Sperm motility assessment using computer assisted semen analysis (CASA): a comparison of standard microscope slides and coverslips and the 20 µm MicroCell™

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Abstract
Computer assisted semen analysis (CASA) uses instrumentation that makes precise measurements of sperm motility, but the values obtained can be influenced by a number of technical aspects. Motility of human sperm was measured using a Sperm Class Analyzer (Microptic S.L., Barcelona, Spain), and the effect of using different counting chamber/slide configurations was investigated. Results for 20µm MicroCell slides (Vitrolife Sweden AB, Göteborg, Sweden) were compared with microscope slides and 22mmx22mm coverslips loaded with either 5µl (CV.5µl) or 10µl (CV.10µl) semen. Operator-correction of readings for all slide configurations resulted in a significantly lower number of sperm assessed due to the elimination of non-sperm bodies. Following operator-correction, the MicroCell chamber and CV.10µl slide gave similar readings for both progressive motility and immotility for up to 5 minutes, whereas the CV.5µl had a progressive increase in immotile sperm. The interval to analysis was therefore standardised at 2 minutes prior to the measurement of kinetic parameters, and the MicroCell values were significantly different to the CV.10µl for curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL), and the CV.5µl for VAP. It is concluded that the same configuration be used within the same study, and that care should be taken when comparing different studies that have used different slide/chamber configurations.

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Introduction

Computer assisted semen analysis (CASA) is used to assess sperm motility using two kinds of instrument (Lu, Huang, & Lü, 2014), namely those that either (i) follow the individual tracks of sperm, or (ii) calculate motility with an algorithm applied to the scatter of light. Instruments following individual sperm are also able to measure a number of additional motility parameters, such as amplitude of lateral head displacement and curvilinear velocity, which are particularly useful in research applications (Mortimer, 2000). Whilst the results obtained with these automated systems are said to be more objective and precise than manual methods (Vyt et al., 2004), the values obtained are influenced by a variety of technical factors such as the chambers used (Gloria et al., 2013) and the settings of the instrument (Boryshpolets, Kowalski, Dietrich, Dzyuba, & Ciereszko, 2013).

Before using a CASA system, one must decide upon the different components and settings that will best serve the purpose. There are many options available when selecting the chamber/slide holding the sperm suspension that is used in conjunction with CASA systems.

A cheap and convenient option often recommended by the manufacturers of CASA systems in measuring motility is the use of a simple microscope slide with coverslip, although the volume of semen added and hence the depth of the sample does need to be standardised as the coverslip is effectively floating on the sample (Del Gallego et al., 2017). When using purpose-made chambers of fixed depth that use capillary action to load the sample, there are different depths that can be purchased which also affect results (Spiropoulos, 2001). Furthermore, an additional variable that must be taken in to account is the time after filling of the chamber that the measurements are made (Ibănescu et al., 2016).

The aim of the present study was therefore to systematically evaluate the use of a standard microscope slide and coverslip with a MicroCell™ chamber of 20µm depth when measuring sperm motility and associated kinematic parameters. Variables investigated included (a) the impact of operator-adjusted identification of sperm, (b) the volume of semen applied to the standard microscope slide, and (c) the time the slide/chamber was left to equilibrate prior to the measurements being made.

Materials and Methods

Ethical approval, consent and semen samples
Consent was given in accordance with the study protocol that had been approved by both the Joondalup Health Campus and Edith Cowan University Ethics Committees. Twenty men attending for routine semen analysis as part of their fertility investigations were recruited and each provided one semen sample. The men each signed a consent form agreeing for their sample to be used in the study.

Study design
Three chamber/slide configurations were evaluated for their effect of sperm motility parameters, namely the MicroCell 20µm chamber (Vitrolife Sweden AB, Göteborg, Sweden) loaded with 3µl semen as per manufacturer's instructions, and standard 76.2 x 25.4 mm slides and 22x22mm coverslips (Livingstone, Roseberry, NSW, Australia) loaded with semen volumes of 5µl (CV.5µl) and 10µl (CV.10µl). Initially, samples were observed at time intervals of 0 minutes, 2.5 minutes, 5 minutes and 20 minutes to observe the effect of time on each chamber in terms of (a) the number of sperm viewed by the CASA system before and after operator correction, and (b) the sperm motility. Once an optimum time was selected for the assessment of the chamber/slides after loading, the effect of the chamber/slide configuration upon sperm kinetics was determined.

Instrumentation and slides
The CASA system used in this study was the Sperm Class Analyzer (Microptic S.L., Barcelona, Spain) coupled through an acA780-75gc GigE camera (Basler AG, Ahrensburg, Germany) to a Nikon microscope (ECLIPSE E200MV R, Nikon, Tokyo, Japan) using a x10 phase contrast objective. Prior to the slides being measured, the microscope was configured to account for the measured chambers depth. Minimum sperm head area to be classified as a sperm head was 1µm2 and the maximum size was 100µm2. Drift was accounted for and a cut-off speed was adjusted at 5µm/s to differentiate between motile cells and immotile cells, operator corrections were applied here if immotile cell's velocity was greater than 5µm/s. Images were captured at 25 frames per second, and 5 fields of view were captured in order to analyze motility.

Motility measurements
Each sample was loaded onto the three chamber/slide configurations in a staggered manner to allow ease of ensuring an accurate time for each slide. Five random fields of a sample were captured by the CASA and the fields were individually reviewed and corrections were applied by the operator (CR) where the CASA software had misclassified a sperm cell or its motility status. Operator bias was minimised by having the same operator take each measurement and apply corrections where necessary. The corrected results were then separately recorded to allow for comparisons between the original CASA report and an operator corrected score. Sperm motility parameters assessed were immotile (IM), non-progressive (NP) and progressive (PR) sperm motility. In addition, kinetic parameters assessed were curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), wobble

(WOB) and amplitude of lateral head displacement (ALH).

Statistical analysis

Statistical comparisons using the SPSS statistics package (IBM) were made between chambers for measured parameters. Data sets were first explored and considered to be either normally or abnormally distributed based on the Shapiro-Wilk test for normality score ($\alpha=0.05$). If these normally distributed data sets contained no outliers, and met Mauchly's tests for sphericity, repeated measures ANOVA testing with Bonferroni post-hoc analysis was applied to investigate where differences occurred between groups. If data sets failed to meet the assumptions required for repeated measures ANOVA, non-parametric Friedman's test was employed to identify possible significant differences between data groups. A Sign pair-wise comparison test was then used to identify where the differences occurred between measurements. For all tests, differences were considered significant if $p<0.05$.

Results

Sperm number in operator-corrected and uncorrected fields

The number of sperm counted in 5 fields of view at 0 minutes, 2.5 minutes, 5 minutes and 20 minutes after being loaded are shown in Table 1, with both the uncorrected and operator-corrected results. At each time interval for each chamber type, there was a statistically significant reduction observed between the CASA's original measurement (uncorrected) and an operators applied corrections (corrected) (all $p=<0.05$). The number of sperm counted on the CV.10µl was significantly higher for both corrected and uncorrected counts when compared to the CV.5µl at 0 minutes, 2.5 minutes and 5 minutes.

Influence of time on sperm motility between chamber types

After semen was loaded on the MicroCell chamber, the CV.5µl and CV.10µl slides, operator-corrected measurements of sperm motility were made at time intervals of 0 minutes, 2.5 minutes, 5 minutes and 20 minutes, and the results are shown in Table 2. At 0 minutes, there were no statistical differences observed between slide types for IM, NP or PR motility. Microcell chambers, CV.5µl and CV.10µl slides all gave stable readings for all categories of motility up to 5 mins. A number of changes were then seen after 20 minutes with both the Microcell and CV.5µl showing a significant reduction in motility compared to time 0 minute. CV.5µl and CV.10µl differed significantly to each other at 20 minutes for IM and NP, the MicroCell and CV.10µl differed significantly from each other at the 20 minute time interval for both PR and IM cell proportions, and the CV.5µl and CV.10µl differed in IM and NP motility.

Sperm motility and kinetic parameters

Based upon the results above showing stability of motility readings over the first 5 minutes, motility measurements (motility types plus kinetic parameters) were made at a standard 2 minutes after loading for each chamber type with operator corrections applied, and the results are shown in Table 3. Neither total motility nor progressive motility had statistically significant difference between chamber types, consistent with the previous round of tests above. However, the MicroCell values were significantly different to the CV.5µl for VAP, and the CV.10µl for VCL, VAP and VSL. No significant differences were observed between the Microcell chamber and the slides with either 5µl or 10µl in the STR, LIN, WOB and ALH.

Discussion

Semen analysis is a routine screening test used to assess male fertility, but a large degree of technical variability has been revealed in external quality assurance programmes (Álvarez et al., 2005; Keel et al., 2000; Matson, 1995). An improvement in precision and accuracy of laboratories performing semen analysis is the basic goal of standardised protocols produced by the World Health Organization, culminating in the recent 5th edition (2010). However, an alternative approach is to use computerised systems that make objective assessments, and a number of CASA systems are now being proposed to undertake routine analysis (Akashi, Mizuno, Okumura, & Fuse, 2005; Tomlinson et al., 2010). Whilst CASA systems have the potential for making objective measurements of sperm motility patterns, results obtained are not absolute but can be affected by a range of

Table 1. The number of sperm (mean ± sem) counted by the analyser in 5 fields of view immediately (0 mins), after 2.5 mins, after 5 mins and after 20 mins when the recognition of sperm was uncorrected or corrected. The three configurations used where a 20µm Microcell chamber, and slides/coverslips with either 5µl (CV.5µl) or 10µl (CV.5µl) semen applied.

	0 mins		**2.5 mins**		**5 mins**		**20 mins**	
Slide	**Uncorrected**	**Corrected**	**Uncorrected**	**Corrected**	**Uncorrected**	**Corrected**	**Uncorrected**	**Corrected**
MicroCell	291 ± 53	228 ± 48[b]	302 ± 59	244 ± 52	308 ± 55	237 ± 45[g]	305 ± 65	241 ± 54
CV.5µl	176 ± 36[a]	135 ± 29[bc]	177 ± 21[d]	131 ± 19[e]	206 ± 30[f]	147 ± 26[gh]	188 ± 28	135 ± 22
CV.10µl	374 ± 59[a2]	281 ± 49[c3]	344 ± 50[d]	261 ± 44[e]	330 ± 45[f]	257 ± 40[h]	269 ± 50[2]	207 ± 44[3]

All values of corrected vs uncorrected are significantly different to each other and hence do not have superscripts. Other values with the same superscript are significantly different from each other.

Table 2. The proportion (mean ± sem) of sperm that were immotile (IM.), non-progressively motile (NP) or progressively motile (PR.) immediately, 2.5 mins and 5 mins after loading. The three configurations used where a 20µm Microcell chamber, and slides/coverslips with either 5µl (CV.5µl) or 10µl (CV.5µl) semen applied.

	0 mins			2.5 mins			5 mins			20 mins		
Slide	IM (%)	NP (%)	PR (%)	IM (%)	NP (%)	PR (%)	IM (%)	NP (%)	PR (%)	IM (%)	NP (%)	PR (%)
MicroCell	56 ± 8	12 ± 2	32 ± 9[1]	56 ± 7	14 ± 2	30 ± 9[2]	54 ± 7[3]	16 ± 2[a]	30 ± 9	60 ± 8[3b]	15 ± 2	25 ± 9[12e]
CV.5µl	52 ± 8	13 ± 4	36 ± 8[4]	54 ± 8[56]	10 ± 2	35 ± 9[7]	58 ± 9[58]	10 ± 1[a]	32 ± 9[9]	63 ± 8[68c]	11 ± 1[d]	26 ± 8[479]
CV.10µl	54 ± 9	11 ± 1	35 ± 8	52 ± 9	10 ± 2	38 ± 9	51 ± 8	12 ± 2	37 ± 8	52 ± 8[bc]	17 ± 2[d]	31 ± 9[e]

Values with the same superscript are significantly different from each other.

Table 3. The motility results (mean ± sem) for 10 semen samples obtained with 20μm Microcell chambers and microscope slides with coverslips (CV.5μl: 5μl semen; CV.10μl: 10μl semen) at two minutes.

Motility and kinetic parameters	Counting chamber/slide		
	Microcell	**CV. 5μl**	**CV. 10μl**
Total motility (%)	49.8 ± 6.2	50.1 ± 7.7	57.0 ± 7.5
Progressive motility (%)	30.6 ± 7.3	34.1 ± 9.3	39.9 ± 9.1
VCL (μm/s)	36.7 ± 4.9[a]	41.4 ± 5.9	42.4 ± 5.5[a]
VAP (μm/s)	20.7 ± 2.6[bc]	24.2 ± 30[b]	24.4 ± 2.8[c]
VSL (μm/s)	13.6 ± 2.1[d]	15.3 ± 1.8	16.6 ± 2.1[d]
STR (%)	58.9 ± 2.5	58.2 ± 1.7	62.5 ± 1.7
LIN (%)	33.9 ± 2.4	35.6 ± 1.9	38.0 ± 20
WOB (%)	55.6 ± 2.0	57.5 ± 1.6	58.6 ± 20
ALH (μm)	01.9 ± 0.3	02.0 ± 0.3	02.2 ± 0.2

Values with the same superscript are significantly different from each other.

technical factors. Data from the present study have shown that the chamber/slide type and configuration has a significant impact on the kinetic measurements, confirming that the counting chamber or slide used in a study or clinical setting should not be changed.

The depth of the chamber is important in being sufficient to allow the free movement of sperm whilst maintaining the cells within focus. A professional consensus paper (ESHRE, 1998) recommended that chambers or slides used for human sperm be 10-20μm deep when assessing motility and kinetics, but be 30μm or more when assessing hyperactivated motility. The WHO 5th edition (2010) recommends disposable chambers of 20μm depth when measuring human sperm motility by CASA, thereby providing a monolayer of sperm cells that allows rotational flagella action (Kraemer, Fillion, Martin-Pont, & Auger, 1998; Le Lannou, Griveau, Le Pichon, & Quero, 1992). This depth is also recommended for assessing sperm motility on a wet preparation (World Health Organization, 2010), and can be achieved by placing 10μl semen on a microscope slide and covering with a 22mm x 22mm coverslip with the weight of the coverslip spreading the sample. The current study used both a fixed 20μm depth chamber (Microcell) and a 22mm x 22mm coverslip with 10μl semen (CV.10μl) which should have been equivalent (ie 20.7μm). The smaller volume of semen added to the microscope slides and coverslips of 5μL would have had smaller depth of 10.3μm.

The capillary-loaded MicroCell chambers in the present study gave constantly reduced measurements of some sperm kinetics compared to the drop-loaded microscope slide and coverslip, consistent with other studies (Hoogewijs et al., 2012; Lenz, Kjelland, VonderHaar, Swannack, & Moreno, 2011; Peng, Zou, & Li, 2015). Although there was no significant difference observed for progressive motility and total motility between chamber types, there were significant differences observed for other sperm kinetic measurements such as VCL, VAP, and VSL. This effect of capillary-loaded chambers is thought to occur due to a number of unique factors that are absent in drop-loaded chambers. These can include the type of glue or adhesive used to fix the coverslip for these set-depth chambers possibly being toxic to sperm cells (Gloria, et al., 2013), whilst fluid dynamics of samples loaded into capillary-action chambers are also known to influence sperm concentration and motility parameters (Douglas-Hamilton, Smith, Kuster, Vermeiden, & Althouse, 2005).
The time between setting up each slide and performing the measurements was investigated in the first part of the present study. Curiously, the PR, NR and IM motility was fairly stable for all the chambers during the first 5 minutes, which contrasts to work with goat sperm (Del

Gallego, et al., 2017) where total motility of a capillary-loaded chamber was influenced just after 2 minutes, and bull sperm (Contri, Valorz, Faustini, Wegher, & Carluccio, 2010) where a capillary-loaded chamber's motility status suffered from time deterioration more severely than a droplet-loaded chamber. Taking into account these different findings, an interval of 2 minutes from set-up to measurement was used to give sufficient time to allow the chamber/slides to settle and equilibrate but to minimise the risk of it becoming unstable.

The number of sperm counted by the CASA machine in the motility assessments was noted to determine if there were differences between the non-uniform chamber depth of the CV slides and the fixed-depth MicroCell, and Table 1 shows the CASA's original count for total sperm counted across five fields of view compared to the results of an operator-corrected count. At each time point for each chamber, there was a statistically significant difference between the operator's corrected count and the original CASA count, with most of the errors the CASA system made being the misclassification of non-sperm cells as sperm cells (data not shown), including abrasions on the slide and cellular debris. Given the differences in the geometry of each chamber arising from different sample depths, it is not surprising in the current study that significant differences in the operator-corrected count were observed between the MicroCell chamber and CV.10µl (both 20µm deep) and the CV5µl (10µm deep). As time progressed, the MicroCell and CV.5µl slide did not have any observed significant differences in corrected count at 0 minutes and 20 minutes. The CV.10µl however showed a significantly reduced corrected count at 20 minutes. A possible explanation for this significant reduction is the effect of evaporation from the edges of the coverslip negatively influencing the apparent count in some way, whereas the MicroCell chamber and CV.5µl slide have less exposure to the atmosphere providing some sort of protection. These observations of significant changes in count between an operator-corrected score and the original CASA score highlights the need for post-analysis corrections to be applied by a human operator, as miscounted non-sperm cells can result in an increased proportion of immotile cells, giving skewed motility parameters.

Conclusion

The present study has shown that measurements made on different aspects of sperm motility can vary according to the chamber configuration used such that the time taken to observe slides after they are loaded must also be standardised, and a manual correction be made for the recognition of non-sperm cells. It is therefore vital that the same configuration be used within the same study, and that care should be taken when comparing different studies that have used different slides/chambers.

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