Journal of Andrology, Vol. 26, No. 1, January/February 2005

Capillary-Loaded Particle Fluid Dynamics: Effect on Estimation of Sperm Concentration

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ABSTRACT: Capillary loaded chambers are frequently used for semen analysis. Poiseuille flow of specimen into these chambers causes migration of suspended particles or cells in a direction transverse to the flow, which results in their preferential accumulation in the Segre-Silberberg (SS) planes. This SS effect depends on the transverse velocity gradient in the laminar flow. For semen analysis in thin capillary-loaded slides, the SS effect can lead to erroneous estimation of sample sperm-cell concentration. To better understand chamber flow dynamics and SS effect significance, we assessed flow uniformity, inflow cell velocity, and results of concentration measurements under different flow conditions for latex bead and porcine and human sperm suspensions. Overall, a concentration peak was present at the meniscus, which continued through chamber loading. High-velocity SS preferred planes, which channeled particles toward the meniscus, were located at the fractional positions of $\beta = .27$ and $\beta = .73$, where β is the distance from wall to plane normalized to the chamber depth. In computer-automated semen analysis, a standard 20-μm $\times$ 18-mm $\times$ 6-mm chamber is commonly used, and these studies supported our previously published fluid-flow theory for this type of chamber. Conversely, the SS effect does not appear to have time to develop in the 100-μm-depth hemacytometer, which is deeper than the standard slide, has lower transverse velocity gradient, and consequently does not exhibit concentration variation due to the SS effect. These findings provide further support that hemacytometry, when performed properly, remains the gold standard. Applicability of our findings to routine semen analyses was then tested in 2 studies performed with independent boar studs. These studies compared diluted boar semen concentrations estimated by standard hemacytometry and in capillary-loaded 20-μm slides, using a computer-automated semen-analysis system designed to compensate for the SS effect. Good numerical agreement for sperm concentration with a high degree of correlation ($r^2 = .936$) was found between the 2 techniques. These findings reaffirm the need to critically assess new technologies for accuracy, repeatability, and precision.

Key words: Spermatozoa, capillary slide, concentration, hemacytometer, Poiseuille, Segre-Silberberg.

J Androl 2005;26:115–122

Capillary-loading slides and hemacytometers are widely used for estimating sperm concentration in neat or diluted samples. Typically used capillary-loaded slides accept very low volumes and are of approximately 20-μm depth to allow for a monolayer of cells while not impeding sperm movement. In capillary loading of slides and hemacytometers, a drop of sample is placed at the entry port, allowing for the formation of a meniscus, which proceeds rapidly by capillary attraction into the chamber, filling it with sample. The cell concentration is then estimated by counting the cells per unit area.

During capillary loading, particles suspended in the flow are exposed to a velocity gradient, which causes particles to move transversely to the flow direction (Vasseur and Cox, 1976). This phenomenon, first reported by Segre and Silberberg (1961) and called the Segre-Silberberg (SS) effect, was observed for particles initially evenly suspended in a cylindrical and planar Poiseuille flow, which then became unevenly concentrated at a well-defined distance from the walls. For a sample of semen (neat or diluted) entering a capillary chamber, sperm can therefore be expected to cross the flow and congregate in faster flowing layers of fluid. The transfer of sperm into the faster flowing layers causes a concomitant reduction in unit area sperm concentration and, thus, an accumulation of sperm at the meniscus. Consequently the measured concentration at the meniscus will be higher, and behind the meniscus (during or after capillary flow) will be lower, than in the original sample. Indeed, previous works using 20-μm capillary slides (Johnson et al, 1996b; Mahmoud et al, 1997) have noted differences in latex-bead concentration across the slide.

We have predicted previously the expected difference between actual sample concentration and that measured in low-volume capillary-loaded slides, similar to those

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This manuscript is part of a patent application on Segre-Silberberg corrected capillary filled slides by these authors.

Received for publication May 25, 2004; accepted for publication September 8, 2004.

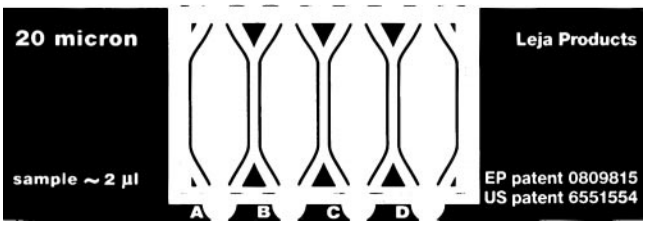

Figure 1. Leja 4-chamber slide. The chambers are each 6 mm wide × 20 mm long × 20 μm depth, with an overall slide size of 25 × 75 mm.

used in computer-automated semen-analysis systems (Douglas-Hamilton et al, 2005). We found that the velocity of transverse flow and hence significance of the SS effect depends on the sample viscosity, surface tension, chamber depth, and cell size. For low-viscosity samples in which the SS effect is completely developed, our model predicted that a factor of 1.30 is necessary to compensate for the SS effect. The purpose of this study was to test and validate our prior predictions on the measured sperm concentration in capillary-loaded slides. Three experiments were designed using low-viscosity samples. Experiment I was performed to verify the predicted meniscus concentration peak in diluted boar and human sperm concentrations within a chamber. In experiment II, inflow velocities of entrained beads and sperm into a capillary chamber were assessed. Last, experiment III was a field study of 2 boar studs, in which boar-sperm concentrations measured using hemacytometry and capillary slides were compared. We found the predicted compensation factor allowed for consistent and accurate agreement between the 2 methodologies.

Materials and Methods

For this study, 20-μm 4-chamber slides (Figure 1; Leja Products B. V., Nieuw-Vennep, The Netherlands) and Improved Neubauer hemacytometers (Fisher Scientific Inc, Pittsburgh, Pa) were used to determine cell concentration. Improved Neubauer hemacytometers were chosen due to previous recommendations regarding reliability (Mortimer et al, 1986; Mahmoud et al, 1997; WHO, 1999). In the 20-μm 4-chamber slides, each chamber under the coverslip measured 18 mm in length and 6 mm in width with tapered ends; chamber volume was approximately 1.9 μL. Because there is a linear relationship between concentration and chamber depth, chamber depth was verified preuse for all sample chambers using an interferometer (Hamilton Thorne Biosciences, Beverly, Mass). In this specially designed interferometer, chamber depth was determined using polarized light of wavelength λ = 630 nm incident at the Brewster angle, which is reflected from the top and bottom walls of the chamber and the beams recombined. Loaded and unloaded chamber depths are determined with accuracy to within 0.5 μm of actual depth. Along with verifying chamber depth, interferometry was also used to ensure that the hemacytometer depth did not significantly change when repeatedly loaded with a solution of viscosity 1.0 centipoise to a level that visually filled the chamber.

Both porcine and human semen samples were used in the initial experiments. Porcine semen was obtained from Monsanto Choice Genetics (St Louis, Mo), where the semen was diluted to 40 × 10^6 sperm per mL (abbreviated as 40 M/mL) and shipped overnight at approximately 17°C to the laboratory for subsequent analyses. Donor human semen samples were washed and resuspended to 22 M/mL using a modified HTF medium with 0.5% human serum albumin (Biowhittaker Europe, Verviers, Belgium). For the field studies, both diluted-chilled (trial A) and fresh-extended (trial B) porcine semen was used, specifics of which are further outlined below.

Experiment I. Intrachamber Sperm Concentration

Porcine extended semen samples (N = 4 boars) were subdivided by placing 1.0-mL aliquots of sample into each of 5 covered test tubes, and by placing 0.5 mL into a sixth test tube. Extender media was added to each tube to achieve final predicted sperm concentrations from the measured weight dilution relative to samples of 1.0, 0.837, 0.672, 0.498, 0.332, and 0.165, respectively. Weights were verified using a calibrated digital balance (Mettler; Fisher Sci). Human semen samples (N = 3), suspended to 22 M/mL in a modified HTF medium, were immobilized by heat inactivation following loading into the Leja slide and prior to data acquisition. All subsequent procedures remained the same.

Capillary chambers were loaded using an adjustable digital pipette (P-10, Rainin Instrument Co Inc, Woburn, Mass) to place 1.5–1.7 μL of sample at the capillary slide entry port. Our observation has been that the sample moves smoothly into the chamber until the rear meniscus of the sample globule reaches the entry port, at which point further flow ceases. The forward meniscus is located 10.3–14.8 mm from the entry port. Sample load time for diluted boar semen with a viscosity of 1.2 cP (determined by viscometry [Gilmont GV-2100; VWR, Boston, Mass]) is 1.3–2.5 seconds, with this time variation due to small differences in the glass-sample capillary attraction. The cessation of flow is abrupt, and the distribution of nonmotile sperm inside the flow will not change significantly thereafter. All sperm introduced into the chamber are visible. Nonmotile sperm can only be moved by drift or Brownian motion: because both of these are negligible, the sperm remain in their positions, and a frozen picture is obtained of particle distribution at the moment when flow ceases. For motile sperm, the inflow velocity (~8 000 μm/s) is greater than the sperm swimming velocity (~200 μm/s), thus, sperm distribution is controlled by the chamber flow dynamics. Rapid analysis after cessation of flow ensures that motile sperm do not have time to change their positions significantly.

Photomicroscopic capture of sperm concentration in the 20-μm slide following the loading operation was measured using a CEROS analyzer (Version 12.1; Hamilton-Thorne Biosciences) with a 10× negative phase contrast objective on an Olympus CH-2 microscope (Optical Analysis, Nashua, NH) and a Sony XC-75 camera (Sony Corp, Tokyo) interfaced with a computer. Each field was immediately checked visually for correct sperm acquisition and recognition by CEROS using the playback feature. Standard setup values for diluted boar sperm were used (Table 1). Because the main variation in concentration is in the

Table 1. *Ultimate standard setup values for boar sperm analysis**

Frames acquired	45/s
Frame rate	60 Hz
Minimum contrast	46
Minimum cell size	7 pixels
Default cell size	7 pixels
Default cell intensity	30
Progressive VAP	45 μm/s
Progressive straightness threshold	45%
Slow VAP cutoff	20 μm/s
Slow VSL cutoff	5 μm/s
Static intensity gates	0.5–2.5
Static size gates	0.65–4.90
Static elongation gates	0–87

* VAP indicates path velocity; VSL, progressive velocity.

direction of flow (and lateral concentration variation was shown to be negligible in preliminary experiments), we measured concentration down the centerline of the chamber at 1-mm intervals, starting at 1 mm from the entry and continuing up to the forward meniscus. In each of the samples, sequential measurements were replicated 4 times in each of 4 separately loaded chambers.

Partial chamber filling through the control of load volume was used to allow for viewing of the entire loaded sample in the chamber and derivation of sample concentration through counting the total number of particles present. Total concentration was defined as the count of all particles divided by the volume of all fields counted in the chamber. Similarly, partial concentration was defined as the count of all particles from each field except the 3 fields nearest the meniscus and the 1 field nearest the entry port, divided by the volume of the fields counted. Partial concentration represented the concentration a fully loaded 20-μm chamber would give with the meniscus wave concentration no longer visible on the slide.

To determine the dependence of the SS effect on capillary flow velocity, we reduced the flow rate into the 20-μm 4-chambered slide. This was achieved by plugging the chamber outlet with 0.2 μL of white ink correction fluid, which is composed of suspended particles in a volatile liquid. When the correction fluid evaporated, the slightly porous material left (which extended about 2 mm into the exit port) provided an effective porous plug. Air could still escape slowly through the pores. Sperm density of 4 aliquots from a porcine extended semen sample (26.4 M/mL) was measured as a function of position, normalized to the meniscus, which was set to 18 mm. Measurements were obtained from each of 4 separately loaded chambers, with the mean and standard deviation at each position computed.

The effect of using a deeper capillary chamber was briefly examined. Three aliquots (each 7.0 μL of boar semen, concentration ~1 M/mL) were loaded into three 100-μm-deep chambers (courtesy of Leja Products) of identical X and Y dimensions as the 4-chamber slide described above. The chamber was filled to allow the meniscus to reach close to 18 mm from entry, and then sperm counted down the centerline using the same CEROS setup with a 4× objective. Cell acquisition was verified by playback for each field. This technique was performed on 3 separate chambers, with the concentrations at each position relative to the meniscus averaged across the chambers.

Experiment II. Entrained Sperm Velocity During Loading

Stroboscopic illumination techniques were utilized to determine particle velocity at inflow (Figure 2). The 20-μm 4-chambered slide was placed on the stage of an Olympus CH 30 microscope with a 10× NH objective, and a Sony XC-75 camera (Sony Corp, Tokyo) interfaced with a CEROS sperm-motion analyzer. The microscope was focused on the central plane of the chamber between the slide and its fixed coverslip. Illumination was provided by a 1 j/pulse Xenon source (Perkin Elmer, Salem, Mass) directed at the phase plate and strobed at 60 Hz. Strobe-flash duration was set to less than 5 μs so that clear images of the

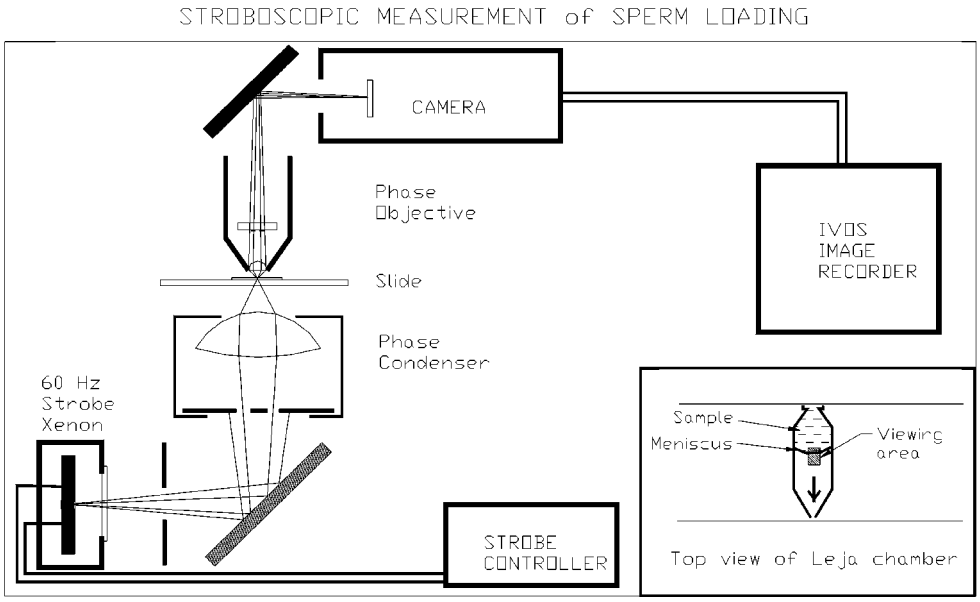

Figure 2. Diagram outlining strobe illumination setup for measuring chamber inflow velocities.

moving particle could be obtained. After setup, the chamber was loaded with 3 μL of sample, and 100 images are acquired at 60 Hz, digitized, and stored using the interfaced software (IVOS V12.2c; Hamilton Thorne Biosciences). The interval between each image was 16.67 msecond, during which the flow travels approximately 130 μm. Images were printed out on vugraph acetate and sequentially compared to determine particle velocity. With proper timing, the meniscus is captured as it passes through the field of view (350 × 550 μm), and, thus, its velocity derived. Initial velocity determinations were made on 2 types of latex beads during their inflow into the 20-μm chamber. A mixture of 4-μm and 1.9-μm beads (Bangs Laboratories, Fishers, Ind) was made in an aqueous medium with viscosity of 1.0 cP and at a concentration of 5 M/mL and 3 M/mL, respectively; at these densities, bead size can easily be distinguished by intensity and individual beads tracked without ambiguity between successive frames. Inflow velocities in the 20-μm slide were then determined for extended boar semen (~6 M/mL) utilizing the same procedure.

Experiment III. Field Studies

Two studies were undertaken to compare porcine sperm concentration generated from use of the 4-chamber slide on a computer-automated semen analysis system internally corrected for the SS effect (UltiMate [ULT], Version 12.1; Hamilton Thorne Biosciences) and with a hemacytometer (Fisher Sci) using standard techniques (Althouse et al, 1995; WHO, 1999). In both studies and with each technique, each sample/ejaculate was analyzed in duplicate. The analysis was double blind; that is, the ULT and hemacytometer results were each unknown to the other technician and were only compared by an independent third party at the conclusion of the trial.

In the first field trial, diluted (~40 M/mL) and cooled specimens (N = 12 boars) less than 6 hours postprocessing were hand delivered to the laboratory for further analysis. Each sample was given an arbitrary identifying number and then subdivided into 2 equal aliquots for determination of sperm concentration using the ULT and hemacytometer. The second field trial took place on-site at a commercial boar stud farm (Iowa Select Farms, Iowa Falls, Ia). Gel-free ejaculates (N = 47) were collected from boars using the gloved-hand technique. The ejaculate was given an arbitrary identifying number and then a subsample diluted 1:40 using isothermal extender (IMV Intl, Minneapolis, Minn). For ULT measurement in each trial, prepared samples (1.9 μL each) were loaded into two 20-μm chambers. After slide loading into the ULT, concentration was determined in each chamber by obtaining concentration measurements in 6 adjacent horizontal fields, with these 6 fields averaged to obtain sample concentration in that chamber. The playback feature was used to confirm proper cell identification and absence of agglutination. Readings from the 2 chambers within 10% of each other were averaged and the final concentration ascribed for that sample. For both field trials, hemacytometry was performed from subsamples of each sample used in the 20-μm chamber and further diluted 1:4 using isothermal extender. Estimation of sample concentration was then performed as previously outlined (Althouse et al, 1995). Counts from both sides of the hemacytometer were required to be within 10% of one another to be accepted, with the reported sperm concentrations being the average of the 2 counts.

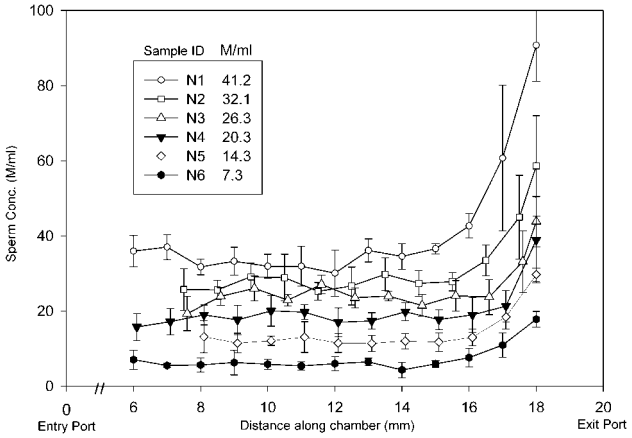

Figure 3. Concentration readings (±SD) of motile boar sperm along centerline of 20-μm Leja chamber (n = 4) for sample concentrations 6–40 M/mL. Measured from entry: meniscus superimposed at 18 mm.

Statistical Analysis

Fundamental descriptive statistics were utilized in all experiments to describe quantitative data. Student's *t* test for paired samples was used for performing pairwise comparisons using a 1-tailed distribution. A 1-tailed distribution was chosen for our analysis based on our understanding of the SS effect and the results of the theoretical model (Douglas-Hamilton et al, 2005). The power of the *t* test for detecting differences was established at $P < .05$ in this study.

Results

Experiment I. Intrachamber Sperm Concentration

Results of this study are presented in Figure 3. The concentration peak at the meniscus is clearly visible starting at 14–16 mm into the chamber. The meniscus wave was higher at greater concentrations, as one might expect, but its relative height was comparable for the different concentrations. The meniscus concentration wave appears to exist within the 3 positions (mm) closest to the meniscus. As such, for complete sample loading, the wave would normally disappear into the exit port, leaving a high-concentration region in the inaccessible and invisible exit port and a lower concentration region in the rest of the chamber behind the wave.

Values of the mean total concentrations averaged over the 4 chambers for each dilution are provided in Figure 3 and summarized in Table 2. Also given in Table 2 is the partial concentration, averaged over 4 chambers. The theoretical weight dilution concentration agrees well with the total concentration estimated by CASA on the 20-μm slide, confirming that all the sperm in the 20-μm chamber are accounted for. Our results show that the average total

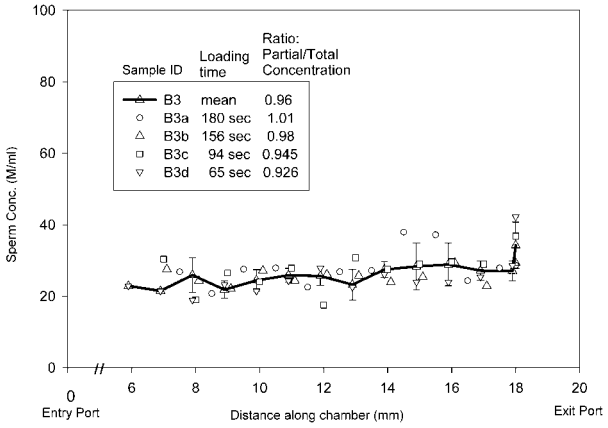

Figure 4. Boar sperm concentration (26.4 M/mL) (±SD) along centerline of slow-Loaded 20-μm Leja chamber. The loading time and ratio of partial-to-total concentration is given (see text).

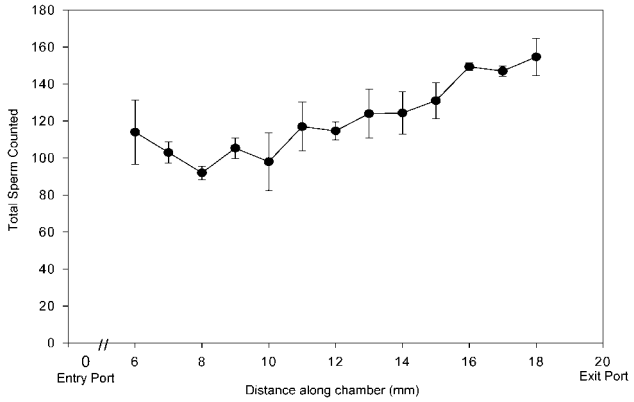

Figure 5. Concentration of boar sperm (±SD) along centerline of a 100-μm-deep chamber (n = 3). Meniscus is located at 18 mm.

to partial concentration ratio can be estimated at approximately 1.17, indicating that the actual concentration is approximately 17% higher than the value measured from a fully loaded 20-μm chamber.

Blocking the exit port with a porous plug effectively reduced inflow and prolonged filling time from approximately 2 seconds to 65–180 seconds. This change greatly diminished the size of the concentration peak at the meniscus, as expected from the analysis. The concentration averaged over 4 chambers is presented in Figure 4, showing almost flat concentration in the partially filled 20-μm slides. The meniscus concentration wave was greatly reduced, as predicted, and the measured partial and total concentrations are much closer, supporting the concept that the SS effect has not had time to develop at the low sample-flow rates.

Sperm concentration in the 100-μm chamber when flow ceases after 18 mm is shown in Figure 5 as a function of position. No sharp meniscus wave was observed in the 100-μm chamber, although cell concentration did increase gradually from entry to exit.

Assessment for an SS effect on human sperm samples (N = 3) with a viscosity of 1.02 cP, is presented in Figure 6. The high-concentration wave at the meniscus is clearly visible in all cases and is similar to that seen in the case of motile boar sperm (see Figure 3).

Experiment II. Inflow Entrained Sperm Velocity

Velocity distribution of the 4-μm-diameter beads is shown in Figure 7. A strong velocity peak for the beads was found near the position where flow velocity approximates 0.8 cm/s, with no slow-velocity beads present. This finding suggests a distinct transport mechanism that draws beads from the low-velocity to the high-velocity planes in the Poiseuille flow. It is consistent with beads

Table 2. *Total, partial, and predicted dilution concentration for boar sperm in Leja 4 chamber analyzed by CEROS (mean and SD)**

Dilution Number	Total Concentration†	Partial Concentration†	Diluted Concentration	Ratio Total : Partial	Ratio Total : Dilution
N1	41.05[a] (2.60)	33.78[b] (1.11)	39.28	1.22	1.05
N2	32.09[a] (1.38)	27.51[b] (1.67)	32.88	1.17	0.98
N3	26.31[a] (1.75)	24.03[b] (1.22)	26.40	1.09	1.00
N4	20.29[a] (0.68)	18.60[a] (1.20)	19.56	1.09	1.04
N5	14.30[a] (0.38)	11.92[b] (0.67)	13.04	1.20	1.10
N6	7.31[a] (0.91)	5.87[b] (0.50)	6.48	1.24	1.13
				1.17	1.05
D1	39.58[a] (2.25)	35.30[b] (2.93)	39.28	1.12	1.01
D2	34.45[a] (1.61)	29.76[b] (1.63)	32.88	1.16	1.05
D3	28.26[a] (1.32)	24.68[b] (0.98)	26.40	1.15	1.07
				1.14	1.04
B3	25.92[a] (1.56)	24.68[a] (2.55)		1.05	

* Total indicates count of all particles divided by the volume of all fields counted in the chamber; partial, count of all particles from each field except the 3 fields nearest the meniscus and the 1 field nearest the entry port, divided by the volume of the fields counted; dilution, weight % dilution: the mass dilution as measured on the Mettler balance is given normalized to the hemacytometer measurement, which was made on sample N2; N1–N6, live (motile) boar sperm samples; and D1–D3, dead (glutaraldehyde immobilized) samples of N1, N2, N3; B3, live sperm used in 4 chambers with porous plug-blocked exits.

† Different superscript letters denote significant differences ($P < .05$) within rows.

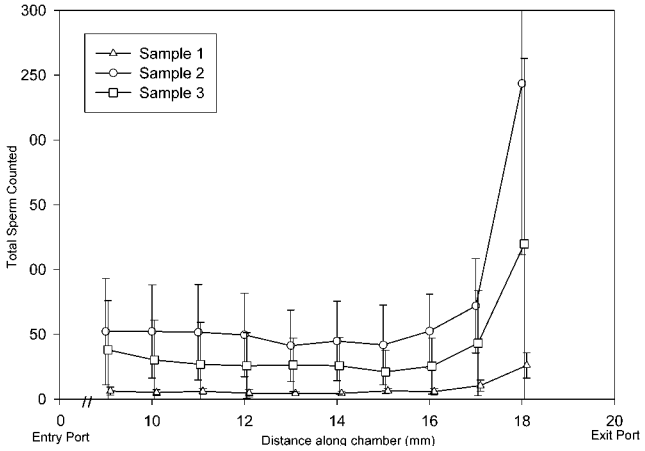

Figure 6. Concentration readings (±SD) of human semen (n = 3) along centerline of 20-μm Leja chamber (n = 4).

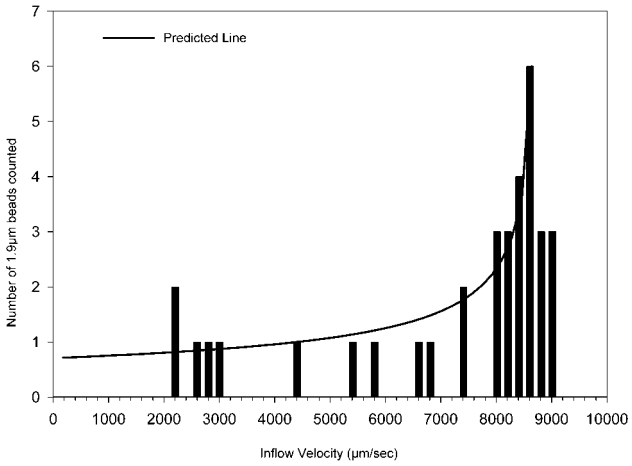

Figure 8. Inflow velocity of 1.9-μm beads flowing into 20-μm Leja 4 chamber (n = 4), velocity measured at 1 cm from entry port.

transported in the high-velocity SS-preferred planes in the 20-μm slide, at the fractional positions of β = .27 and β = .73, where β is the distance from plane to wall normalized to the chamber depth.

Velocity distribution of the 1.9-μm beads is shown in Figure 8. Although only 34 beads were counted at the 3 M/mL concentration, there is a clear trend for the 1.9-μm beads to establish low- and high-velocity regions. The solid line represents a prediction of the velocity frequency curve for uniformly distributed beads in Poiseuille flow. The distribution of beads observed in this experiment is close to uniform: that is, the 1.9-μm beads have not had enough time to segregate in the SS planes because the transverse velocity varies as the cube of the bead radius, showing large standard deviation in velocity. No leading-edge concentration peak was observed.

The inflow velocity distribution of live, motile boar sperm is shown in Figure 9. The sperm show the same distribution as the 4-μm latex-bead spheres, with the sperm concentration increasing sharply near the meniscus and decreasing behind it. The mean inflow velocity (ie, meniscus velocity) is shown as a solid vertical line. As observed, almost all the sperm in the postmeniscus region are moving at a velocity higher than the mean fluid flow. These high-velocity sperm accumulate at the meniscus leading edge thereby reducing the concentration of sperm measured behind the meniscus. Denoting the maximum velocity along the central plane of the chamber as V_{max}, the velocity corresponding to the SS layer is approximately 0.8 V_{max}, or about 7400 μm/s.

Experiment III. Field Studies

Results of the 2 field studies are shown in Figure 10. When comparing concentrations determined by hemacytometry to the ULT that has been corrected for the SS effect, the correlation coefficient was r^2 = .936, with a regression gradient of 0.984.

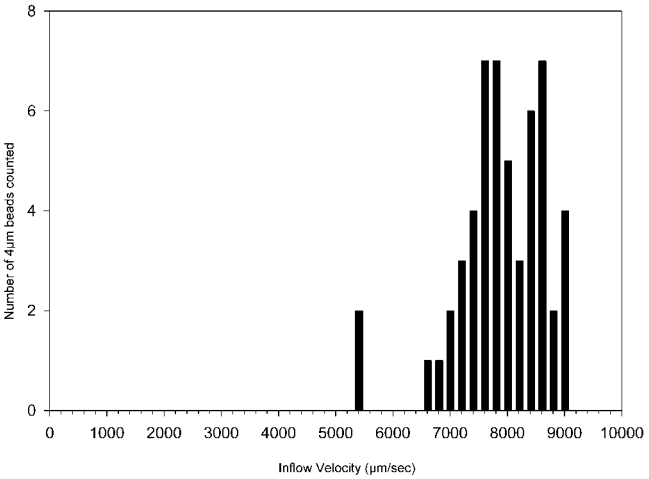

Figure 7. Inflow-velocity distribution of 4-μm beads flowing into 20-μm Leja 4 chamber (n = 4), measured 1 cm from entry port.

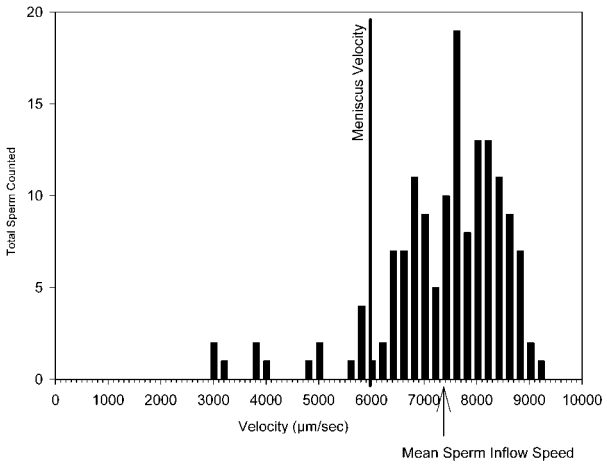

Figure 9. Velocity distribution of motile boar sperm flowing into 20-μm Leja 4 chamber (n = 4), measured 1 cm from entry port.

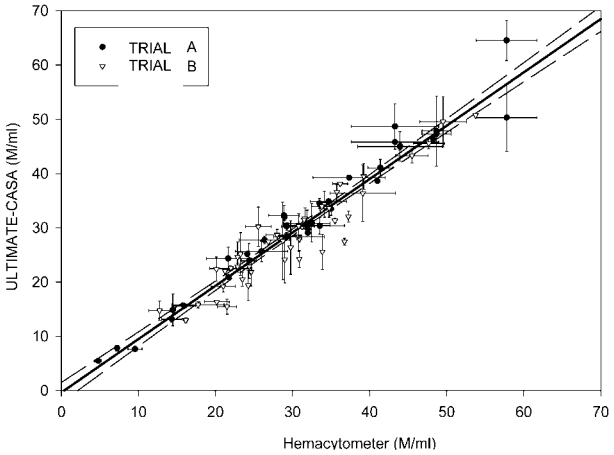

Figure 10. Boar semen analysis data from trials A (solid circles) and B (open triangles). Error bars represent standard deviations for repeat observations of the same sample. Ninety-five percent confidence limits for the regression line are shown. $r^2 = .936$. Regression gradient = .984.

Discussion

The results of experiment I show that sperm cells in a plane parallel capillary flow tend to collect near the leading edge of the flow, with the concentration in the trailing part of the flow concomitantly reduced. This effect was most pronounced for rapid flow into narrow chambers. These findings are in accordance with predictions from the SS effect and were observed with boar (Figure 3) and human (Figure 6) sperm.

When determining total, partial, and dilution concentrations within chambers using total counts, we expect that the ratio of the total mean concentration to the expected weight percentage dilution concentration approaches 1, if a representative sample of sperm was counted. Therefore, the ratio of the total to the partial measured concentration reflects the difference between the actual number of sperm present and what would normally be counted in a capillary slide. A total-to-partial concentration ratio of 1.17 was found, establishing that the actual concentration was approximately 17% higher than the value measured from a fully loaded 20-μm chamber. Note that this ratio is only approximate because it depends on estimation of the number of cells in the meniscus peak. This number is of limited accuracy because the size of the measuring area (~½ mm) is similar to the width of the meniscus concentration peak (~1 mm; see Figure 3), and the cells in the concentration peak will be undercounted. We can, however, conclude that these results provide direct evidence that the meniscus high-concentration wave exists and that the concentration behind the wave is lower than the sample concentration.

Blocking the exit port with a porous plug was performed to greatly reduce inflow velocity. As expected, reduction of velocity effectively eliminated the SS effect (B3 in Table 2; Figure 4) in the 20-μm chamber. Likewise, particle concentration did not develop a significant meniscus wave in the 100-μm slide because particles had not had time to migrate sufficiently to the faster regions of flow. This is in accordance with the prediction that the time to develop the SS segregation effect varies as $1/L^2$, where L is chamber depth. Therefore, a negligible SS effect can also be expected in the 100-μm depth hemacytometer, in which the flow travels less than 10 mm from point of entry. Indeed, when we repeated these experiments using boar sperm in a hemacytometer, we found that the inflow was irregular because of the V loading incision in the Improved Neubauer hemacytometer. Nevertheless, no concentration increase at the leading edge of the meniscus wave was observed.

In experiment II, inflow velocity distribution of boar sperm and 2.0-μm- and 0.95-μm-radius beads was assessed. Results confirmed that the SS effect occurs for the larger sperm and 2.0-μm beads as they flow into the chamber and show that, as expected, the beads and sperm are in fact entrained into more rapidly moving layers of the flow, move in more rapidly than the mean fluid velocity, and as a result are measured behind the meniscus with reduced concentration. The SS-stable planes are expected to be at the fractional positions of $\beta = 0.27$ and $\beta = 0.73$. On the other hand, the larger spread in velocity of the 1.9-μm beads is expected because the predicted degree of segregation increases monotonically with the figure of merit, which is proportional to the cube of the particle radius (Douglas-Hamilton et al, 2005). The smaller particles do not have time to segregate into a particular stream, and they remain spread through all velocities.

Last, 2 field studies were performed comparing diluted semen concentrations estimated by standard hemacytometry and in capillary-loaded 20-μm slides using a computer-automated semen-analysis system designed to account for the SS effect (eg, ULT). A very high degree of correlation ($r^2 = .936$) was found between estimates obtained from the ULT and hemacytometer. Additionally, high amounts of precision, accuracy, and repeatability were observed between the 2 sites.

Sperm segregation can be expected to occur whenever a sperm sample of low viscosity is introduced into a narrow capillary-loaded slide. Therefore, a correction is required if these slides are to be used to estimate sperm concentration. This segregation is due to the SS effect and is important to consider in industries where samples examined are always diluted and of low viscosity. Oversight in accounting for this SS effect can lead to faulty estimation of sperm concentration when using thin, capillary-loaded slides, which induce the SS effect. The oversight of the SS effect in capillary-loaded slides most likely accounts for the differences reported in prior published articles in which variability existed in estimates of sperm

concentration between hemacytometry and CASA (Johnson et al, 1996a; Seaman et al, 1996; Mahmoud et al, 1997; Brazil et al, 2004; and others).

We have derived a simple model to predict the concentration change and compared it with experiments using 20-μm chambers. The model derived is based on spheres suspended in Poiseuille flow. Sperm behavior in gradient flow is complicated by the presence of the tail: this suppresses rotation and increases the sperm effective area. However, the model derived for spheres gives relatively good prediction of sperm behavior in the experiments, and SS segregation of sperm is observed similar to that expected for 4-μm-diameter spheres. Our model and observations also show that the SS effect is insignificant in hemacytometers due to the larger chamber depth and small size. The hemacytometer remains the gold standard. Using SS-compensated UltiMate CASA analysis, good agreement was obtained in a field test between hemacytometer and UltiMate for boar sperm analysis.

Conclusion

We have developed and tested a model of capillary-loading fluid flow and its effect on sperm concentration for typical 20-μm slides. We find that a SS segregation of particles causes a change in the observed particle concentration. The predicted high-concentration wave at the meniscus and the prediction that sperm entry velocity is greater than the mean fluid flow velocity were both confirmed. Stroboscopic measurement of entrained sperm velocity during loading has supported the hypothesis that the sperm congregate in a rapid-flow layer and are pumped to the forward end of the flow. Analysis of the experiments using 100-μm slides showed that the SS effect is not significant in hemacytometers, as predicted, and the hemacytometer therefore remains the gold standard for concentration determination. These experiments do demonstrate, however, the necessity to account for the SS effect in sperm in thin capillary-loaded slides. The SS compensation factor derived for boar sperm with viscosity close to 1.0 cP was 1.30. Application of this compensation factor along with inherent flow dynamics of the slide allowed for good correspondence between CASA and hemacytometry. Experimental results from 2 field studies using diluted live boar sperm agreed well with the model derived from the SS effect. A similar compensation factor would be expected for washed (low viscosity) human semen, while viscous samples are predicted to require a lower compensation factor.

Acknowledgments

We would like to record our indebtedness to Irakli Shekriladze, of the Georgian Academy of Sciences, who pointed out the relevance of the SS effect to the slide-filling problem, for many useful discussions. We would also like to thank Von Miller-Lux for assistance in specimen counts, Brian Qualley for his hospitality at Iowa Select Farms, and Rick Vigus for providing materials for this study.

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