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The Influence of Oxidative Damage on Viscosity of Seminal Fluid in Infertile Men

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ABSTRACT: Increased oxidative damage has been suggested to play an important role in the viscosity changes of blood. However, changes in levels of oxidative damage products in semen and their relationship to seminal fluid viscosity are unknown. The aim of our study was to investigate whether oxidative damage was associated with seminal plasma viscosity in infertile subjects. The levels of malondialdehyde, and protein carbonyls were measured in sperm and seminal plasma from 102 individuals, including 60 infertile patients. Seminal fluid viscosity and semen viscosity were studied by use of capillary viscometer and glass pipettes, respectively. Significantly higher levels of oxidative stress and damage markers were found in subfertile subjects compared with the control subjects. The seminal fluid viscosities of patients were found to be significantly higher, although all of the control and patient subjects had normal viscoelasticity when semen samples were assessed according to World Health Organization guidelines. From Pearson correlation analysis, there were significant positive correlations between seminal fluid viscosity and seminal malondialdehyde and carbonyl levels in infertile males ($r = .676$, $P < .01$; $r = .276$, $P < .05$, respectively). Our results suggest that increased oxidative damage might be a factor for hyperviscosity of seminal plasma in infertile males.

Key words: Male infertility, malondialdehyde, protein carbonyls.
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Oxidative stress is believed to underlie the etiology of numerous human conditions. Organisms are subject to oxidative stress from endogenous and exogenous sources, including exposure to solvents, other chemicals, and environmental pollutants. All these potential hazards contain components that can induce severe macromolecular, cellular, and tissue damage through 1) direct cytotoxic effects, 2) promotion of primary genotoxic events, or 3) generation of reactive oxygen intermediates (Halliwell and Gutteridge, 1999; Fraczek and Kurpisz, 2007). Reactive oxygen species (ROSs), such as the superoxide anion and hydroxyl radical, can be produced by human spermatozoa (Aitken et al, 1989, 1993). Because of high polyunsaturated fatty acid content, human spermatozoa plasma membranes are highly sensitive to ROS-induced damage, and hydrogen peroxide appears to be the most toxic ROS for human spermatozoa. There is growing evidence that peroxidative damage to the human spermatozoa membrane is an important pathophysiological mechanism in human male infertility (Agarwal et al, 2003). It has been also reported that seminal plasma and spermatozoa from men with male infertility have higher ROS levels than fertile men (Alkan et al, 1997).

On the other hand, recent studies have shown that oxidative stress is associated with blood viscosity (Jain et al, 1990; Chung and Ho, 1999; Liu et al, 2004). It has been found that blood viscosity increases were correlated with certain diseases associated with oxidative damage, such as diabetes mellitus, coronary artery disease, hyperlipoproteinemia and chronic kidney disease (Vaya et al, 1993; Kesmarky et al, 2006; Marcinkowska-Gapinska and Kowal, 2006). Serum levels of malondialdehyde (MDA), one of the most studied unsaturated carbonyl products of oxidative stress, were also correlated with blood viscosity (Jain et al, 1990; Chung and Ho, 1999; Liu et al, 2004) and other oxidative stress parameters, such as serum protein carbonyl content (Turkoglu et al, 2000). Several research groups have studied the elevated blood viscosity of erythrocyte membrane proteins induced by oxidative stress and MDA and have suggested that MDA, by leading to protein cross-links, might be the cause of viscosity changes (Pfafferott et al, 1982; Pasini et al, 1991; Uyesaka et al, 1992).

If the increased oxidative damage in blood is correlated with increases in the viscosity of blood and plasma of certain diseases associated with oxidative damage, then it is possible that there is an association between seminal fluid viscosity and seminal oxidative

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damage in infertile males, although compositions of blood and seminal fluid are different.

Increased viscosity of ejaculate was reported to occur more frequently among infertile couples than in fertile males (Bunge, 1970; Hubner et al, 1985; Elzanaty et al, 2004). Several conditions, such as concentrations of prostate-specific antigen, zinc and calcium, and activity of neutral α-glucosidase in seminal plasma, were found to be correlated with changed semen viscosity (Mendeluk et al, 2000; Elzanaty et al, 2004; Andrade-Rocha, 2005). Siciliano et al (2001) has demonstrated a severe impairment of both the high– and low–molecular weight antioxidative systems in semen with hyperviscosity. However, there are no reports studying the relationship of oxidative damage and semen viscosity in the context of male infertility. Therefore, the general aim of this study was to determine whether the viscosity of seminal fluid is associated with MDA and protein carbonyl levels of sperm and seminal plasma in infertile patients.

Materials and Methods

This study was approved by the institutional review board of the Infertility Central Urology Department of Cerrahpaşa Medical Faculty, İstanbul, Turkey. Semen specimens were obtained from 60 men aged 26–48 years with infertility between 2005 and 2006. Specimens were also obtained from 42 male volunteers aged 24–49 years with normal semen analysis according to World Health Organization (WHO, 1999) guidelines to serve as the fertile control. Institutional Ethical Committee approval was taken in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from each study subject. Individuals with a significant medical history, signs suggestive of defective androgenization, or abnormal testicular examinations were excluded from this study. Further exclusion criteria for both groups included chromosomal disorders related to a fertility disorder, cryptorchidism, vasectomy, abnormal liver function and hormone tests, cigarette smoking, alcohol consumption, or the use of folic acid, glutathione, vitamin C, or vitamin E supplements or medication within 3 months before recruitment. Criteria for study inclusion were infertility for at least 12 months, with at least 1 semen parameter abnormality, semen leukocyte count less than 1×10^6/mL, and negative semen anti-sperm antibody on a mixed agglutination reaction test. Semen specimens were collected by masturbation into a sterile wide-mouth metal-free plastic container after at least 3 days (3–5 days) of abstinence and liquefied at 25°C for 30 minutes.

Semen Analysis

A semen analysis was carried out according to WHO guidelines to obtain volume, pH, sperm concentration, motility, and morphology. Sperm concentration was determined with a Makler Counting Chamber (Seti-Medical Instruments, Haifa, Israel). Motility was expressed as a percentage of motile spermatozoa and their mean velocity. Morphology was determined according to the WHO criteria after incubation of the sample with trypsin for 10 minutes at 25°C according to the methylene blue eosin staining procedure, feathering and fixation by flame. At least 100 cells were examined at a final magnification of 1000×. Semen visco-elasticity was assessed using glass pipettes as recommended in the WHO guidelines and semen samples showing a thread of more than 2 cm long were considered highly visco-elastic, whereas the visco-elasticity was considered normal when the thread length was 2 cm or less.

Measurement of Seminal Plasma Viscosity

After liquefaction, at least 1 mL of semen was centrifuged at $400 \times g$ for 15 minutes at 25°C. Seminal plasma was collected at the top. Manual viscosity measurements were made according to the recommendations of the International Committee for Standardization in Haematology (1984) with the use of a Harkness capillary viscometer (Coulter Electronics Ltd, serial No: 6083, Luton, United Kingdom) and were evaluated in relation to distilled water (relative viscosity), the water bath of which was maintained at 35°C (Harkness, 1963). This system comprises a glass capillary tube 0.30 mm in internal diameter and 200 mm long through which a sample of 0.5 mL is forced at a constant positive pressure of 17.2 kPa. Seminal fluid viscosity is proportional to its flow time through the capillary. The seminal fluid viscosities were expressed in millipascal seconds (mPa·s). The intra-assay coefficients of variation for seminal plasma viscosity was 6.7%. With this device, we calculated the viscosity of seminal fluid according to the formula of Poiseuille (1840),

$$\eta = \frac{\Delta P t \pi r^4}{8LQ}$$

where η is viscosity (mPa·s), ΔP is the pressure gradient (pascals), r is the radius of the capillary (m), L is length (m), Q is flow rate (m^3/s), and t is time (s).

Spermatozoa Preparation

After liquefaction, spermatozoa were fractionated on Percoll gradients (40%–95%) according to WHO guidelines. Semen was layered on top of the gradient and centrifuged at $400 \times g$ for 20 minutes at 25°C. Spermatozoa in the 95% Percoll layer were collected and washed twice at $400 \times g$ for 6 minutes at 25°C with added Tris, sodium, and EDTA (TNE) buffer (0.15 mol/L NaCl, 0.01 mol/L Tris-HCl, 1 mmol/L Na_2-EDTA, pH 7.4) (Alkan et al, 1997). The remaining spermatozoa were frozen without preservatives and stored for up to 1 month at −70°C before being assayed for malondialdehyde and protein carbonyls.

Measurement of Lipid Peroxidation

The lipid peroxide levels in the seminal plasma and spermatozoa were measured with a thiobarbituric acid (TBA)–reactive substance assay, which monitors MDA production on the basis of the method of Buege and Aust (1978). Briefly,

Table 1. *The population characteristics and semen variables in infertile men**

	Age, y	Abstinence period, d	Sperm concentration, $\times 10^6$/mL	Sperm motility, %	Sperm morphology, %
Control group (n = 42)	39.88 ± 9.05	3.90 ± 0.82	64.69 ± 25.00	53.29 ± 8.63	49.40 ± 4.93
Infertile group (n = 60)	38.98 ± 6.75	3.68 ± 0.77	19.85 ± 11.88†	46.33 ± 8.44†	43.97 ± 6.62†

* Values ($\bar{x}$ ± SD) are significantly different compared with the control donors at † $P < .001$ (Student's *t* test).

to a 100-µL sample of seminal plasma (or 1×10^6 spermatozoa/mL), 200 µL of cold 1.15% (wt/vol) KCl was sonicated for 30 seconds on ice and added to 1.8 mL of 3% phosphoric acid and 0.6 mL of 0.6% TBA. These mixtures were heated in boiling water for 45 minutes. After cooling, the MDA was extracted by centrifugation at $1500 \times g$ for 10 minutes at 25°C, and the intensity was measured at 535 nm by ultraviolet-visible spectrophotometry (Shimadzu UV-1601, Tokyo, Japan). The MDA level was determined from the molar absorption coefficient of the MDA at 535 nm, 1.56×10^5 mol/L cm^{-1}.

Measurement of Protein Carbonyls

Because carbonyl groups (aldehydes and ketones) might be introduced into proteins by ROS and free radicals, quantitation of protein carbonyls was carried out by incubating equal volumes of the sample (seminal plasma or 1×10^6 spermatozoa/mL) and 2,4-dinitrophenylhydrazine (3.4 mg/10 mL of 1 mol/L HCl) at 50°C for 1 hour. After the reaction, proteins were precipitated with 20% trichloroacetic acid, and the unreacted dye was removed by centrifugation. The pellet was dissolved in 1 mol/L NaOH, and the absorbance at 450 nm was recorded. The molar absorbance coefficient (ε = 25 500 mol/L cm^{-1}) was used to calculate the carbonyl content (Levine et al, 1990). Protein concentrations were determined by the Lowry method, with bovine serum albumin as the standard (Lowry et al, 1951).

Statistical Methods

Values reported are $\bar{x}$ ± SD. All data were normally distributed and underwent equal variance testing. Statistical significance of differences was determined by SPSS program version 11.5 for Windows (SPSS Inc, Chicago; Ill). Average comparison between 2 subgroups was made by Student's *t* test. Correlation between seminal fluid viscosity, and seminal malondialdehyde and carbonyl levels was tested with a Pearson correlation model. Data were considered statistically significant at $P < .05$.

Results

We determined the seminal fluid and semen viscosities and the concentrations of MDA and protein carbonyls in sperm and seminal plasma from 60 infertile and 42 fertile men. Table 1 shows the population characteristics and results of classic semen analysis in subfertile patients and control donors.

Considering the oxidative stress biomarkers, we compared the levels of MDA and protein carbonyls in spermatozoa and seminal plasma between infertile patients and controls. As shown in Table 2, protein carbonyls and MDA levels in the seminal plasma and spermatozoa were significantly higher in the infertile group.

When viscoelasticity of semen samples was assessed according to the WHO guidelines, all of the control and patient subjects had normal viscoelasticity. However, when the seminal viscosity of patients and controls were compared after the capillary viscometer was used to study seminal plasma viscosity changes, the mean seminal plasma viscosity value of the patient group was significantly higher then the control group ($P < .05$) (Table 2). Additionally, oxidative damage was associated with seminal plasma viscosity in subfertile and control subjects. Correlation analysis (Pearson test) revealed a significant positive relationship of seminal fluid viscosity with seminal plasma MDA and sperm MDA ($r = .676$, $P < .01$; $r = .482$, $P < .01$, respectively) in the subfertile group (Table 3; Figure). In addition, seminal plasma viscosity was significantly but weakly correlated with sperm and seminal plasma protein carbonyl concentrations ($r = .276$, $P < .05$; $r = .308$, $P < .05$, respectively). However, these observed correlations were not found in the control groups (Table 3).

Table 2. *Spermatozoa (10^6 spermatozoa/mL) and seminal plasma malondialdehyde (MDA), protein carbonyl levels, and seminal fluid viscosity in infertile men**

	Spermatozoa MDA, nmol/10^6 spermatozoa	Seminal Plasma MDA, nmol/mL	Spermatozoa Protein Carbonyls, nmol/10^6 spermatozoa	Seminal Plasma Protein Carbonyls, nmol/mg protein	Seminal fluid viscosity, mPa·s
Control group (n = 42)	0.32 ± 0.11	0.49 ± 0.10	1.78 ± 0.38	1.83 ± 0.36	1.59 ± 0.15
Infertile group (n = 60)	0.41 ± 0.12†	0.90 ± 0.20†	3.27 ± 0.59†	3.55 ± 0.63†	1.99 ± 0.31†

* Values ($\bar{x}$ ± SD) are significantly different compared with the control donors at † $P < .001$ (Student's *t* test).

Table 3. *Relationship between seminal fluid viscosity and spermatozoa malondialdehyde (MDA), seminal plasma MDA, spermatozoa protein carbonyls, and seminal plasma protein carbonyls**

	Correlation Coefficient (r)	
	Control Group (n = 42)	Infertile Group (n = 60)
Spermatozoa MDA	0.193	0.482†
Seminal plasma MDA	0.272	0.676†
Spermatozoa protein carbonyls	0.140	0.276‡
Seminal plasma protein carbonyls	0.256	0.308‡

* Values are significantly different from the control group at † $P < .01$ and ‡ $P < .05$.

Discussion

The importance of semen viscosity lies in the fact that the spermatozoa are tangled in the fibrous or mucoid mass in the semen and prevented from migrating properly from the seminal plasma into the cervical track fluids to ascend to the site of fertilization. Seminal viscopathy was shown to be associated with male infertility (Bunge, 1970; Hubner et al, 1985; Elzanaty et al, 2004). From previous studies, serum MDA levels were correlated with blood viscosity (Pfafferott et al, 1982; Chung and Ho, 1999; Liu et al, 2004). As it is known, free radicals can also react with proteins, in addition to lipids. A positive correlation has been shown between serum protein carbonyl content and blood viscosity (Liu et al, 2004). Protein carbonyl formation has been known to be an early marker for protein oxidation (Reznick and Packer, 1994). Although oxidative stress, most frequently represented as an MDA value and protein oxidation formation, has been recognized in accordance with male fertility (Aitken et al 1989; Agarwal et al, 2003; Aydemir et al, 2007), how

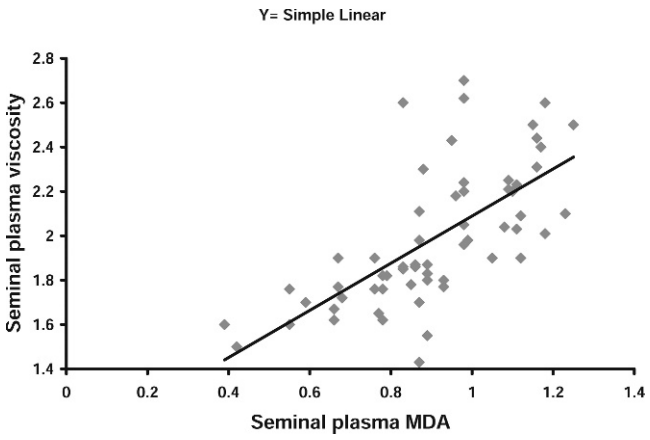


Figure. Correlation between seminal fluid viscosity (mPa·s) and seminal malondialdehyde levels (nmol/mL) for the 60 infertile males ($r^2 = .45698$; $y = 1.0627x + 1.0262$).

pivotal MDA, other unsaturated carbonyls, and protein carbonyls are in contributing to an increase in seminal viscosity has not been quantified. To our knowledge, this study is unique in examining relationships between oxidative stress markers and seminal fluid viscosity, although a severe impairment of the low– and high–molecular weight seminal antioxidative capacities has been reported to be associated with semen hyperviscosity (Siciliano et al, 2001).

On the basis of semen samples from a group of 60 patients, seminal plasma MDA and protein carbonyl levels were found to be increased in infertile subjects. Our findings are in accordance with other investigators' reports (Chen et al, 1997; Aydemir et al, 2007). When we applied the WHO standard method for assessment of semen viscoelasticity, all subjects were normoviscous for semen. However, analysis of the viscosity values measured with a capillary viscometer (Table 2) showed a statistically significant difference in levels of seminal plasma viscosity between the group of healthy patients and control subjects with normal viscoelasticity. The simplest explanation for lack of difference in semen viscosity between our infertile and control subjects, when viscoelasticity was assessed with glass pipettes, might be because this method is qualitative and might be inefficient for estimating the differences in semen viscosity. Moreover, sperm concentration might be an important determinant of semen viscosity because the mean sperm concentration values were significantly higher in control subjects than in infertile subjects. However, an exact explanation for this situation could not be made by Hubner et al (1985) and Lin et al (1992), who found increased viscosity in the semen samples of infertile subjects by rotational viscometer. Viscometry by capillary viscometers is a well-accepted method frequently used for analysis of non-Newtonian liquids in the laboratory and is a good indicator of samples containing macromolecules. To our knowledge, our results are the first to report seminal fluid viscosity measured by capillary viscometer.

Furthermore, our study demonstrated significant associations between viscosity of seminal fluid and MDA levels in seminal plasma and sperm of infertile subjects. Again, seminal fluid viscosity was also correlated positively with protein carbonyl levels of sperm and seminal plasma in infertile patients, although it was weakly correlated. Some investigators have found that some conditions, including concentrations of prostate-specific antigens zinc and calcium and activity of neutral α-glucosidase in seminal fluid, were correlated with changed semen viscosity (Bunge, 1970; Mendeluk et al, 2000; Elzanaty et al, 2004; Andrade-Rocha, 2005). Siciliano et al (2001) have reported that the lowering of catalase activity and total antioxidant status values appeared to be associated with semen hyperviscosity.

The present experimental data suggest that MDA and protein oxidation might contribute to viscosity increase in seminal fluid. The possible explanation for high seminal viscosity could be a change in protein-protein interactions in the seminal plasma. MDA, which is an end product of lipid peroxidation, is known to cross-bind or induce secondary oxidative damage in the plasma proteins (Traverso et al, 2004). Small molecular aldehydes can react with membrane proteins and modify their structure. Malondialdehyde also causes decreased protein solubility, which is related to changes in viscosity (Ingemansson et al 1995). In addition, it has suggested that proteins modified directly by ROSs with the eventual formation of oxidized amino acids and proteins modified indirectly with reactive carbonyl compounds formed by the autoxidation of carbohydrates and lipids might be cause of the viscosity changes (Esterbauer et al, 1991). Such a biological side-reaction inevitably might cause alterations in the physicochemical properties of biological materials, resulting in changes in the membrane fluidity of sperm, leading to viscosity alteration. However, the high seminal plasma viscosity observed in our patients could be the result of a rise in many other products of oxidative damage (eg, unsaturated aldehydes) and other factors. On the other hand, Mendeluk et al (2000) reported that protein disulfide bonds in the gel network of seminal plasma are responsible for the differential rheological properties observed in the hyperviscous group. It is known that oxidative stress leads to disulfide bond formation of sulfoxidation in sulfhydryl residues in proteins.

Therefore, a further study is desired to assess how oxidative damage might contribute to seminal viscosity changes.

The data from our study by capillary viscometer demonstrated positive relationships between seminal fluid viscosity and seminal malondialdehyde and carbonyl levels in infertile males. These results suggest that seminal fluid viscosity is influenced by the products of oxidative damage in the seminal plasma.

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