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Andrology Lab Corner*

Filling Time of a Lamellar Capillary-Filling Semen Analysis Chamber Is a Rapid, Precise, and Accurate Method to Assess Viscosity of Seminal Plasma

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Among men from infertile couples, increased viscosity of the ejaculate has been reported to occur more frequently than in fertile men (Moon and Bunge, 1968; Bunge, 1970). During standard semen analysis, several semen variables are evaluated (Oehninger, 2000), but viscosity is rarely quantified. Since the current seminal variables have limited prognostic value (Castilla et al, 2006), extra variables are needed to improve male fertility diagnostics.

Viscosity is a semen variable that has received relatively little attention in the literature. Hyperviscosity might be due to dysfunction of accessory sex glands. Associations have been made between hypofunction of seminal vesicles and hyperviscosity (Gonzales et al, 1993), and altered prostate function is associated with low zinc content in sperm chromatin and low chromatin stability (Björndahl and Kvist, 1990). When this integrity is altered, sperm quality declines. Seminal viscosity abnormality has been shown to be associated with male infertility and was found to accompany poor semen quality (Elzanaty et al, 2004) and altered sperm motility (Eliasson, 1973, Gonzales et al, 1993). It also has been shown that the epididymis and accessory sex glands play a role in the function of male gametes (Gonzales et al, 1992). Deviant viscosity can affect the quality of sperm cells. Hyperviscosity can attribute to biophysical alterations or chemical changes of the ejaculate that could impact sperm quality despite other normal sperm variables seen during

semen analysis (Gonzales and Sanchez, 1994).

To make viscosity a diagnostic variable, the relationship between viscosity and fertility needs to be determined. First the viscosity of semen must be quantified properly. Currently this remains a time-consuming job because specialized machinery is required. The method for viscosity assessment suggested by the World Health Organization (1999) is semiquantitative and in our opinion, inadequate for good quantitative assessments. A method that can be widely used and that expresses its results in centipoise (cP), the international unit of viscosity, is needed.

It is known that the flow velocity in a capillary depends on the diameter of the capillary, the angle of contact, and the viscosity of the fluid. For this study, we used capillary-loaded semen analysis chambers from Leja. The properties of these chambers are well defined. According to theoretic assumptions, a linear relationship exists between filling time of the capillary and viscosity of the sample (Douglas-Hamilton et al, 2005).

The aims of this study were to assess the relationship between the filling time of a capillary-loaded chamber and the viscosity of seminal plasma, to express the viscosity in cP, and to assess the accuracy and precision of the proposed method. Such a method will make it possible to describe the relationship between fertility and the semen variable viscosity. This will extend the number of variables available for the assessment of male fertility and will contribute to better models of male fertility.

Materials and Methods

Seminal Plasma Samples and Processing— Samples of seminal plasma were collected from 248 men who participated in a fertility program of the IVF Center of the Vrije Universiteit Medical Center (Amsterdam, The Netherlands). The samples were collected between June and December 2005. Semen was collected by masturbation, liquefied for at least 30 minutes, and separated into seminal plasma and spermatozoa by density-centrifugation Puresperm (NidaCon International AB, Mölndal, Sweden). The spermatozoa were used for the fertility program. Normally the seminal plasma is discarded. For the purpose of this study, samples were numbered, patient-specific information was removed, and the samples were given to the investigator. The samples were stored frozen until use. Removal of the spermatozoa does not alter the viscosity of the seminal plasma (Hubner et al, 1985; Mendeluk et al, 1992).

Assessment of Viscosity and Filling Time— The viscosities of the samples of human semen plasma were analyzed by the Vilastic 3 (Vilastic Scientific Inc, Austin, Tex). This apparatus is designed for the assessment of the viscosity of various kinds of biologic fluids. For 1 viscosity measurement, a minimum volume of 0.5 mL of seminal plasma was needed. All samples were measured at room temperature.

Two types of disposable semen analysis chambers were used, Leja 4- and Leja 2-chamber slides (Leja, Nieuw-Vennep, The Netherlands). Each chamber has a depth of 20 µm and a length of 21 mm. The width of the Leja 4-chamber is 6 mm, and the width of the Leja 2-chamber is 15 mm. An Eppendorf 10-µL micropipette was used. It is essential to overload the filling area of the chamber to get a proper assessment of the filling time. The tip of the pipette was placed at the filling area of the chamber without touching the entrance. The piston and stopwatch were pushed at the same time. As soon as the liquid reached the air outlet of the chamber, the stopwatch was stopped.

Assessment of Precision and Accuracy— For the assessment of precision, at least 4 mL per sample was needed. Samples of seminal plasma with filling times shorter than 5 seconds (previously

measured in the 4-chamber slide) were pooled, filtered with a 0.21-µm filter, and stored frozen until use. This pooled sample represented lower viscosity. A second pooled sample contained seminal plasma with filling times longer than 17 seconds (4-chamber slide), was filtered with a 0.21-µm filter, and was stored frozen until use. The second sample represented high viscosity. Six repeated measurements each were taken for the 2 pooled and filtered seminal plasma samples, with distilled water and with culture medium (human tubal fluid [Cambrex, Verviers, Belgium] with 0.5% human serum albumin [Sanguin, Amsterdam, The Netherlands]) with the Vilastic 3 and with the 2 types of Leja slides. To assess the accuracy of the method, distilled water and culture medium were measured 6 times. They have a known viscosity of 1 cP and served as reference fluids.

Statistical Analysis— Regression analyses were carried out to investigate the relationship between viscosity and filling times. The 95% confidence interval (CI) of the prediction was also determined. The statistical analysis was preformed with the statistical package STATA 9.0 (DPC Nederland, Bredor, The Netherlands).

Ethics— According to the laws of The Netherlands, no permission is needed from the ethical review board for this type of research.

Results

Initial Assessment and Sample Selection— Seminal plasma samples were thawed, used for assessments, and refrozen for further use. Initially the filling times of all 248 samples were assessed with Leja 4 chamber slides. These results are depicted in Figure 1. The filling times of the 248 initial samples showed a bimodal distribution. The first elevation is skewed and represents the semen samples of which the viscosity could be assessed by measuring the filling times of the capillary slide. The second elevation, representing 27 samples at the end of the distribution, is formed by very viscous samples. Of these 27 samples, 10 did not fill the chamber at all and were considered to be very viscous. Therefore, of the 248 samples, 221 (89.1%) filled the Leja 4 chamber slide within 20 seconds.

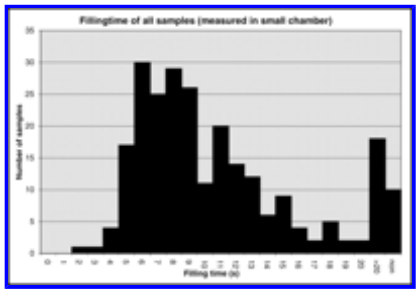

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Figure 1. Filling times of 248 seminal plasma samples assessed in the Leja 4-chamber slide.

Second Experiment— The next experiment was performed with the 148 samples representing all filling times shorter than 20 seconds. These 148 sample were reassessed with the Leja 4-chamber slides (Figure 2A) and Leja 2-chamber slides (Figure 3A) using the Vilastic 3. The filling times of the 4-chamber slide varied between 3.6 and 29.8 seconds. Using the Leja 2-chamber slide, filling times between 4.4 and 43.5 seconds were observed. The Vilastic 3 revealed a lowest viscosity of 1.3 cP and highest viscosity of 10.0 cP; the median value was 4.1 cP. The viscosity of 95% of all

samples was between 2.8 and 6.8 cP.

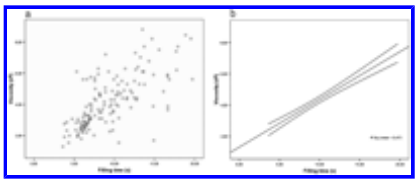

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Figure 2. **(A)** Scatter diagram and **(B)** regression analysis with accompanying 95% confidence intervals showing the relationship between filling time and viscosity (cP) of seminal plasma (*P* < .01; Leja 4-chamber slide). The cut-off value of filling times was above 20 seconds.

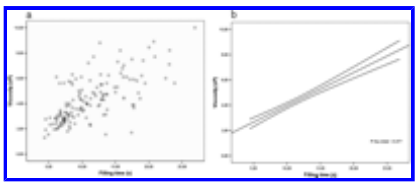

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Figure 3. **(A)** Scatter diagram and **(B)** regression analysis with accompanying 95% confidence intervals showing the relationship between filling time and viscosity (cP) of seminal plasma (*P* < .01, Leja 2-chamber slide). The cut-off value of filling times was above 27 seconds.

Statistical analysis revealed that there was a good linear relationship between filling time and viscosity (*P* < .01). However, there appeared to be cut-off values for filling times. There was a linear relationship between viscosities and filling times of 3.6 to 20.0 seconds (regression line: y = 0.30x + 1.34 [y = viscosity in cP, x = filling time in seconds]; Leja 4-chamber slide) or filling times between 4.4 and 27.0 seconds (regression line: y = 0.24x + 1.68; Leja 2-chamber slide). Six samples were omitted using the Leja 4-chamber slide or 2 samples using the Leja 2-chamber slide. Figure 2B shows the regression line on the data from the Leja 4-chamber slide with accompanying CI. Figure 3B shows the regression analysis on the data from the Leja 2-chamber slide with accompanying CI.

Precision and Accuracy— Precision and accuracy assessments were performed with the 2 pooled and filtered samples of seminal plasma with culture medium and water as reference fluids. The viscosities of all fluids were assessed with the Vilastic 3. The filling times of the Leja 2- and 4-chamber slides were assessed with a stopwatch. As expected, distilled water and culture medium had viscosities near 1 cP; mean ± SD (coefficient of variation [CV]) = 1.03 ± 0.03 cP (0.03) and 1.07 ± 0.03 cP (0.03), respectively. The values of the pooled sample with lower viscosities were 1.97 ± 0.05 cP (0.05). The values of the pooled sample with higher viscosities were 5.38 ± 0.19 cP (0.04).

The filling times in the Leja 4-chamber slide were: distilled water, 3.33 ± 0.17 seconds (CV, 0.05); culture medium, 3.73 ± 0.09 seconds (0.02); low-viscosity pooled seminal plasma sample, 5.30 ± 0.25 seconds (0.03); and high-viscosity pooled seminal plasma sample, 12.78 ± 0.29 seconds (0.02). The filling times in the Leja 2-chamber slide were: distilled water, 3.08 ± 0.13 seconds (0.04); culture medium, 3.45 ± 0.10 seconds (0.03); low-viscosity pooled seminal plasma sample, 5.95 ± 0.18 seconds (0.03); high-viscosity pooled seminal plasma sample 14.21 ± 0.55 seconds

(0.04).The CVs of the filling times were between 0.02 and 0.05. The CVs of the viscosity values were 0.03 to 0.04. These coefficients do not differ, indicating that the precision of both methods is the same.

The regression lines that describe the relationship between viscosity of filtered seminal plasma and filling time were y = 0.45x − 0.38 (y = viscosity in cP, x = filling time in seconds; Leja 4 chamber slide) and y = 0.40x − 0.36 (Leja 2-chamber slide). All regression lines were significantly different than each other ($P < .01$; see also previous paragraph).

Discussion

We have found a well-defined and statistically significant relationship between the filling time of 20-µm capillary slides and the viscosity of seminal plasma assessed by the viscosity meter Vilastic 3 ($P < .01$). Twenty-seven samples had filling times longer than 20 seconds (out of 248); of these, 10 were very viscous and did not fill the chamber at all. This means that in 89.1% of seminal plasma samples, the viscosity can be assessed by measuring the filling time of a disposable capillary used for semen analysis. The use of only 2 tools, a stopwatch and a Leja slide, provides an extra semen variable to use for the evaluation of male fertility.

The accuracy of this method is based on the accuracy of the Vilastic 3, which was calibrated with distilled water. Distilled water has by definition a viscosity of 1 cP. The CV (the ratio of SD to mean) is an indication of the precision of a method. We used 2 pooled samples and filtered seminal plasma. The CVs of these samples were 0.02 to 0.05 for the filling times of the capillaries and 0.03 to 0.04 for the viscosities assessed by the Vilastic 3. Although both methods have comparable precisions, we have to keep in mind that unfiltered seminal plasma will often not be homogeneous, which will result in higher CVs. The best results will be achieved with well-liquefied seminal plasma. Besides the homogeneity of the sample and the present particles, the filling time of a capillary like the Leja chamber depends on the angle of contact between fluid and surface of the chamber, the chamber depth, and the capillary length. This is demonstrated by the significant differences among the regression lines of the 3 experiments ($P < .01$). This means that when the filling time of a capillary is used to assess the viscosity of a fluid, calibration with a viscosity meter like the Vilastic 3 will be essential for each bodily fluid.

A viscosity meter like the Vilastic 3 is not very handy for daily use in a fertility center. One needs a 0.5-mL sample, which is used up during the assessment. The assessment with a Vilastic takes about 10 minutes. After the Leja slide is filled and the filling time assessed, the slide can be used for further semen analysis (motility, concentration, and other microscopic characteristics). One needs only 10 µL and as short as 20 seconds' waiting time for this purpose.

When one uses unfiltered seminal plasma and the Leja 4-chamber slide, the relationship between filling time and viscosity is expressed by the regression line y = 0.34x + 1.34 (y = viscosity in cP, x = filling time t in seconds, $t \geq 3.6$ and $t \leq 20.0$ seconds). When one uses the Leja 2-chamber slide the formula is y = 0.24 + 1.68 ($t \geq 4.4$ and $t \leq 27.0$ seconds), and no further calibration is needed.

In this study, we used samples of seminal plasma from men with fertility problems; our results do not represent a normal population. In our population, the lowest measured viscosity was 1.3 cP and the highest viscosity was 10.0 cP; the median value was 4.1 cP. The viscosity of 95% of all samples was between 2.8 and 6.8 cP. Samples selected for a pooled sample with low viscosity had, after filtration, a viscosity of 2.0 cP. Samples selected and pooled for the assessment of higher viscosity had, after filtration, a viscosity of 5.4 cP. Mendeluk et al (1992) reported that semen

with a "normal consistency" had a viscosity of 4.3 ± 0.2 cP and that semen with a "high consistency" had a viscosity of 5.4 ± 0.4 cP. We can conclude that the values reported by us are in the range of the viscosity of normal semen samples. However, the distribution we found will be typical for a male population seeking help for fertility problems.

Deviant viscosity of semen is associated with male infertility (Gonzales and Sanchez, 1994; Elzanaty et al, 2004), but large clinical studies on this topic are sparse. There is ample evidence that factors of seminal plasma are involved in male fertility (Diekman, 2003; Gwathmey et al, 2006). But how this relates with normal agglutination and liquefaction of seminal plasma is unknown. It can be presumed that disturbed function of male accessory sex glands, resulting in deviant viscosity and deviant interaction between seminal plasma glycoproteins and sperm membranes, can diminish male fertility. Further studies are needed to elucidate such relationships.

This proposed method represents a simple but accurate and precise method for quantification of viscosity of seminal plasma, allowing large-scale research into the relationship between viscosity and male fertility.

Footnotes

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