerating antioxidants, and preserving mitochondrial function, CoQ10 is an important factor in overall sperm health (40). Our results showed that asthenozoospermic patients had lower levels of CoQ10 compared to normozoospermic controls. These findings are consistent with other reports that found a negative association between these measures (41), and a positive effect of CoQ10 supplementation on semen parameters (42).

NQO1 enzyme, a major quinone reductase, plays an important role in the reduction of its substrate, the CoQ10 (43). The reduced form of CoQ10 (ubiquinol) is known for its role in inhibiting lipid peroxidation and generating other antioxidants (44). A mutation in the *NQO1* gene that leads to a substitution (P187S) from proline to serine ($NQO1^{P187S}$), can result in destabilization of the NQO1 enzyme, which results in decreased or even absence of its activity (45).

In this study, we investigated the association between *NQO1* polymorphism $NQO1^{P187S}$ (rs1800566) and CoQ10 levels in the semen of patients with asthenozoospermia. Our results showed no statistically significant difference between CoQ10 levels among carriers of different genotype categories of $NQO1^{P187S}$ (stratification was performed according to a dominant model of inheritance) in the total population. However, asthenozoospermic patients with the CT/TT genotypes had lower levels of CoQ10 compared to normozoospermic controls with the same genotype. One explanation of this could be that other factors not tested in this report are affecting the CoQ10 levels (i.e. other than $NQO1^{P187S}$ polymorphism). Another viewpoint refers to the potential role of the disease process itself in reducing the levels of CoQ10 resulting in lower levels among cases compared to controls carrying the same genotype.

We found no difference in the distribution of CC or CT/TT genotypes between asthenozoospermic and normozoospermic subjects. However, our regression model showed that following adjustment for age, BMI, and CoQ10, CT/TT carriers had an 8-fold higher risk of asthenzoospermia compared to CC genotype carriers. Moreover, an increase in CoQ10 alleviated the negative impact of CT/TT genotype in our sample and reduced the risk by 3%. This aligns with the literature suggesting that CoQ10 supplementation might be beneficial in treating or reducing the risk of asthenozoospermia, even in individuals with a genetic predisposition to the disease. The results of previous studies highlight the possibility of treating asthenozoospermia by the exogenous administration of CoQ10 (46, 47).

Several factors influence the levels/activity of CoQ10 in humans. It is known that CoQ10 production decreases with age (48). Optimal production occurs at approximately 25 years of age, and then production steadily declines, reaching approximately 50% of its optimal level at the age of 65 years (49). Furthermore, the levels of CoQ10 are reported to be reduced by medications, notably statins, and various disorders, such as cardiovascular and neurological disorders (50).

There is a limited number of studies on the influence of *NQO1* polymorphisms on the levels of ubiquinone and ubiquinol (CoQ10). A previous study found that heterozygous carriers (CT) exhibited significantly decreased CoQ10 levels compared to homozygous individuals (TT). However, the number of individuals in the above study with the homozygous TT genotype was only two (51).

A previous study showed that the frequency of the *NQO1* polymorphism varies by ethnicity, with Asians having the highest frequency of the homozygous variant allele (20.3%) and Caucasians having the lowest (5%) (52). Jordan is a country in the Middle East and North Africa (MENA) region. Literature on the frequency of the T allele in the MENA region is scarce. Considering our findings that the CT/TT genotypes could be a risk factor for infertility, future studies that estimate the frequency of the T allele in countries of the MENA region could be of utility for health personnel working in the urology/fertility sectors.

One of the main limitations of this report is the limited number of patients with the TT genotype tested for CoQ10 levels. Only eight individuals had the homozygous TT genotype, among them five individuals were from the control group. Another limitation is the inherent inability of case-control studies

to establish causal relationships and/or temporality between the variables. Indeed, based on the design of this study it cannot be concluded whether low CoQ10 levels are a cause or a symptom of asthenzoospermia.

Future clinical trials that examine the effect of CoQ10 pharmacological supplementation on sperm parameters are of better utility in examining causal relationships. Such studies could be designed in a way that allows the research team to examine if genetic variation at the *NQO1* locus modifies the effectiveness of CoQ10 supplementation. The above clinical trials could be complemented with longitudinal studies that track CoQ10 levels over time and correlate these levels with semen parameters.

Conclusion

In conclusion, the findings of this study demonstrate the presence of an association between asthenozoospermia and CoQ10 levels in seminal plasma. Moreover, it reveals that the CT/TT genotypes of the $NQO1^{P187S}$ polymorphism increase the risk of asthenozoospermia. Additionally, it is suggested that CoQ10 exerts its protective role in individuals who carry the CT/TT genotype of $NQO1^{P187S}$. Collectively, these results highlight an interaction between environmental and genetic factors in determining asthenozoospermia risk.

Ethics Approval: The research was approved by the Institutional Review Board (IRB) at Jordan University of Science and Technology and King Abdullah University Hospital in July 2022 (Ref: 2022/149/44) and followed the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964 and Declaration of Tokyo, 1975, as revised in 1983). Participants obtained consent forms before their inclusion in the study.

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Authors Contribution: OB: Obtained funding and major supervision for the research, including significant guidance in the study design, data interpretation, and manuscript preparation. D.H and HK: Collaborated on designing the work, conducting experiments, writing and revising the manuscript. M.A.A: co-supervised the work, performed the data analysis, helped in manuscript writing, and critically revised the work. E.Z: performed the statistical analysis and reviewed the article. All authors had read and approved the manuscript before submission.

Declaration on the Use of AI: No chatbot has been used in the preparation of this work.

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