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Investigating the Relationship Between Semen Paraoxonase-3 Concentration, Sperm Parameters, and DNA Fragmentation in Fresh or Post-Thaw Semen of Normozoospermic Men

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Abstract

Background: PON3 is associated with high-density lipoprotein (HDL) and plays a protective role against oxidative damage to low-density lipoprotein (LDL). The purpose of the current study was to investigate the effect of semen paraoxonase-3 (PON3) concentration on sperm parameters and DNA fragmentation in both fresh or post-thaw semen.

Methods: This study analyzed PON3 levels in 30 normospermic semen samples obtained from men who attended Dezful Infertility Center and provided informed consent. The samples were analyzed before and after cryopreservation for sperm motility, DNA fragmentation, and seminal PON3 levels. Data analysis was performed using SPSS software and statistical differences were evaluated using ANOVA. A $p \leq 0.05$ was considered statistically significant.

Results: There was no significant difference in sperm morphology (p=0.37) and count (p=0.25) before and after freezing at different levels of PON3. The highest levels of progressive motility were observed in samples with the highest PON3 concentrations both before (p=0.01) and after freezing (p=0.02), whereas non-progressive motility was significantly greater in samples with the lowest PON3 concentrations at both time points (p=0.01). Sperm DNA fragmentation significantly decreased before or after freezing as PON3 levels increased (p=0.03).

Conclusion: This study suggests that PON3 levels may serve as an indicator for both sperm motility and DNA fragmentation pre- or post-cryopreservation, potentially contributing to future clinical research.

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Introduction

Antioxidants are categorized into two types of enzymatic and non-enzymatic. Enzymatic antioxidants, also known as natural types, are present in various forms, one of which is paraoxonase. Paraoxonase is an enzyme associated with HDL and plays a role in preventing oxidative modifications of LDL. With its antioxidant properties, paraoxonase protects cells against oxidative stress (1).

The paraoxonase (PON) gene family includes PON1, PON2, and PON3. These genes are located on chromosome 7 and have been found to be expressed in various types of spermatogenic cells. While all the three genes were expressed in sper-

matogonia, spermatocytes, and round and elongated spermatids, only PON1 and PON3 were detected in Sertoli and Leydig cells. The location of PON proteins at different stages of sperm development suggests they may play a role in sperm function and development. This information was corroborated through immunohistochemical analysis of mouse testicular tissue (2).

This enzyme is also present in seminal plasma and can affect the quality and parameters of sperm. Freezing is an operational process that, with the help of assisted reproductive techniques, preserves and maintains cells at a very low temperature for a long period of time without reducing their viability and performance. It is generally considered an important part of fertility treatment and techniques (3).

Despite the recognized advantages of sperm cryopreservation in fertility preservation, extensive research has demonstrated that the process—comprising cooling to the freezing point followed by thawing—induces substantial and deleterious effects on sperm viability and motility (4). These damages may include alterations to the lipids in the cell membrane and acrosome, as well as impairments in sperm motility, sperm viability, and also DNA integrity (5).

The mechanism of the mentioned damages can also be related to osmotic pressure difference, cold shock, formation of crystals inside the cell, and overproduction of reactive oxygen species (ROS) or a combination of these factors (6). During sperm cryopreservation, the movement of water and salts across the plasma membrane, resulting in changes in cell volume, induces variations in osmotic pressure, which can consequently lead to the generation of ROS (7).

Furthermore, cold shock induces the production of ROS (8). Although the production of ROS plays an important and vital role in sperm function such as capacitation, acrosome reaction, and binding to the zona pellucida, excessive levels of these species can damage mitochondria, cell membranes, and DNA, thereby reducing sperm motility and viability (9).

Under normal conditions, sperm and seminal plasma possess an antioxidant system that counteracts ROS and ultimately prevents damage to sperm. However, an imbalance between the levels of antioxidants and ROS can lead to cellular damage (10).

The distinctive cellular characteristics of spermatocytes, including their cellular membrane composition, high mitochondrial content, low cytoplasmic volume, and relatively low levels of antioxidants, render sperm particularly susceptible to oxidative damage from free radicals. Antioxidants represent the primary defense mechanism against oxidative stress caused by free radicals (11). Despite the known importance of oxidative stress in sperm cryodamage, no studies have investigated whether PON3 levels influence post-thaw sperm quality. This study is the first to independently examine the effects of varying levels of PON3 on sperm motility, DNA fragmentation, and morphology both before freezing and after thawing. Therefore, the aim of this study was to determine the relationship between semen PON3 concentration and sperm parameters, as well as DNA fragmentation, in both fresh and post-thaw semen samples of normozoospermic men.

Methods

This research was approved by the Ethics Committee of Dezful University of Medical Sciences (IR.DUMS.REC.1398.024). Prior to the study's initiation, all participants provided written informed consent. The subjects were selected from patients attending Omolbanin Infertility Center at Ganjavian Hospital, Dezful, Iran following the acquisition of their informed consent.

This study was conducted on a randomly selected group of 30 normozoospermic semen men, in accordance with the World Health Organization (WHO) standards. The samples were collected in sterile containers by the participants via self-ejaculation. The semen samples were incubated at 37°*C* for 20 to 30 *min* to liquefy. All procedures were performed on two aliquots of each sample, prior to freezing, and after thawing. A 0.5 *ml* aliquot of semen was taken and mixed with an equal volume of cryoprotectant solution in a sterile cryovial, resulting in a final volume of 1 *ml* for freezing. It was then kept for 10 *min* at room temperature and 20 *min* in nitrogen vapor, before finally being placed in a nitrogen tank at -196°*C*.

Regarding sampling procedure, 30 semen samples were collected. Each sample was divided into two equal aliquots; one was subjected to freezing and thawing, while the other was used for fresh semen analysis. After measuring the paraoxonase levels in each of the 30 samples from both groups, the samples were categorized into distinct paraoxonase concentration ranges (0–10, 10–20, 20–

30, and 30–40 *pg/ml*), based on empirical data. The quality of semen and DNA fragmentation were then assessed within each range.

A 1 *ml* aliquot of semen was examined under a light microscope at 400x magnification by a single observer. Subsequently, sperm count, motility (progressive, non-progressive, and immotile sperm), and morphology (shape and size) were assessed using a Neubauer chamber and a counting device.

For this purpose, sperm were randomly selected and examined under several microscopes, with the resulting data averaged. Sperm morphology was assessed using the standard Papanicolaou (Pap) staining method. Additionally, eosin staining (0.5% in saline) was employed to distinguish live and immotile sperm from non-viable sperm. Fresh samples were tested at room temperature (24-20°*C*), and frozen samples were thawed after two weeks and underwent all the aforementioned stages.

To separate the seminal plasma from the sperm fraction, samples were centrifuged at 2000 *rpm* for 10 *min*, and the supernatant was collected and stored at –70°*C* until all samples were accumulated. The quantitative measurement of PON3 in semen was performed using an ELISA kit (My BioSource, USA). The remaining pellet was then washed twice with VitaSperm washing medium (centrifuged at 300×*g* for 5–10 *min* each), and subsequently centrifuged again. Finally, a sperm suspension containing 15–20 million spermatozoa was prepared. The sample was subsequently subjected to sperm DNA fragmentation analysis using the SDF kit (Avicenna Research Institute, Iran) protocol, followed by staining on a microscope slide for evaluation. The level of DNA fragmentation in sperm was assessed using the sperm DNA fragmentation (SDF) test. In this experiment, sperm chromatin and DNA were exposed to acidic denaturation on a microgel platform. Subsequently, following the removal of chromatin proteins, the DNA strands were allowed to disperse maximally around the sperm head, forming a halo that was visualized through specific staining. In contrast, sperm with fragmented DNA exhibited limited or no extension of DNA strands, resulting in the absence or formation of only very small halos around the sperm head.

Statistical analysis: Data analysis was performed using SPSS software, version 20 (IBM, USA). One-way ANOVA was employed to compare sperm parameters across PON3 concentration subgroups (0–10, 10–20, 20–30, 30–40 *pg/ml*), followed by Fisher's least significant difference (LSD) test to identify statistically significant differences between the groups. In all the statistical analyses, $p \leq 0.05$ was considered significant.

Results

Based on measured PON3 concentrations, samples were divided into four groups: 0-10 *pg/ml* (n=7), 10-20 *pg/ml* (n=9), 20-30 *pg/ml* (n=8), and 30-40 *pg/ml* (n=6). Based on the results of table 1, sperm count and normal morphology did not differ significantly across the various concentrations of paraxonase in fresh semen. However, progressive motility was significantly increased at higher levels of paraxonase ($p \leq 0.05$). The percentage of immotile sperm was also significantly lower in samples with higher levels of paraxonase concentrations compared to other groups ($p \leq 0.05$), with the highest proportion of immotile sperm observed in the of 0-10 *pg/ml* paraxonase group ($p \leq 0.05$).

According to the results presented in table 2, the lowest level of sperm DNA fragmentation was observed in the highest paraxonase concentration range (30-40 *pg/ml*) ($p \leq 0.05$). According to the results presented in table 3, sperm count and normal morphology did not differ significantly across the various paraxonase concentrations in semen after thawing. Post-thaw progressive motility was highest at 30-40 *pg/ml* paraxonase levels, showing a significant difference ($p \leq 0.05$). The greatest proportion of immotile sperm was observed at the

Table 1. The relationship between paraoxonase level in fresh semen before freezing and sperm quality parameters

Paraoxonase (*pg/ml*)	Count	Normal morphology	Progressive motility	Sluggish progressive motility	Non-progressive motility
0-10	65.5±2.11 [a]	15.25±2.14 [a]	20.5±2.77 [c]	10.5±2.4 [a]	15±2.12 [a]
10-20	70.5±1.27 [a]	15.55±1.25 [a]	22.5±3.28 [c]	10.5±3.3 [a]	10±3.3 [b]
20-30	70±2.51 [a]	12.9±3.4 [a]	45±3.29 [b]	10±3.13 [a]	10.5±2.75 [b]
30-40	65.5±2.32 [a]	15.75±1.5 [a]	60.5±2.21 [a]	12.5±.2.2 [a]	5.5±2.3 [c]

Letters a, b, and c denote significant differences between groups ($p \leq 0.05$)

Table 2. The relationship between paraoxonase level in fresh semen before freezing and sperm DNA fragmentation

Paraoxonase (*pg/ml*)	DFI <15%	DFI 15-30%	DFI >30%
0-10	39.12±2.33 [b]	15.37±2.17 [a]	52.21±1.87 [a]
10-20	45.5±2.46 [b]	25.24±2.77 [a]	34.5±2.36 [b]
20-30	43.26±2.47 [b]	42.65±3.14 [a]	30.45±3.13 [b]
30-40	65.2±3.14 [a]	45.18±2.51 [a]	29.8±2.95 [b]

Letters a, b and c denote significant differences between groups ($p \leq 0.05$)

Table 3. The relationship between paraoxonase level in semen after freezing and sperm quality parameters

Paraoxonase (*pg/ml*)	Count	Normal morphology	Progressive motility	Sluggish progressive motility	Non-progressive motility
0-10	55±2.81 [a]	10.5±2.17 [a]	10.25±2.7 [c]	5.5±3.27 [a]	15±2.41 [a]
10-20	50.5±2.30 [a]	10.5±3.22 [a]	25.5±3.16 [b]	5±2.36 [a]	10.5±2.12 [b]
20-30	50.5±3.12 [a]	10±2.37 [a]	25±2.65 [b]	5.5±2.71 [a]	10.9±3.5 [b]
30-40	55.5±2.55 [a]	10.25±1.91 [a]	45.5±3.78 [a]	5.5±1.66 [a]	5.5±2.3 [c]

Letters a, b, and c denote significant differences between groups ($p \leq 0.05$)

Table 4. The relationship between paraoxonase level in semen after freezing and sperm DNA fragmentation

Paraoxonase (*pg/ml*)	Less than 15% fragmentation	Fragmentation between 15-30%	More than 30% fragmentation
0-10	30.15±2.71 [b]	15.25±2.28 [b]	44.15±2.11 [a]
10-20	31.4±3.97 [b]	18.21±2.15 [b]	42.88±1.37 [a]
20-30	34.23±2.16 [b]	36.24±2.44 [a]	31.15±2.21 [b]
30-40	45.55±2.78 [a]	40.12±3.26 [a]	28.21±1.66 [b]

Letters a, b, and c denote significant differences between groups ($p \leq 0.05$)

0-10 *pg/ml* paraoxonase range (the lowest concentration), following thawing ($p \leq 0.05$).

In table 4, sperm DNA fragmentation did not show a significant difference across 0-10, 10-20, and 20-30 paraxonase levels after freezing, with DNA fragmentation remaining below 15%. However, the lowest level of sperm DNA fragmentation after freezing was observed in the highest paraxonase concentration range (30-40 *pg/ml*) ($p \leq 0.05$).

Discussion

In recent years, extensive research has been conducted on the impact of oxidative stress and reactive oxygen species on human sperm function and their role in male infertility. Sperm cells are constantly exposed to oxidative environments from the time of their formation in the testes through their journey into the female reproductive tract. Oxidative stress has been identified as a leading cause of male infertility and has also been linked to higher levels of spontaneous hyperactivity of sperm. These findings highlight the importance of understanding the role of oxidative stress in male fertility and developing effective treatments to address the issue (12, 13).

PON is an enzyme that is known to play a role in protecting cells against oxidative stress. When it comes to sperm quality and DNA fragmentation, oxidative stress can be a major factor in reducing fertility (2). Some studies have shown that PON activity is correlated with sperm quality, and that lower levels of PON activity are associated with higher levels of sperm DNA fragmentation (2, 3, 14).

Paraxonase is present in seminal plasma and it can affect the quality and parameters of sperm. Therefore, in this study, the levels of this enzyme were measured in both fresh and thawed semen samples. Also, the quality and sperm parameters as well as sperm DNA fragmentation were evaluated before and after freezing and compared with different levels of paraxonase. Based on the results of this study, sperm progressive motility was significantly higher in semen samples with higher levels of paraxonase. Additionally, sperm DNA fragmentation was significantly lower in patients who had higher levels of paraxonase in their semen.

In a study conducted in 2009, the level of paraxonase 1 was measured in the seminal plasma of men and its relationship with sperm parameters

was examined. The researchers stated that in men with abnormal semen parameters, the level of paraxonase was significantly lower and that paraxonase may play a key role in male infertility, which is consistent with the findings of our study (15). In a study by Aitken et al., it was reported that a decrease in paraxonase was associated with an increase in homocysteine thiolactone, which interfered with lysine residues in sperm. Ultimately, this compromises sperm integrity, which is partially consistent with the findings of our study (16).

In a study by Efrat et al., it was demonstrated that paraoxonase 1 possesses antioxidant properties that influence sperm quality; however, the relationship between PON1 and semen parameters, as well as its impact on semen quality, remains unclear. The results showed that purified PON1 inhibits rapid sperm motility in a dose-dependent manner. Seminal fluid PON1 activity was positively correlated with sperm count but negatively associated with acrosome reaction. These findings are not consistent with our study, although our study specifically focused on paraoxonase 3 (17).

In a study conducted by Gong et al., the activity of seminal PON1 was examined in liquid samples from 270 infertile men and 50 healthy fertile men using spectrophotometric methods. The findings showed that in the infertile group, PON1 activity in semen was significantly lower than that in the control group. In patients with decreased sperm motility, a decrease in seminal PON1 activity was also observed. Seminal PON1 activity was positively correlated with sperm viability but inversely correlated with the percentage of sperm exhibiting abnormal morphology (18).

Catalán et al. indicated that oxidative stress effects cryopreserved horse sperm, showing that ejaculates with poor post-thaw motility and membrane integrity had higher lipid peroxidation and DNA fragmentation, which were associated with elevated ROS and oxidative stress indices. Conversely, post-thaw sperm quality showed a negative correlation with seminal plasma antioxidants. Their results suggest that variability in cryopreservation success depends on the balance of seminal plasma antioxidants, emphasizing the role of PON1 and other antioxidants in mitigating oxidative stress damage during freezing (19).

Efrat et al. investigated the relationship between PON1 activity and human sperm hyperactivity and spontaneous acrosome reaction (sAR). They found that PON1 inhibited both sperm hyperactivity and sAR in a dose-dependent manner, suggesting that it may have a positive effect on fertility by preventing premature spontaneous sperm capacitation and acrosome reaction before reaching the female genital tract (20).

Conclusion

Overall, our findings suggest that PON level may be an important biomarker for assessing sperm quality and DNA fragmentation, particularly in the context of frozen-thawed semen. Further research is needed to fully understand the relationship between PON level and male fertility; however, these preliminary findings are promising.

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Conflict of Interest

Authors reported no conflict of interests.

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