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Effect of the numbers of frames analyzed on sperm density and motility using Hamilton-Thorn Motility Analyzer (HTMA).

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Eleven semen samples from volunteer donors were analyzed to assess the influence of the number of frames analyzed on the result of human sperm density the percentage of sperm motility and sperm movement characteristics when using the Hamilton-Thorn Motility Analyzer (HTMA). These semen samples were analyzed by the HTMA Model 2030, version 7 at different sperm concentrations (10×10^6 - 80×10^6 sperm/ml) and different numbers of frames analyzed (5-20 frames). The data indicates that values obtained for sperm density, percentage of sperm motility and sperm movement characteristics depend on the number of frames analyzed. It is concluded that 20 frames is recommended for assessing sperm movement characteristics, but 5 frames is suitable for the assessments of sperm densities and percentage of sperm motility to minimize errors due to collisions.

Key words : Hamilton-Thorn Motility Analyzer (HTMA), Number of frames analyzed, Sperm density, Percentage of sperm motility, Sperm movement characteristics.

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นเรศร สุขเจริญ, เอนก อารีพรรค. ผลของจำนวนภาพที่ใช้วิเคราะห์ด้วยเครื่องวิเคราะห์การเคลื่อนไหวของอสุจิของเฮมิลตัน ธอร์น ต่อความเข้มข้นของอสุจิและการเคลื่อนไหวของอสุจิ. จุฬาลงกรณ์เวชสาร 2538 กรกฎาคม; 39(7): 521-530

เพื่อศึกษาผลของจำนวนภาพที่ใช้วิเคราะห์ด้วย Hamilton-Thorn Motility Analyzer (HTMA) ต่อความเข้มข้นของอสุจิ ร้อยละของการเคลื่อนไหวของอสุจิและลักษณะการเคลื่อนไหวของอสุจิ ได้ศึกษาน้ำอสุจิ 11 ตัวอย่างจากผู้บริจาค ตรวจน้ำอสุจิดังกล่าวด้วย Hamilton-Thorn Motility Analyzer Model 2030 version 7 ที่ระดับความเข้มข้นต่าง ๆ (10 x 10⁶ - 80 x 10⁶/ มล.) และจำนวนภาพที่ใช้วิเคราะห์ต่าง ๆ (5-20 ภาพ) จากข้อมูลที่ได้พบว่าความเข้มข้นของอสุจิ ร้อยละของอสุจิที่เคลื่อนไหวและลักษณะการเคลื่อนไหวของอสุจิขึ้นกับจำนวนภาพที่ใช้วิเคราะห์สรุปว่าการวิเคราะห์จำนวน 20 ภาพ เหมาะสำหรับการตรวจลักษณะการเคลื่อนไหวของอสุจิ แต่การวิเคราะห์จำนวน 5 ภาพ เหมาะสำหรับการตรวจความเข้มข้นของอสุจิและร้อยละของการเคลื่อนไหวของอสุจิเพื่อลดความผิดพลาดจากการชนกันของอสุจิ

Semen analysis is the procedure most commonly used in the evaluation of male fertility potential. Traditionally, the parameters analyzed include sperm density, percentage of sperm motility and morphology. It is generally believed that standardized, accurate,and precise analysis of sperm motion in semen will improve the predictivity of male fertility. The measurements and analysis of sperm motion are kinematically complex, and are optically difficult to image with high resolution light microscope. Historically, the clinical asessment of sperm motility was based on subjective visual impressions with limited accuracy and precision. This realization has led to the development of numerous systems for manual and semi-automated analysis which is acceptable for routine applications. More recently, fully automated analysis intended purely for research has been introduced. Video and computer vision technology has produced new instruments that are able to identify and track individual sperm cells and thence to calculate a number of parameters characterizing sperm movement.[1-4] Such instruments use a computer to process the electrical signal obtained by a video camera integrated to a microscope , either by direct analysis of that signal, or by analysis of the corresponding signal from a video tape recorder.[1]

In recent years, the evolution of computer vision technology has given rise to a new generation of instruments that have overcome the historical limitations and are becoming amenable to practical clinical utilization. Computer-aided semen analysis (CASA) technology has become commercialized and is now used in hundreds of laboratories worldwide. These laboratories are, for the most part, oriented towards research as well as clinical practice. A challenge to contemporary CASA is to define its role as a routine tool in the clinical analysis and treatment of human infertility. Nowadays, CASA systems are designed to obtain measurements of sperm density, the percentage of sperm motility, and sperm movement characteristics.[1]At least six CASA systems are currently available. The Hamilton Thorn Motility Analyzer (HTMA) used in this study is one of the most popular CASA systems. However, to a great extent, results seem to depend on parameter settings which are completely at the discretion of the user. The number of frames analyzed is one of the most important such parameters. This study was undertaken to investigate the influence of the number of frames analyzed on the result of the sperm density, the percentage of motile sperm and their movement characteristics.

Materials and methods
Semen samples and semen preparation

Semen samples were provided by 11 donor volunteers. After allowing at least 30 minutes for liquefaction to occur, the sperm were then separated from the seminal plasma by discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation using a 2-step gradient comprising a 3 ml layer of 80% Percoll overlaid with 3 ml of 40% Percoll. Isotonic Percoll was created by supplementing 10 ml of 10 x concentrated medium 199 (Flow Laboratories, Irvine, UK) with 300 mg bovine serum albumin (BSA), or the human serum albumin preparation "Albuminar " (Armour Pharmaceutical Co., Wastbourne, UK), 3 mg sodium pyruvate and 0.37 ml of a sodium lactate syrup and adding 90 ml of Percoll. This preparation was designated 100% Percoll[5] and was subsequently diluted with HEPES-buffered Biggers-

Whitten-Whittingham medium (BWW)[6] supplemented with bovine serum albumin (BSA) or Albuminar, as indicated. After a 20 min period of centrifugation at 500 g, the highest quality sperm, located at the base of the 80% fraction were collected, washed with a 5 ml volume of medium BWW and finally resuspended at concentrations of 10, 20, 40, 60 and 80 x10⁶/ml which were confirmed by conventional semen analysis for sperm density.[7] These sperm suspensions were used for sperm density and sperm motility assessment by the HTMA and determination of percentage of sperm motility by a conventional method.

Determination of sperm motility by HTMA

Sperm motility assessment was performed immediately after preparation using the European (25 Hz) version of the HTMA Model 2030, version 7 (Hamilton Thorn Research, Danvers, MA, USA) at a temperature of 37°C using the following settings; minimum contrast 90; minimum size 5; low and high size gates 0.5 and 2.0, respectively; low and high intensity gates 0.5 and 1.45, respectively; nonmotile intensity 90. The numbers of frames analyzed were varied from 5-20 to evaluate the influence of the numbers of frames on the outcome of the automated semen analysis. The measurements were conducted in 20 μm depth MicroCell slides (Fertility Technologies Inc., Natick, MA) and at least 100 motile cells were assessed for each determination. These determinations were carried out in duplicate and the results were averaged.

The criteria of movement assessed in this study were curvilinear velocity (VCL), straight line velocity (VSL); average path velocity (VAP); percentage of motile sperm (the percentage of cells exhibiting a VAP of ≥ 10 μm/S) and ALH (the amplitude of lateral sperm head displacement in mm.). Linearity (LIN) was defined as VSL/VCL x 100, while straightness (STR) was VSL/VAP x 100. The playback function of the HTMA was used to accurately identify motile cells as a red dot and immotile cells as a blue dot to verify the validity of the cell identification process and was used to check the threshold settings during each use.

Determination of sperm motility by a conventional method

For each sample, a 10 μl aliquot of sperm suspension was mounted between a warmed slide and a 22 x 22 mm. coverslip. With the aid of a grid on an eyepiece graticule using phase contrast optics these preparations were then scored at 400 x magnification by counting the numbers of motile sperm in several randomly selected fields away from the ceverslip edge. Sufficient fields were scored so that at least 200 sperms were counted.

In each field the number of progressively motile sperm was counted first, counting only those sperm that were present in the field at a given moment. If the number of sperm in an entire field was too great for rapid visual counting then a small area of the field was delineated using an eyepiece graticule. Immotile sperm were those which showed no flagellar movement at all.

Statistical analysis

All data are presented as means ± standard deviation (SD). The data were analyzed by the paired t-test or repeated analysis of variance (ANOVA) where applicable, using the Statview

programme (Abacus Concepts Inc., Berkeley, CA). Data were considered statistically significant when the P value was less than 0.05.

Graphical displays use box plots for displaying the detailed distribution of a variable. Each box plot is composed of five horizontal lines that display the 10th, 25th, 50th, 75th and 90th percentiles of a variable. All values for the variables above the 90th percentile and below the 10th percentile were plotted separately.

Results

The influence of the number of frames analyzed on the result of sperm densities at different sperm concentrations is shown in Figure 1. At all sperm concentrations, the sperm densities obtained from the HTMA were higher when the number of frames analyzed was increased. The sperm concentrations obtained from the HTMA at 20 frames were significantly higher than those at 5 frames or by conventional semen analysis ($p < 0.05$).
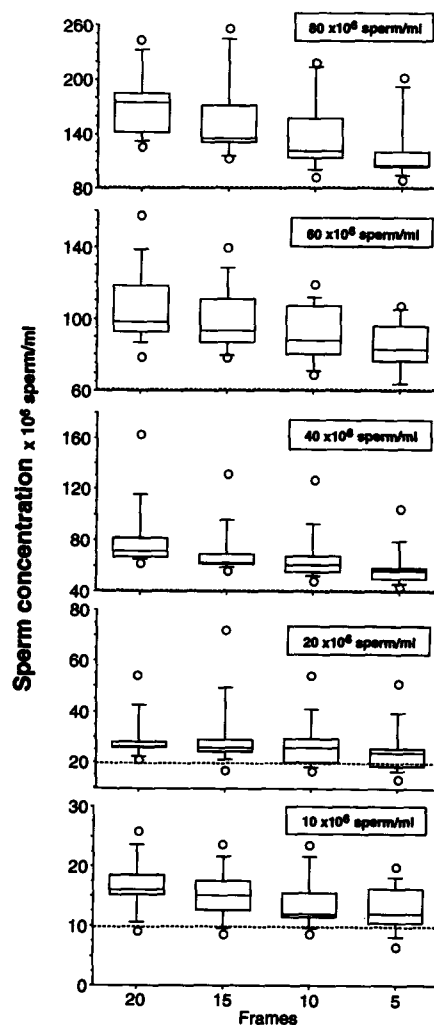
Figure 1. The influence of the numbers of frames analyzed on the result of sperm concentration at different sperm concentrations. Dashed lines represent the sperm concentration measured by conventional method. (Boxplot shows the 10th, 25th, 50th (median), 75th and 90th percentiles. The values above the 90th and below 10th percentile are plotted as open circles.)

The mean sperm concentration obtained from HTMA at 5 frames at 10, 20, 40, 60, 80 million sperms per ml concentrations were 12.2 ± 4.0,25.0 ± 10.3, 58.3 ± 16.6, 84.8 ± 13.9 and 122.3 ± 40.1, respectively. There were no significant differences between sperm densities obtained from HTMA at 5 frames and conventional semen analysis at 10 and 20 million sperm per ml. In contrast, HTMA at 5 frames significantly overestimated sperm density (p < 0.05), when there were higher sperm concentrations (> 20 million sperms per ml.).

Table 1 shows the influence of the number of frames analyzed on the percentage of sperm motility and sperm movement characteristics. The percentage of sperm motility were significantly higher when the number of frames analyzed increased from 5 to 20 as shown in table 1. The mean percentage of sperm motility determined by the conventional method was 70.2 ± 6.3% which is not significantly different from the mean percentage of sperm motility determined by HTMA at 5 frames. All of the sperm movement characteristics were significantly different between the 5 frames and the 20 frames settings. VCL, VSL, VAP, STR and LIN measured were significantly lower, when a smaller number of frames was analyzed. However, measured ALH and BCF showed an opposite effect with higher readings when increasing numbers of frames were analyzed.

In conclusion, all values measured for sperm density, percentage of sperm motility and sperm movement characteristics were dependent on the number of frames used for analysis (Figure 1 and Table 1).

Table 1. Mean ± SEM values for the various movement charactersitics determined by HTMA at the 5, 10, 15 and 20 frames, respectively.

Sperm movement characteristics	5 frames	10 frames	15 frames	20 frames
Percentage of sperm motility	71.5 ± 5.3	72.9 ± 5.6	74.0 ± 5.6*	74.6 ± 6.0*@
VCL (μm/s)	59.4 ± 5.0	59.7 ± 5.0	58.0 ± 5.0*@	54.3 ± 5.0*@#
VSL (μm/s)	35.3 ± 2.3	31.8 ± 2.7*	29.9 ± 2.7*@	28.0 ± 2.3*@#
VAP (μm/s)	42.3 ± 2.7	40.3 ± 3.0*	39.0 ± 2.7*@	36.7 ± 2.7*@#
ALH (μm)	4.6 ± 5.0	5.3 ± 4.6*	5.6 ± 4.6*@	5.5 ± 4.6*
STR (%)	81.6 ± 3.0	77.6 ± 3.3*	76.5 ± 3.6*@	75.4 ± 3.6*@#
LIN (%)	60.0 ± 2.3	54.1 ± 2.7*	52.9 ± 2.7*@	52.2 ± 2.7*@
BCF (Hz)	6.7 ± 0.7	12.8 ± 0.7*	12.8 ± 0.7*	12.5 ± 0.7*

* Significant difference from 5 frames (p<0.05)
@ Significant difference from 10 frames (p<0.05)
# Significant difference from 15 frames (p<0.05)

Discussion

In this study, HTMA consistently overestimated sperm densities especially at higher sperm concentnations. However, at the lower concentrations of 10 and 20 million sperms per ml., HTMA still overestimated sperm density, but not significantly different from the conventional semen analysis. This overestimation may be caused by the phenomenon called "collision effect".[8] Generally, the HTMA identifies sperms by sperm head size and their luminosity. If two swimming sperms collide, they interfere with each other's movement and the sperm cell size recognition by HTMA (Figure 2). At the collision point (Figure 2B), HTMA will recognize both sperms as debris because its size is too big for a typical sperm head and it will be rejected from the analysis. These sperms will be recognized as sperms again in the next frames (Figure 2C). Therefore, HTMA will finally construct all of the images as 4 sperm tracks as shown in the diagram (Figure 2D). A similar problem exists if a swimming sperm collides with an immotile one or with a large piece of debris or another cell (e.g., a leukocyte). Therefore, overestimation of sperm density is increased when the number of frames analyzed is increased because increased analyzing of frames will increase the chance of identification of sperm collision. In addition, overstimation of sperm density is decreased when the sperm concentration of the specimens is low. Dealing with this concept has been a difficult problem for all those working on CASA. However, assessment of sperm densityat the appropriate concentration (10-20 million per ml.) and using the smallest possible numbers of frames analyzed (5 frames) can reduce the overestimation from the HTMA system.

In general, the total motile sperm count is higher than the total sperm count because of the collision effect. This causes an overestimation of the percentage of sperm motility. Higher numbers of motile cells and higher numbers of frames analyzed will increase the percentage of sperm motility. However, the percentage of sperm motility determined by HTMA at 5 frames is not significantly more than the percentage of sperm motility determined by conventional semen analysis. Therefore,the smallest number of frames analyzed (5 frames) is suitable for sperm motility assessment.

Accurate determinations of sperm movement characteristics require the largest number of frames analyzed possible to obtain the most precise information as to the actual curvilinear path followed by a sperm. These differences in numbers of frames analyzed could be of great importance since they cause significant changes in the perceived movement caracteristics, as shown in the results. When we increased this setting from 5 to 20 frames, a significantly lower velocity was recorded. There are 2 explanations for these findings. Firstly, sperms with high velocity seem to have a greater chance of leaving the analysis area before the inclusion criteria of a high number of frames is met. This would bias the results toward the slower-moving sperm who remain in the field longer. Secondly, at high sperm concentrations, there is exclusion of sperm tracks interrupted by cell-cell collisions when the larger number of frames analyzed is used. This significantly decreases the amount of data available for HTMA calculations and excludes the faster moving sperm from extimates of VCL and LIN.[9] Since the heterogeneity of sperm motion in semen requires relatively long
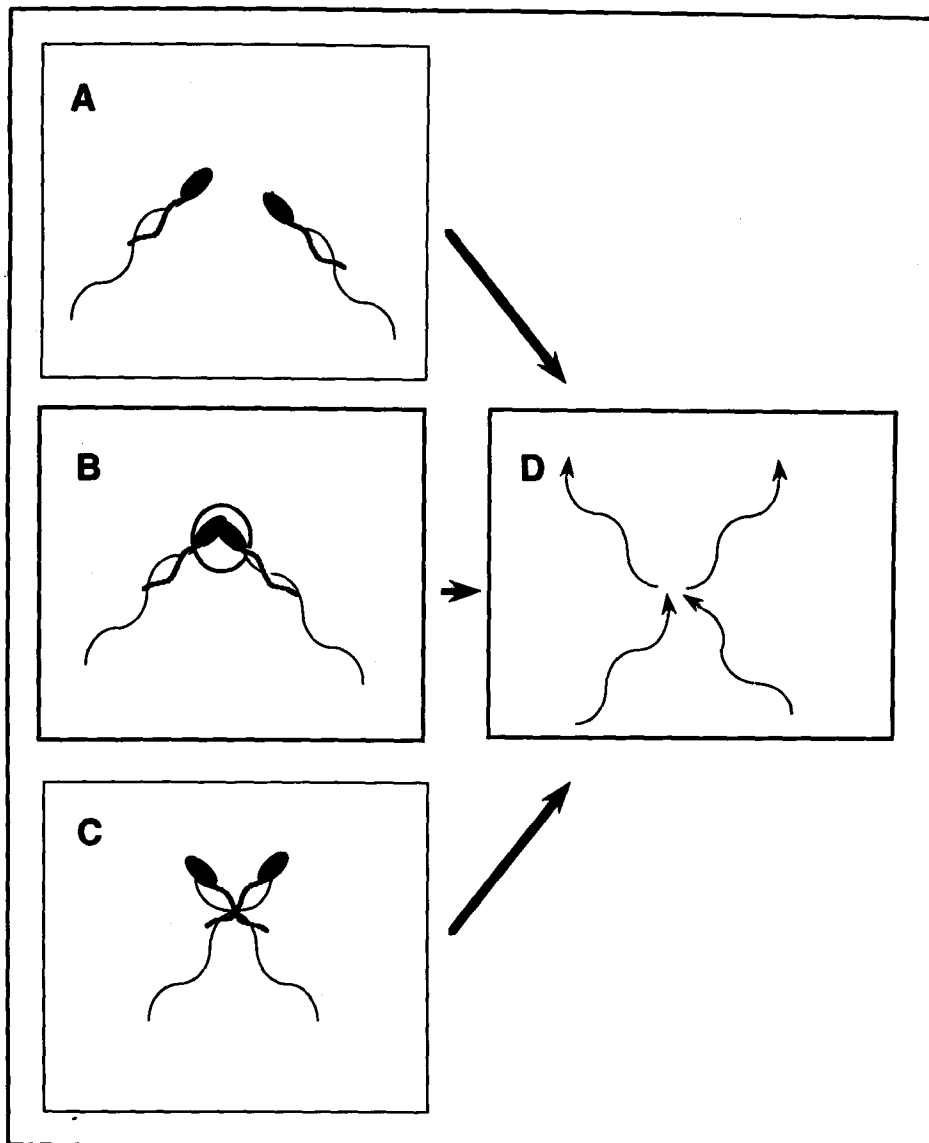
Figure 2. Diagrams depicting "collision effect".

sperm tracking observations to provide accurate measures of sperm movement characteristics, expecially VCL and LIN, dilution of semen to below 20 million/ml is recommended to avoid concentration-induced bias. To avoid changing rheological properties and other aspects of the seminal milieu, semen should be diluted with an aliquot of the subject's own seminal plasma (10-100) μl aliquot centrifuged at 16,000 x g for 5 minutes to obtain cell-free seminal fluid).[10] In addition, a too small number of frames

analyzed may not give a representative value for the velocity and linearity over a certain period. This problem becomes particularly important when hyperactivated sperm with much higher velocity values are measured. This is the case, for example, with sperm washed and prepared for in vitro fertilization.

For determination of characteristics related to the lateral displacement of the sperm head about its axis of progression (ALH and BCF), the data obtained from 5 frames were significantly

different from the larger number of frames analyzed because it contained very little information for analysis. The results showed that the measurements of ALH and BCF at 5 frames were inaccurate. Serious errors can arise because the measurements of sperm head oscillation (BCF) and amplitude (ALH) are constrained by the number of frames analyzed, expecially if the number of frames analyzed is too low.[11] From our data, the accurate computation of both parameters requires at least 10 frames.

Theoretically, a larger number of frames will improve the accuracy of sperm movement characteristics. In combination with an appropriate duration of analysis, single sampling points will reflect the actual path of the sperm even more precisely. Too small numbers of frames analyzed, however, leads to an erroneously high linearity reading. From basic geometry it is evident that an extremely small number of frames analyzed, such as only two sampling frames, would lead to a linearity of 100%, which clearly cannot represent the motion pattern of a single sperm cell. The more points that are sampled, the better the real track will be characterized, even though analysis is slowed down. Therefore, a small number of frames analyzed will lead to some increases in linearity and higher values for velocity.

From our studies, the number of frames analyzed significantly affects calculated sperm movement characteristics (VCL, LIN and ALH).[9,12] A progressive decline in apparent curvilinear velocity occurs when utilizing increasing numbers of frames analyzed.[9] At a reduced number of frames, most sperms appear to be moving in straight lines, while at higher numbers of frames, a full spectrum of linearities

is recorded.[9] In conclusion, use of small numbers of frames cannot provide reliable measures of any sperm movement characteristics.

Accurate determinations of sperm movement characteristics require the largest possible numbers of frames analyzed to obtain the most precise information as to the actual curvilinear path followed by a sperm. Any sperm movement characteristics determined by smaller numbers of frames will be significantly different from the sperm movement characteristics obtained from larger numbers of frames. In conclusion, the analysis of human sperm movement at the highest numbers of frames will give useful values for all sperm movement characteristics.

The data presented demonstrates that the results of automated semen analysis depend, to a great extent, on the number of frames analyzed. As expected from theoretical considerations, sperm, densities percentage of sperm motility and sperm movement characteristics were influenced by the number of frames analyzed. Therefore, adjustment of the number of frames is of crucial importance since even small variations of this parameter seems to influence the measurement of sperm density and the percentage of sperm motility considerably.

Although the CASA system is supposed to allow the objective evaluation of semen samples, results depend to a great degree on parameter settings, which so far have not been standardized.[13] Thus, results for sperm density, percentage of sperm motility and sperm movement characteristics should not be looked upon as absolute values but must be interpreted in the light of parameter settings and samples analyzed. The results of automated semen analysis may not be comparable among different laboratories unless

identical parameter settings are used. The data indicate that values obtained for sperm density and, percentage of motile sperm depend on the numbers of frames analyzed. Our study has demonstrated that the number of frames analyzed is a very important parameter setting and the appropriate number of frames analyzed for HTMA has been recommended to provide the best correspondence with conventional semen analysis. It is concluded that 20 frames is recommended for assessing sperm movement characteristics but 5 frames is suitable for sperm densities and the percentage of sperm motility assessment to minimize errors due to collisions.

References

1. Boyers SP, Davis RO, Katz DF. Automated semen analysis. Curr Probl Obstet Gynecol Fertil 1989 Jan-Mar; 12(1):165-200

2. Katz DF, Drobnis EZ, Overstreet JW. Factors regulating mamalian sperm migration through the female reproductive tract and oocyte investments. Gamete Res 1989 Apr; 22(4):443-69

3. Gagnon C. Controls of Sperm Motility : Biological and Clinical Aspects. Boca Raton : CRC Press, 1990.

4. Mortimer D. Objective analysis of sperm motility and kinematics. In : Keel BA, Webster BW, eds. Handbook of the Laboratory Diagnosis and Treatment of Infertility. Boca Raton : CRC Press, 1990 : 97-133

5. Lessley BA, Garner DL. Isolation of motile sprematozoa by density gradient centrifutation in Percoll. Gamete Res 1983 Jan; 7(1):49-61

6. Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos in vitro. In : Daniel JC Jr, ed. Methods in Mammalian Embryology. Freeman : San Francisco, 1971: 86-102

7. World Health Organization. Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. 3rd ed. Cambridge ; Cambridge University Press, 1992.

8. Douglas-Hamilton DH. Effect of sperm cell concentration and collisions on concentration and velocity measurements. HTM Res Rep 1988 Jan; 14(1):1-17

9. Vantman D, Koukoulis G, Dennison L, Zinaman M, Sherins RJ. Computer-assisted semen analysis : evaluatiton of method and assessment of the influence of sperm concentration on linear velocity determination. Fertil Steril 1988 Mar; 49(3):510-15

10. Sherins RJ. Clinical use and misuse of automated semen analysis. Ann NY Acad Sci 1991 Dec; 637(6):424-35

11. Davis RO, Katz DF. Quantitative analysis of sperm motion kinematics from realtime video-edge images. Proc Internat Soc Optical Eng 1987 Jan; 832(1):14-20

12. Mack SO, Wolf DP, Tash JS. Quantitation of specific parameters of motility in large numbers of human sperm by digital image processing. Biol Reprod 1988 Mar; 38(3): 270-81

13. Knuth UA, Yeung CH, Nieschlag E. Computerized semen analysis; objective measurement of semen characteristics is biased by subjective parameter setting. Fertil Steril 1987 Jul; 48(1):118-24