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Levels of DNA, Protein, Lipid Oxidation and Apoptosis Biomarkers in Semen of Men with Hyperviscous Semen: A Cross-Sectional Study

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Abstract

Background: Semen hyperviscosity is a threatening cause of abnormal spermatozoa and infertility in men. We aimed to evaluate oxidative stress, antioxidants depletion and sperm apoptosis as main reasons for poor quality of spermatozoa in men with hyperviscous semen.

Materials and Methods: In this cross-sectional study, ejaculate specimens were collected from fertile (n=102) and infertile men with hyperviscous semen (n=123) and without semen hyperviscosity (n=143). Total antioxidant capacity (TAC), glutathione (GSH), malondialdehyde (MDA), protein carbonyl (PC), 8-hydroxydeoxyguanosine (8-OHdG), and were measured in semen samples to estimate oxidative stress status. Gene expression pattern of *BAX*, *CASPASE-9*, *CASPASE-3*, and *BCL2* was assessed to estimate sperm apoptosis.

Results: The average of sperm count, normal morphology, normal motility, and sperm vitality in men with hyperviscous semen was significantly lower than infertile subjects without hyperviscous semen (P<0.01). Men with hyperviscous semen exhibited higher levels of PC (8.34 ± 1.03 nmol/mg vs. 6.01 ± 0.93 nmol/mg, P=0.008), MDA (1.14 ± 0.27 nmol/ml vs. 0.89 ± 0.22 nmol/ml, P=0.031), 8-OHdG (259.71 ± 24.59 ng/ml vs. 197.13 ± 18.47 ng/ml, P=0.009), but lower TAC contents (1250.44 ± 66.23 µM/L vs. 1784.31 ± 89.87 µM/L, P=0.018) and GSH (3.82 ± 1.05 µM vs. 5.89 ± 0.87 µM, P=0.021) than men with non-viscous semen. The expression of *BAX*, *CASPASE-3* and *CASPASE-9* genes in men with hyperviscous semen was significantly increased by 1.39-fold (P=0.041), 1.47-fold (P=0.046), 1.29-fold (P=0.048), respectively, as compared with the infertile subjects without hyperviscous semen. However, *BCL2* expression in infertile men without hyperviscous semen was higher compared to those with hyperviscous semen (1.36-fold, P=0.044).

Conclusion: Hyperviscous semen is markedly associated with depletion of seminal plasma antioxidants, sperm membrane lipid peroxidation, DNA and protein oxidation, and sperm apoptosis. Antioxidant therapy might be considered as a valuable strategy to protect sperm cells against oxidative damage in cases with seminal fluid hyperviscosity.

Keywords: DNA Oxidation, Lipid Peroxidation, Protein Oxidation

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Introduction

Human infertility is a main health problem that has affected many couples worldwide (1). Approximately 40-50% of all infertility cases are due to male factor infertility (2). In general, impaired spermatogenesis and poor sperm quality are important causes of male infertility (3). Therefore, identification of factors that impose either direct or indirect effects on human spermatogenesis and spermatozoa functioning, is essential. A wide range of risk factors from environmental pollutants and toxicants to epigenetic changes and genetic disorders have been identified that affect human spermatozoa and cause male infertility (4, 5). However, there are many risk factors with underlying mechanisms on sperm function and fertility potency that have remained unclear (3).

Semen hyperviscosity or failure in liquefaction of semen is an important cause of idiopathic male infertility (6). It is characterized by an unusual and very thick appearance of semen that can cause serious impairment of sperm maturation and function (7). Hyperviscous semen can also reduce sperm movement and subsequently inhibits normal sperm

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progression through the female genital tract (8). Until now, some studies have reported the adverse effects of semen hyperviscosity on sperm motility and count, but the underlying mechanisms, in which it affects sperm function remain unclear (9, 10). Since semen viscosity is a critical factor to achieve fertilization, in-depth studies are essential to consider the effect of semen hyperviscosity and its underlying mechanisms on sperm function (11). Recent evidence has recommended that oxidative stress caused by massive production of reactive oxygen species (ROS) and antioxidant depletion may be a main mechanism of action, disturbing hyperviscous sperm function and spermatozoa quality (7, 12); however, there are very limited studies to support this theory.

Oxidative stress is a pathological condition that occurs as the loss of balance between ROS generation and the contents of antioxidants defense systems against them (13). ROS like hydroxyl radicals ($OH^{\cdot}$) and superoxide anion ($O2^{\cdot}$) are highly reactive oxidizing agents that easily react with biomolecules to compensate their deficit electron (14). However, human semen contains various antioxidants such as glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), vitamins E, C, copper, zinc and glutathione (GSH) that are referred to as total antioxidant capacity (TAC) (15). These antioxidant defense systems neutralize the detrimental effects of ROS. During uncontrolled generation of ROS, the effective concentration of seminal antioxidants will be reduced and consequently ROS impair normal sperm function, causing sperm apoptosis by peroxidation of sperm membrane unsaturated fatty acids, as well as oxidation of sperm DNA and proteins (16, 17). ROS may also attack the membrane fluidity of spermatozoa, which is subsequently associated with reduced ability of spermatozoa for oocyte fusion and fertilization (18). We assume that oxidative stress and antioxidant depletion may be a major mechanism, by which semen hyperviscosity affects sperm quality and function, and enhances sperm apoptosis. Since the relationship between seminal fluid viscosity and changes in levels of oxidative stress and sperm apoptosis is not well-elucidated, we designed this research to determine whether the hyperviscous semen is associated with seminal plasma antioxidant depletion, oxidative stress and sperm apoptosis.

Material and Methods

Semen samples

In this cross-sectional study, 368 ejaculate samples were collected from normal individuals (n=102), infertile men with hyperviscous semen (n=123) and infertile men without semen hyperviscosity (n=143) at Behta Clinical Laboratory in Rasht (Guilan, Iran) from April 2019 to May 2021. After obtaining institutional review board approval (IR.BMSU.REC.1396.75) and informed consent, semen samples were collected by masturbation within a sexual abstinence for three days. Before samples collection, a checklist was provided, in which basic demographic information of men, including age, weight, smoking habits or opiate using were recorded. Men with history of opiate using, testicular damage or developmental disorders in sex organs, infectious diseases, varicocele, cryptorchism, as well as those using antioxidant supplementation during at least 3 weeks prior to sample collection, were excluded from the study. Only healthy individuals with normal sperm parameters (normozoospermia) and men with idiopathic infertility and hyperviscous semen were entered into the study. The consistency of semen was assessed by placing a glass rod into the liquefied semen samples and measuring the length of the thread on withdrawal of the rod. Semen with a thread length above 2 cm were considered as normal consistency, but hyperviscous ejaculates showed a thread length >2 cm (19).

Semen analysis

Following liquefaction of ejaculates at room temperature (after 25-30 minutes), semen volume was measured. One hundred microliter (µl) of each semen sample was applied to examine the sperm count, sperm motility, morphology, and viability under a light microscopic (Nikon TS100, USA). Sperm quality parameters (sperm counts, motility and viability) were evaluated according to the World Health Organization (WHO) criteria (20), but normal morphology of spermatozoa was examined according to Kruger's strict criteria (21). Sperm viability was determined via eosin staining method (22). Briefly, 10 µl of semen samples were placed on sterile slides and each sample was mixed with 10 µl of 5% eosin and white cells were counted to determine the live cells.

Total antioxidant capacity measurement

Seminal plasma TAC was assessed by Ferric Reducing of Antioxidant Power method (FRAP) described by Benize (1996) (23). FRAP is a colorimetric assay that uses antioxidants as reductants for the conversion of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion and estimates the antioxidant capacity of a particular sample. Approximately, 500 µl of ejaculates were centrifuged at 1400 × g, at 4°C for 7 minutes, and the supernatants were diluted with distilled water (10-fold). The solution was urgently used for TAC assay using the FRAP reagent [300 mM Acetate buffer, pH=3.6, 10 mM 2,4,6-Tri(2-pyridinyl)-S-triazine (TPTZ) and 20 mM Ferric chloride].

Glutathione assessment

Reduced GSH was determined using the Tietz method (24). Approximately, 400 µl of NaH2PO4 (3 mM) with 50 µl of 0.04% 5,5-dithiobis 2-nitrobenzoic acid (DTNB) in 0.1% sodium citrate was added to seminal plasma samples. The absorbance of the solution was recorded at 410 nm using a UV/vis spectrophotometer (UV-1600PC, USA).

Malondialdehyde measurement

Seminal plasma malondialdehyde (MDA) level was measured as a biomarker of membrane lipid peroxidation. It was determined using the thiobarbituric acid reaction method

(TBAR) previously described by Hosseinzadeh Colagar et al. (25). Briefly, semen specimens were centrifuged at 2000 × g for 7 minutes. Supernatants (100 µl) were diluted with distilled water (900 µl) and then transferred into a sterilized glass tube containing 500 µl of TBA reagent. The mixture was then incubated for 1 hour in boiling water and then centrifuged at 4000 × g for 8 minutes following cooling at room temperature. The absorbance of each supernatant was read in a UV/vis spectrophotometer at 534 nm.

8-hydroxydeoxyguanosine measurement

8-hydroxydeoxyguanosine (8-OHdG) level was measured as a biomarker of sperm DNA oxidation (26). It was assessed using an enzyme-linked immunosorbent assay kit (ELISA kit, China). The severity of the reaction product color was assessed by ELISA reader (Beckman Coulter, USA) at 450 nm.

Protein carbonyl measurement

Protein carbonyl (PC) was assessed as a biomarker of protein oxidation. Its level in seminal fluids (50 µL) was detected according to a previous method (27, 28). The concentration of carbonyle was measured based on the molar extinction coefficient of 2,4-dinitrophenylhydrazine (DNPH) and expressed as nmol/mg of the protein.

Gene expression analysis

In this study we evaluated the expression of *CASPASE-3*, *BAX*, and *CASPASE-9* genes (as biomarker of apoptosis), as well as *BCL2* (as an anti-apoptosis biomarker). Briefly, 1 ml of liquefied ejaculate specimens were centrifuged at 2500 × g for 10 minutes and then the pellets containing spermatozoa were used for RNA extraction. RNX-Plus Kit (SinaClon, RN7713C) was used to extract the total RNAs of spermatozoa. The quantity of the extracted RNAs was measured by Nanodrop ND-1000 spectrophotometer. For cDNA synthesis at 42°C for 1 hour, a revert aid reverse transcriptase (Thermo Fisher Scientific, USA) along with random hexamer primers were applied. A Rotor Gene 6000 thermocycler in 41 cycles was used for amplifications. Each reaction contained 5 µl master mix and 100 nmol primers. The specific primer sequences for candidate genes are presented in Table 1. The mRNA levels of the study genes were normalized relative to the *GAPDH* mRNA levels. Eventually, the $2^{-\Delta Ct}$ method was applied to calculate the relative expression of the studied genes (29).

Statistical analysis

In this study, parametric data are shown as means ± SD. Comparison of the means of each of the quantitative parameters between the three groups was completed using one-way ANOVA: Post Hoc-Tukey test. SPSS software (IBM, USA, version 22) was applied for data analysis. A $P<0.05$ was considered as significant.

Table 1: Primer sequences for the genes used in the study

Genes	Primer sequence (5′-3′)
BAX	F: GAGGATGATTGCTGATGTGGATA
	R: CAGTTGAAGTTGCCGTCTG
BCL2	F: GGAGCGTCAACAGGGAGATG
	R: ACAGCCAGGAGAAATCAAACAGA
CASP-2	F: AAGCCGAAACTCTTCATCATTCA
	R: GCCATATCATCGTCAGTTCCAC
CASP-9	F: ATGACCACCACAAAGCAGTCC
	R: CGTGACCATTTTCTTGGCAG
GAPDH	F: AAGTTCAACGGCACAGTCAAGG
	R: CATACTCAGCACCAGCATCACC

Results

The comparison results of the mean of semen parameters among different groups are summarized in Table 2. No significant differences were found in the means of age and body mass index (BMI) among the three groups. The means of sperm count, normal morphology, vitality and normal morphology were significantly higher in healthy individuals than the infertile men with or without hyperviscous semen ($P<0.001$). While there was no significant difference in the mean of ejaculate volume between men with and without hyperviscous semen, a significant decrease was observed in the mean of sperm count (1.69-fold, $P=0.038$), sperm vitality (1.68-fold, $P=0.041$), sperm motility (1.44-fold, $P=0.045$) and normal morphology of spermatozoa (2.70-fold, $P=0.018$) in men with hyperviscous semen as compared to infertile individuals without hyperviscous semen (Table 2).

Table 2: Comparison of the semen analysis between three groups

Sperm parameters	Control	Infertile men		P value
		Hyperviscous semen	Non-hyperviscous semen	
Age (Y)	34.67 ± 2.48	32.79 ± 3.59	35.12 ± 3.86	0.76
BMI (Kg/m^2)	24.18 ± 1.09	23.72 ± 1.48	24.77 ± 1.22	0.27
Semen volume (ml)	4.82 ± 1.26	3.14 ± 1.12*	3.26 ± 1.33*	0.043
Sperm count (×10^6/ml)	78.63 ± 18.35	18.41 ± 9.67*	31.27 ± 18.42*	<0.001
Total sperm count (×10^6)	378.99 ± 23.12	57.80 ± 10.83*	98.18 ± 20.63*	<0.001
Sperm vitality (%)	67.44 ± 9.37	21.17 ± 5.39*	35.57 ± 6.82*	<0.001
Sperm motility (%)	62.32 ± 8.72	19.77 ± 5.14*	28.64 ± 6.54*	<0.001
Normal morphology (%)*	14.84 ± 4.65	3.22 ± 2.24*	8.72 ± 1.48*	<0.001

Results are presented as mean ± SD. BMI; Body mass index. According to Kruger's criteria, One-Way ANOVA; Post Hoc-Tukey test was applied to compare mean value of all parameters between all groups, *; P<0.001, compared to control group.

Comparison results of the mean of FRAP value, PC, MDA and 8-OHdG contents in seminal plasma of all groups are shown in Table 3. A significant difference was observed among the mean values of the oxidative biomarkers in the three groups. Collectively, the control fertile group had significantly higher FRAP and GSH mean levels, but lower contents of seminal PC, MDA and 8-OHdG, when compared to both infertile groups (P<0.001). Infertile men without hyperviscous semen exhibited higher FRAP values (1.42-fold, P=0.047) and GSH (1.54-fold, P=0.037) in their seminal plasma when compared to those with hyperviscous semen. In contrast, men with hyperviscous semen showed significantly higher mean levels of MDA (1.28-fold, P=0.048), PC (1.39-fold, P=0.041) and 8-OHdG (1.32-fold, P=0.046) in their seminal plasma than those without semen hyperviscosity (Table 3).

There was a significant difference in expression of *BAX*, *BCL2*, *CASPASE-3* and *CASPASE-9* genes among the groups (P<0.001). Men with hyperviscous semen had significantly higher degree of *BAX* (3.52-fold, P<0.001, Fig.1A), *CASPASE-3* (2.81-fold, P<0.001, Fig.1B) and *CASPASE-9* (2.56-fold, P<0.001, Fig.1C) genes expression, but lower expression of *Bcl2* gene (2.41-fold, P<0.001, Fig.1D) when compared to the control. Moreover, the expression of *BAX*, *CASPASE-3* and CASPASE-9 genes in men with hyperviscous semen was significantly increased by 1.39-fold (P=0.041), 1.47-fold (P=0.046), 1.29-fold (P=0.048), respectively, as compared with infertile subjects without hyperviscous semen. The expression of *BCL2* in infertile men without hyperviscous semen was greater than that in men with hyperviscous semen (1.36-fold, P=0.044, Fig.1).

Table 3: Comparison of oxidative stress biomarkers between three groups

Sperm parameters	Control	Infertile men		P value
		Hyperviscous semen	Non-hyperviscous semen	
FRAP (µM/L)	2651.8 ± 67.38	1250.44 ± 66.23*	1784.31 ± 89.87*	<0.001
GSH (µM/L)	9.42 ± 1.18	3.82 ± 1.05*	5.89 ± 0.87**	<0.001
PC (nmol/mg)	3.89 ± 1.09	8.34 ± 1.03*	6.01 ± 0.93**	<0.001
MDA (nmol/ml)	0.53 ± 0.11	1.14 ± 0.27*	0.89 ± 0.22*	<0.001
8-OHdG (ng/ml)	136.41 ± 16.12	259.71 ± 24.59*	197.13 ± 18.47*	<0.001

Results are presented as mean ± SD. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of all parameters between all groups. FRAP; Ferric reducing of antioxidant power, GSH; Reduced glutathione, PC; Protein carbonyl, MDA; Malondialdehyde, 8-OHdG; 8-hydroxydeoxyguanosine, *; P<0.001, and **; P<0.01 compared to control group.

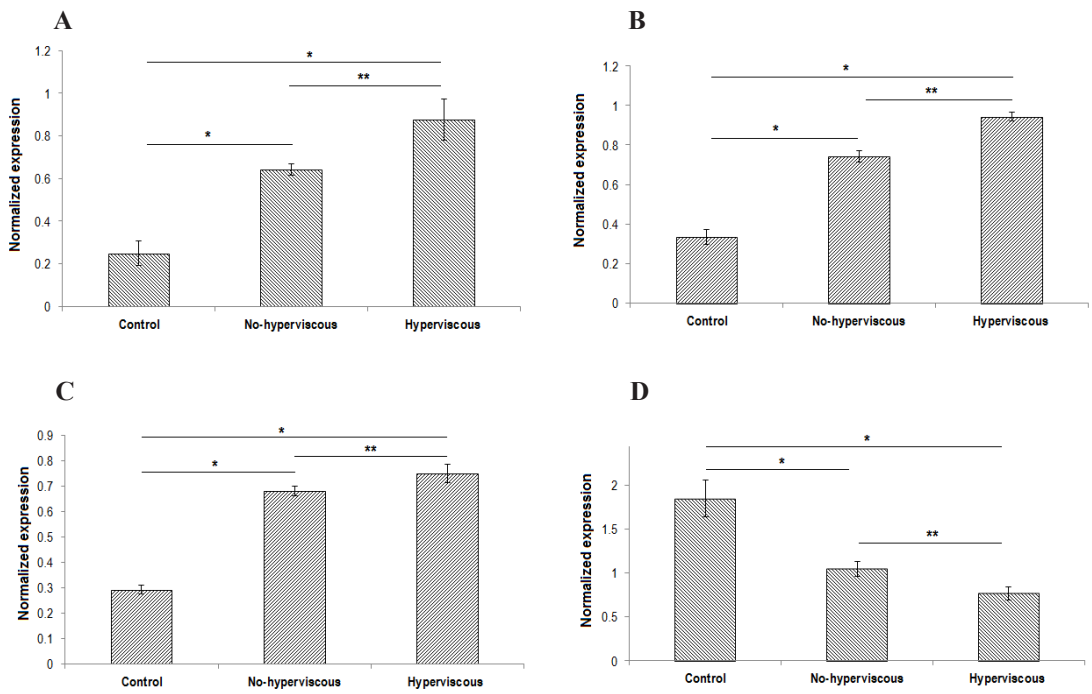

Fig.1: Comparison of the expression of apoptotic genes between the study groups. **A.** mRNA expression of *BAX*, **B.** mRNA expression of *CASPASE-3*, **C.** mRNA expression of *CASPASE-9* and **D.** mRNA expression of *BCL2*. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of all parameters between all groups. *; P<0.001 and **; P<0.01 compared to control group.

Discussion

Hyperviscosity semen can be considered as a leading cause of male infertility as it traps spermatozoa in the coagulated semen and prevents sperm migration through the female reproductive tract. Moreover, it may decrease the success rate of assisted reproductive techniques (ART) as it leads to difficulties in proper separation of spermatozoa during ART (30). In the current research, we investigated the relationship between hyperviscous semen and oxidative stress, antioxidant systems and apoptosis status. Our data illustrated that hyperviscosity is significantly correlated with depletion of TAC and GSH contents in the semen. In contrast, a significant enhancement was found in the mean contents of MDA, PC and 8-OHdG, as the biomarkers of lipid peroxidation, and oxidation of proteins and DNA, respectively. More importantly, we observed overexpression of apoptosis-related genes, *BAX*, and *CASPASES*, while down-regulation of the anti-apoptotic gene, *BCL2*, in spermatozoa of infertile men with hyperviscous semen.

Although oxidative stress, antioxidant depletion and apoptosis were found in both infertile groups, men with hyperviscous semen exhibited a significantly higher degree of these pathological conditions when compared to the non-hyperviscous group. Besides, we found that severe hyperviscosity was closely associated with higher levels of oxidative stress and antioxidant depletion. Therefore, these data emphasize that oxidative stress and apoptosis are among the major underlying mechanisms, by which seminal fluid viscosity leads to production of poor quality of spermatozoa, and thus male infertility. To support this hypothesis, there are some studies that have reported increased oxidative stress in seminal fluids of men with hyperviscous semen. For instance, in a previous study, Siciliano et al. (31) showed that both enzymatic and non-enzymatic antioxidants in semen of men with hyperviscosity are impaired. They concluded that the poor sperm motility in these patients is likely due to oxidative stress caused by depletion of antioxidants. In another study, Aydemir et al. (32) reported enhanced MDA mean contents in seminal fluids of men with hyperviscous semen compared to non-viscous samples. Layali et al. (19) demonstrated depletion of TAC and increased contents of MDA in seminal fluids of infertile men with hyperviscous semen. More recently, Barbagallo et al. (12) reported that seminal fluid viscosity is associated with impairment of antioxidant systems and consequently increases oxidative stress. Our research is in line with these studies; however, our study has advantages compared to previous work. In our study, not only we evaluated antioxidant depletion and lipid peroxidation status, but also, we evaluated oxidation of DNA and proteins, and performed expression analysis of apoptosis-related genes in men with hyperviscous semen, that are not reported in previous studies. According to our findings, men with hyperviscous semen exhibit lower TAC mean value, but higher MDA, 8-OHdG, and PC mean levels. In addition, we observed that overexpression of apoptosis-related genes were found in these cases compared to the non-hyperviscous group.

Therefore, our results indicate that antioxidant defense system in seminal plasma of men with hyperviscous semen is impaired. This defect is associated with increased levels of sperm membrane lipid peroxidation, DNA and proteins oxidation, and subsequently sperm apoptosis and poor quality of spermatozoa. The exact mechanism, in which oxidative stress in the seminal fluids of men with hyperviscous semen is increased has remained unclear at this point. We anticipate that overproduction of ROS is the main reason for antioxidant deficiency and oxidative damage in hyperviscous semen. Previous studies demonstrated that morphologically abnormal spermatozoa and leukocytes are important sources of ROS generation in human ejaculates (11).

It has been reported that the percentage of leukocytes is higher in men with hyperviscous semen than those without hyperviscous semen (12, 18). Leukocytes may be involved in developing and progressing hyperviscous semen, because their numbers are dramatically increased during infection, and produce massive amounts of ROS (33). To support this statement, some studies have demonstrated a significant relationship between leukocytospermia and hyperviscous semen (34). For example, Mahran and Saleh (34) have reported that the prevalence of leukocytospermia in infertile men with hyperviscous semen is about 37.5%. Moreover, they showed that an elevation in the number of leukocytes in men with semen hyperviscosity significantly decreases the percentage of sperm with normal motility and vitality. These researchers concluded that hyperviscous semen may be resulted from a previous ongoing infection or inflammation in 75% of these cases.

In another study, Elia et al. (8) recommended that administration of anti-inflammatory agents might successfully treat patients with mild semen hyperviscosity. Reduced levels of seminal plasma zinc is likely another main reason for poor sperm quality in men with hyperviscous semen. Hyperviscous seminal fluid contains a low level of zinc, which acts as an important cofactor for several antioxidants such as Cu/Zn-superoxide dismutase. Moreover, it has a great contribution to homocysteine trans-sulfuration into glutathione. So, there is a possibility that reduced Zn leads to decreased GSH production as well as SOD activity and consequently elevated oxidative stress (12). Therefore, these data indicate that overproduction of ROS and the subsequent oxidative stress and depletion of antioxidants such as Zn are important underlying mechanisms, in which hyperviscous semen causes sperm apoptosis and poor sperm quality. Given the fact that hyperviscous semen is associated with the risk of sperm DNA oxidation, some epigenetic or genetic abnormalities may be transmitted to offspring through the ART. Therefore, it is very important to evaluate the relationship between hyperviscous semen and the risk of some mutations in certain genes. Furthermore, antioxidant therapy might be helpful in mitigating these abnormalities and protecting sperm cells

against oxidative damages in patients with hyperviscous semen. However, further clinical trials are necessary to investigate the effects of antioxidant supplementation on mitigating oxidative stress and fertility potency in men with hyperviscous semen.

Conclusion

In summary, our findings showed that semen hyperviscosity triggers some cellular events associated with male infertility. Oxidative stress, antioxidant depletion and subsequent apoptosis of spermatozoa are likely the main mechanisms, by which hyperviscous semen induces poor sperm quality, and thus infertility in men. Impairment of seminal plasma antioxidants can be associated with oxidation of sperm DNA, protein, membrane lipids, and consequently low fertilization rate. Antioxidants therapy may be helpful in treating patients presenting with hyperviscous semen.

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Authors' Contributions

N.K., F.G.B., F.H., T.K.Z., H.B.D., S.R.; Clinical samples collection and laboratory phases. A.Sh., S.S.S.; Study concept and design, preparing data and manuscript writing. All authors read and approved the final manuscript.

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