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Factors Involved in the Biochemical Etiology of Human Seminal Plasma Hyperviscosity

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ABSTRACT: Semen rheology was studied to elucidate the biochemical basis of seminal plasma hyperviscosity. Semen proved to fit in with a power law model, by presenting a pseudoplastic behavior. Apparent viscosity at 230 s^{-1} and 25°C (η_a) was 4.3 ± 0.2 cp and 5.4 ± 0.4 cp in normal and high-consistency semen, respectively. The effect of enzymes and mucolytic agents on human seminal plasma viscosity were evaluated by incubating normal and hyperviscous semen pool aliquots with trypsin, dithiothreitol, EDTA, α-amylase and deoxyribonuclease I. After incubation, trypsin treatment reduced η_a by 36% in normal semen and by 44% in hyperviscous semen. There was a decrease in η_a following incubation of hyperviscous samples with dithiothreitol (33%) and α-amylase (44%) that was not observed in the normal consistency samples. No decrease was observed in η_a after EDTA or DNAse treatment of both groups. Comparison of normal and hyperviscous seminal plasmas revealed no difference in the concentration of total proteins, DNA, or in the percentage of water content. These findings indicate that the primary substances responsible for basic normal semen rheologic behavior are proteins. A comparison of rheological properties between normal and hyperviscous semen samples indicates the existence of a highly organized network in the latter group, in which disulfide bonds and oligosaccharide chains complexed to the peptide core may play a key role.

Key words: Semen, viscosity, rheology, proteins, oligosaccharides.

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The diagnosis of male infertility is a rapidly developing field of investigation. A descriptive semen analysis is still at the heart of the diagnostic workup of male patients and important advances have been made to standardize the procedures used to construct the conventional semen profile (Rowe et al, 1993). Basic semen tests contain no variables that may allow certification that a man is fertile. Research into the biology of fertilization has been stimulated by the desire to find a theoretical framework for understanding the basis of human infertility. Despite that, the specific effects of seminal plasma on spermatozoa remain unexplored; thus, only a few studies have investigated the possible relationship between hyperviscosity and natural fertility (see Tjoe and Oestenoeng, 1968; Dunn and Picologlou, 1977; Nag et al, 1979; Hubner et al, 1985; Lin et al, 1992).

A viscous fluid is one in which attractive forces exist between neighboring portions of the fluid. These forces define its flow behavior, which can be studied by a rheogram, a plot of shear stress (τ) as a function of share rate ($\dot{\gamma}$). The shear stress is the force per unit area resulting from the bulk flow; the share rate is the velocity gradient perpendicular to the force (Cantor and Schimmel, 1980).

Several models exist to describe the flow behavior of fluids. The Ostwald-de-Waele model, corresponding to a pseudoplastic behavior (Astarita and Marrucci, 1974), is defined by the following:

$$\tau = K \cdot \dot{\gamma}^n, \quad (1)$$

where K is the structural viscosity and n is the coefficient of consistency (Groupe Français de Rhèologie, 1990).

The slope at each rate of shear of the rheogram represents the changing values of viscosity, termed *apparent viscosity* (η_a). The decrease in apparent viscosity with increasing shear stress is attributed to shear alignment, dekinking, and uncoiling of molecules or to changes in the shape of suspended elastic particles caused by directional shearing action. Numerous examples of pseudoplastic materials exist. Most high molecular weight polymers, lattices, starch pastes, and many emulsions exhibit this type of flow. The shear stress at zero shear (τ^0) is indicated in the flow curve by an intercept on the shearing stress axis. Materials that exhibit τ^0 different from zero do not flow until the applied shearing stress exceeds a minimum value. The τ^0 is believed to be a measure of the force of flocculation per unit area, which exists between the suspended particles. Thus, a suspension of highly flocculated particles shows a high τ^0 value. For many fluids, the plot

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of $\tau = f(\dot{\gamma})$ obtained by increasing $\dot{\gamma}$ is different from that obtained when decreasing $\dot{\gamma}$. The area enclosed by both curves is the ''thixotropy area'' (μ), a rheological parameter that describes the time-dependent behavior of a given sample (Tanner, 1985).

Note that for $n = 1$, equation 1 converts to the following:

$$\tau = K \cdot \dot{\gamma}, \quad (2)$$

corresponding to a Newtonian flow behavior with $K = \eta$ (viscosity). The more n deviates from 1, the more shear-rate dependent the fluid viscosity becomes.

A rheogram is a physical ''fingerprint'' of a given sample and is used to define the melt flow behavior of polymers to characterize their processibility, to understand their molecular structure, and to indicate influences in polymer blending or compounding.

Human semen is ejaculated in a liquid state but immediately coagulates. Liquefaction follows in 15 to 20 minutes (Rowe et al, 1993). Liquefied semen samples may be classified as normal or hyperviscous.

Mucoid debris hinders the evaluation of sperm count and sperm morphology as the spermatozoa become obscured. Different methods have been proposed to improve hyperviscous semen handling. Bunge and Sherman (1954) achieved liquefaction of viscid semen in vitro by adding a 5% solution of α-amylase. Gersh (1970) found pancreatic dornase to be an effective mucolytic agent in semen, whereas sputolysin was found to be so by Upadhyaya et al (1981). A study on sperm recovery techniques reports that semen samples with increased viscosity may benefit from prior dilution with culture medium or even chymotrypsin-induced liquefaction before density gradient centrifugation (Mortimer, 1994). Sharma et al (1993) reported that semen samples with high viscosity were collected in α-chymotrypsin. Despite this observation, the cause of abnormally high residual viscosity after semen has coagulated and then liquefies was not accurately explained.

The aim of this study was to clarify the biochemical etiology of the phenomenon of seminal plasma hyperviscosity.

Material and Methods

Chemicals

All the chemicals used in this work were of analytical grade. Most were purchased from Sigma Chemical Company (St Louis, Mo); bizbenzimidazole to determine DNA concentration was provided by Fluka Biochemika (Buchs, Switzerland).

Sperm Collection

Semen samples were collected and analyzed by World Health Organization (WHO) criteria (Rowe et al, 1993) from men attending the Seminal Laboratory at the University Hospital of Buenos Aires. According to these criteria, a semen specimen was regarded as having high consistency when the length of the thread formed on withdrawal of a glass rod exceeded 2 cm. Only liquefied samples were employed.

Viscosity Measurements

Viscosity measurements were taken at 25°C with a Wells-Brookfield rotational viscosimeter (model DV-I) using a cone angle of 1.565°. Operating it involves rotating a flat cone upon and perpendicular to a planar surface (plate) at different speeds of rotation. The rotation creates constant and uniform rates of shear. The sample, placed in the bottom of the sample chamber, lies between the cone and the plate and resists the rotation of the cone. The torque necessary to overcome the resistance to the rotation of the flat cone is a function of the viscosity of the sample and is measured in absolute values. The viscosimeter allows 8 shear rates. The readings were taken every 3 minutes from 1.15 s^{-1} to 230 s^{-1}; and then, the reverse procedure was followed. Shear stress was calculated from the measured viscosity profiles and the corresponding rheograms were plotted. Rheological variables were assessed from these graphs. (Astarita and Marrucci, 1974).

Treatments with Enzymes and Mucolytic Agents

The effect of enzymes and mucolytic agents were evaluated in normal and high-consistency sperm pools, composed of 18 and 16 samples, respectively. Each semen pool was divided into equal aliquots (1.0 mL). Thus, 4 different treatments were performed as described by Tockman et al (1995) with 1 mg/mL trypsin, 2.95 mM dithiothreitol (DTT), 12 mM EDTA, and 14.36 UK/mL deoxyribonuclease (DNAse I, from bovine pancreas). In addition, digestion was performed with 0.2% α-amylase as described by Bunge (1954). All the assays were performed in triplicate.

Temperature and dilution controls were processed in parallel in tubes containing 1 mL of semen and 1 mL of semen plus 100 μL of phosphate-buffered saline (PBS, pH: 7.2; Mendeluk et al, 1996). The contents of the tubes were thoroughly mixed and incubated for 60 minutes at 37°C. The mixture in each tube and the untreated aliquots (basal smears) were tested for apparent viscosity at 230 s^{-1} (25°C), thus resembling WHO recommendations to determine consistency at room temperature.

Concentration of Seminal Plasma Components

Protein concentration was determined according to a description by Bradford (1976), DNA concentration was measured as described by Brunk et al (1979), and water content was evaluated by mass difference postlyophilization of the individual seminal plasmas.

Analysis

The mean and standard errors (SEMs) were calculated for all the variables studied, except when indicated. A Fisher's test was applied to compare variances. A Student's test was used if variances in 2 populations showed no significant difference according to the Fisher's test; the Welche's test was employed if there was a significant difference. A Clarke's test (average difference)

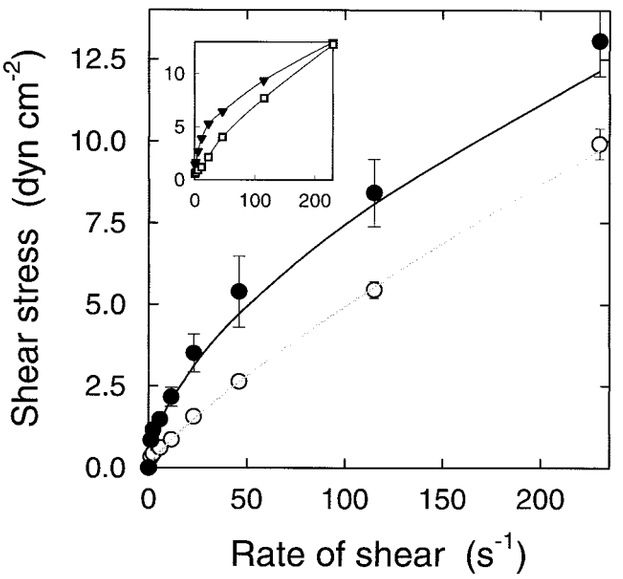

Figure 1. Rheological characterization of semen. Individual normal (n = 19) and hyperviscous (n = 67) semen samples were rheologically studied. Viscosity measurements at different shear rates were performed with a Wells-Brookfield rotational viscosimeter. The readings were taken every 3 minutes starting from: 1.15 s^{-1} to 230 s^{-1} on normal (○) and high-consistency (●) semen samples. The continuous lines correspond to the graphical representation of equation 1 using the best-fit parameter values indicated in the text. The inset includes a typical rheogram showing a thixotropic loop. The upper curve was obtained by increasing the rate of shear (▼), the lower curve was obtained by decreasing the rate of shear (□). The area between both curves is the thixotropy area.

was applied to compare maximum apparent viscosity (η_{am}) obtained after different mucolytic treatments. Values were considered significant at $P < .05$.

The equations were fitted to the experiment data by a nonlinear regression procedure based on the Gauss-Newton algorithm (Seber and Wild, 1989). The dependent variable was assumed to be homoscedastic (constant variance), and the independent variable was assumed to have negligible error. A computer program was used to find the best fit parameters for a chosen equation and the level of uncertainly that described the statistical relevance of the fitted curve to the measured test points (Rossi, 1987). The best equation was considered to be the one that gave the minimal standard deviation of the regression and the least biased fit. Parameters were expressed as mean value ± SEM.

Table 1. *Seminal consistency and rheological parameters determined with a rotational viscosimeter at room temperature†*

Rheological Parameters	Normal Consistency (n = 67)	High Consistency (n = 19)
η_{am}(cp)	4.3 ± 0.2	5.4 ± 0.4*
τ° (dyne/cm^2)	0.32 ± 0.01	0.83 ± 0.08***
μ (dyne/cm^2 s)	47.1 ± 3.8	218.2 ± 36.2**

† Values are means ± SEM.
* Significant difference at $P < .05$.
** Significant difference at $P < .0002$.
*** Significant difference at $P < .0001$.

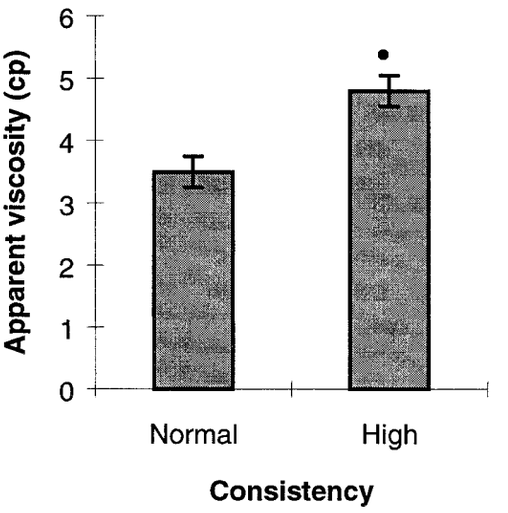

Figure 2. Apparent viscosity in normal and high-consistency semen plasma pools. Determinations were performed with a rotational viscosimeter at 230 s^{-1}. Values represent mean ± SEM for 3 replicates per aliquot. The column marked with a dot is significantly different from the control ($P < .0001$).

Results

Rheological Characterization of Seminal Plasma

Rheological behavior of semen is shown in Figure 1. Data on shear stress (τ) for each shear rate ($\dot{\gamma}$) for normal (n = 19) and hyperviscous semen samples (n = 67) are grouped (ascending curve). Figure 1 shows that shear stress increases when the rate of shear increases in a nonlinear way. The best fit to the data was obtained by applying the Ostwald-de-Waele model (equation 1). The best fitting values of the parameters were $K = 0.12 \pm 0.02$ dyn s/cm^2 and $n = 0.81 \pm 0.03$ in the normal consistency semen group, and $K = 0.58 \pm 0.14$ dyn s/cm^2 and $n = 0.57 \pm 0.05$ in the high-consistency group.

The apparent viscosity at maximum shear rate (η_{am}), the shear stress at zero shear (τ^0), and the thixotropy area (μ) determined in normal and hyperviscous samples revealed significant differences between groups ($P < .05$; see Table 1). These experiments were done at room temperature, and the same statistical differences in all the studied parameters were also found at 37°C (data not shown).

The following experiments were designed to evaluate the effect of enzymes and mucolytic agents on seminal hyperviscosity. We worked with semen plasma instead of whole semen because the contribution of spermatozoa to semen consistency were demonstrated to be negligible (Hubner et al, 1985; Mendeluk et al, 1992). The volume of individual samples was not sufficient for testing all further treatments, so we combined the semen plasmas in normal and high-consistency sperm pools. Figure 2 shows

Table 2. *Seminal consistency and concentration of total proteins, DNA, and the percentage of water content**

Variable	Normal Consistency	High Consistency
Total protein concentration, mg/mL	5.25 ± 0.2 (n = 17)	5.03 ± 0.30 (n = 14)
Free DNA concentration in seminal plasma, μg/mL	23.3 ± 3.2 (n = 23)	28.4 ± 2.0 (n = 60)
Water content, %	90.38 ± 0.45 (n = 19)	90.33 ± 0.35 (n = 18)

* Values are means ± SEM. No significant differences between groups are apparent.

data on η_{am} found in aliquots of normal and hyperviscous semen pools ($P < .0001$).

Composition of Normal and Hyperviscous Seminal Plasma

In order to elucidate the biochemical basis of seminal plasma hyperviscosity we took into account the rheology of other biological fluids such us cervical mucus (Boyers, 1995) and tracheobronchial secretions (Oteo Ochoa and Sueiro Bendito, 1985). Because polymers usually confer high viscosity to a given solution (Han, 1976), we hypothesized that proteins and DNA could be involved in the etiology of the phenomenon under study. However, no difference in total proteins and DNA concentration could be detected while studying normal and hyperviscous semen samples. In addition, there were no differences in water content between the groups (Table 2).

Effect of Enzymes and Mucolytic Agents

Viscosity was reduced by 36% ($P < .0001$) after incubating normal-consistency semen plasma with trypsin (Figure 3). No decreases in η_{am} were observed following other treatments. Figure 4 shows that the addition of trypsin induced a decrease in viscosity in high-consistency semen plasma (44%; $P < .00001$). This behavior was also observed when high-consistency semen was incubated in the presence of DTT (33%) and α-amylase (44%; $P < .00001$). However, no effect on η_{am} was observed after EDTA or DNAse treatment. Values for η_{am} after PBS treatment (60 minutes at 37°C) were similar to those observed in the untreated samples, indicating that the effect of dilution and temperature are not significant in these experiments.

Discussion

We infer that a power law model (pseudoplastic behavior) fits better than a linear model (Newtonian behavior) on individual shear stress data versus shear rate. This pattern was observed both in the normal and in the hyperviscous samples; the hyperviscous samples had higher structural

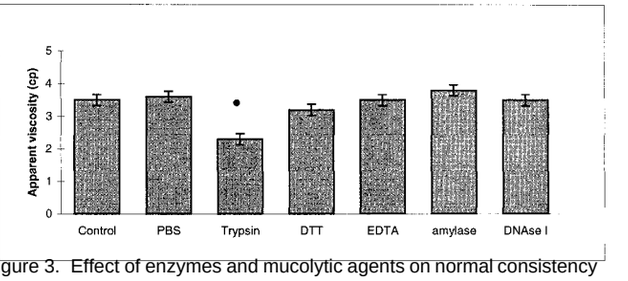

Figure 3. Effect of enzymes and mucolytic agents on normal consistency semen plasma. Aliquots were incubated for 1 hour at 37°C with the enzymes and mucolytic agents indicated. The apparent viscosity was measured at 230 s^{-1}. Values represent mean ± SEM for 3 replicates per aliquot. The column marked with a dot is significantly different from the control ($P < .0001$).

viscosities and lower coefficient of consistencies than the normal samples did. Apparent viscosity decreased with increasing shear rate, indicating that the fluid was exhibiting a shear thinning behavior, probably associated with the presence of structurally complex molecules (Han, 1976).

Comparison of the rheological properties τ^0 and μ between normal and high-consistency semen samples indicates the possibility of an organized network that is more complicated than a simple interaction. The high thixotropy observed in the hyperviscous samples validates the mechanical method employed by Amelar (1962), who proposed the forcible ejection of semen several times through a syringe and needle into a glass container to reduce seminal viscosity.

The experiments on the effect of enzymes and mucolytic agents were designed to be performed on semen pools; thus, our conclusions may explain the etiology of hyperviscosity in the majority of cases. However, individual cases may not fit into this general pattern.

Amelar (1962) proposed that factors that determine the physical characteristics of the coagulated semen are different from those of hyperviscosity. The liquefaction of

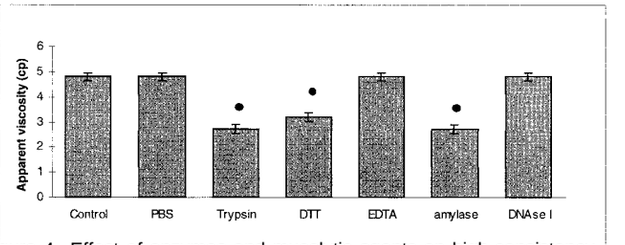

Figure 4. Effect of enzymes and mucolytic agents on high-consistency semen plasma. Aliquots were incubated for 1 hour at 37°C with the enzymes and mucolytic agents indicated. The apparent viscosity was measured at 230 s^{-1}. Values represent mean ± SEM for 3 replicates per aliquot. Columns marked with dots are significantly different from the control ($P < .0001$).

the coagulum is activated in the presence of EDTA and inhibited by nonchelated Zn^{2+} (Lilja and Laurell, 1984). A 2-step confluent process of liquefaction has been suggested. Macroscopic solubilization of the coagulum constitutes the first phase, whereas further lysis of lysed clot represents the second phase of liquefaction (Mann and Lutwak-Mann, 1981). In the last one, prostate-specific antigen (PSA), which is an abundant prostate-derived serine protease in the seminal fluid that has an extensive structural similarity to the glandular kallikreins but has an enzyme action similar to that of chymotrypsin, causes the proteolytic degradation of the coagulum constituted by 2 major gel proteins, semenogelin I and II (Bjartell et al, 1996). Our data showed that EDTA had no detectable effect on seminal viscosity, excluding metal involvement in hyperviscosity, thus supporting the hypothesis of Amelar.

Because free DNA concentration did not differ between normal and high-consistency semen samples, and the addition of DNAse had no effect on viscosity, this study precludes a role for DNA that is not implied in the etiology.

Cystic fibrosis causes defects in active ion transport; the consequent lower water content is the physiological mechanism responsible for the diminished fluidity of the secretions. Hyperviscous cervical mucus has been associated with subfertility in woman with cystic fibrosis because of a decrease in water content (Kredentser et al, 1986). However, we could not find a hydration defect in the hyperviscous semen samples that would suggest that water is involved in the phenomenon under study.

After trypsin digestion, seminal plasma viscosity was reduced by 36% in normal samples and 44% in hyperviscous ones, and there were no statistically significant differences between the viscosities of either group after proteolysis. These results lead us to conclude that proteins are involved in semen rheology. However, it is not possible to explain the different flow behaviors found in semen samples with different consistencies on the basis of standard protein analysis because 1) total protein concentrations showed no difference between groups, which agrees with the results reported by Hubner et al (1985); and 2) protein patterns (SDS-PAGE) showed no differences between both groups (Carpino and Siciliano, 1998).

Our results (Figure 4) show that treatment with DTT and α-amylase decreases the viscosity of the seminal plasma only on hyperviscous samples (33% and 44%, respectively) and leads its value to an apparent viscosity that is not significantly different from that measured in the normal consistency control. These results indicate that this is the maximal possible disruption of the hyperviscous macromolecular structure leading to the normal apparent viscosity. In addition, neither DTT or α-amylase reduced the apparent viscosity of the normal consistency semen pools. This effect suggests that protein disulfide bonds in the gel network are responsible for the differential rheological properties observed in the hyperviscous group. Although our results on the addition of α-amylase are in accordance with those reported by Bunge and Sherman (1954) involving the participation of carbohydrates in the gel network, further studies should be done to find the biochemical basis of this effect.

Sperm surface glycoproteins are involved in sperm–zona pellucida recognition. Contrary to the current understanding of sperm membrane biogenesis, recent evidence shows that some of the coating proteins are not synthesized by the spermatozoa themselves but are secreted by cells of the male genital tract. They are transported in the fluid secretions and then incorporated into the sperm membrane. This novel mechanism, by which proteins can move in vivo from one cell to that of another, may account for a significant proportion of the surface changes that occur during ejaculation. Their expression may interfere in the process of capacitation and acrosome reaction, as well as in cell recognition and fusion (Drisdel et al, 1995). We speculate that molecules that are responsible for the rheologic behavior of hyperviscosity may also play a role in sperm physiology, in which high-consistency semen affects sperm motility. Furthermore, sperm motility alone does not adequately explain differences in male fertility. In addition to motility, sperm must also be able to bind to the zona pellucida, to undergo the acrosome reaction, to penetrate the zona pellucida, to bind to the oocyte membrane, and to activate the oocyte to start normal development. A defect in any of these processes will reduce fertility.

We conclude that a highly organized network is responsible for the rheologic characteristics found in hyperviscous semen samples, where disulfide bonds and oligosaccharide chains complexed to the peptide core may play a key role. Further studies should be performed to understand the real importance of these moieties in reproduction.

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