ACCURATE BOAR SPERM COUNTING USING A DISPOSABLE NON-TOXIC COUNTING CHAMBER

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ABSTRACT AND INTRODUCTION

Artificial insemination has had an exponential growth in the swine industry over the past few years. As a result, there is an increasing demand for extended semen samples containing a defined number of sperm cells with a given quality. Accurate and fast concentration determination of boar semen samples is of major importance to increase the amount of straws per ejaculate and to guarantee a consistent quality of those straws.

Various methods are used to determine the number of sperm cells (counting, photometric technique, opacity measurement) and the quality (motility, viability). As the opacity and photometric techniques have an inherent error of ± 30%, and only measure concentration, there is need for a reliable, precise and objective way to determine concentration and quality. The standard for counting in the US is the hemocytometer, a 100 µm reusable counting chamber, used to count dead sperm. It needs to be cleaned and assembled prior to every counting event, and motility parameters are assessed separately by microscopic investigation. Various disposable counting chambers have entered the veterinary and human reproductive market claiming a fixed height of 20 µm; they can be used for the assessment of the quality and concentration of a semen sample in one step.

Numerous studies have been performed on the accuracy and reproducibility of the results of these disposable chambers compared to the hemocytometer. In this study, we compare the Leja 4-chamber slide (SC-20-01-FA) to the hemocytometer. To have a third and independent concentration measurement, we included flow cytometry (FACS) countings, as a new reference for the absolute number. We compared the concentrations (over a range of 10-80x10^6/ml), the ranges and standard deviations of the three different methods.

Sperm cells in the Leja slide were counted using the Hamilton Thorne CEROS, a computer assisted semen analysis machine. This computer system allows counting a large number of sperm cells, and to determine motility parameters in a very short time span. Counting more sperm cells improves the reliability of the results by reducing Poisson distribution related errors.

Boar sperm is much more sensitive for oxygen and metabolite depletion, and for toxic components than human sperm is. Because of toxic components used for the assembly of disposable counting slides, a rapid loss of motility has been observed in some brands of disposable slides. In this study, we investigated the loss of motility of boar sperm within the Leja 4-chamber slide, by performing motility measurements throughout the chamber in time.

From this study we can conclude that the Leja 4-chamber slide is non-toxic for boar sperm (for at least 10 minutes), yields concentrations equal to the FACS and the hemocytometer, and with a lower deviation and a smaller range than the hemocytometer. Therefore, the Leja 4-chamber slide provides consistent and accurate data on boar sperm analysis, and is more reliable than the hemocytometer.

MATERIALS AND METHODS

General

Boar semen was collected at the "Varkens Activiteiten Centrum" (Swine Activity Center), in Bunnik, the Netherlands. The ejaculates were diluted in X-CELL extender (IMV Technologies, France) to achieve the start concentration, and were further diluted by serial dilution also in X-CELL. During the experiments, the dilutions were kept at room temperature.

Counting

1. Leja 4-chamber slide in combination with the HT CEROS

The slides (SC-20-01-FA, Leja, Nieuw-Vennep, the Netherlands) were pre-heated to 37°C, and were loaded by capillary action using a negative displacement pipette. The sample was allowed to heat up to 37°C for 1 minute. Concentration and motility parameters were determined using the Hamilton Thorne CEROS using the program "Swine AI", with software version 12.1c (settings, as advised by HT, are shown in Table 1, and were the result of extensive investigation to optimize the various parameters for boar sperm: HT research report no. 40). 6 different, manually selected fields per chamber were analyzed. Slides from various batches were used.

2. Hemocytometer counting

The serial dilutions were further diluted in saturated $NaHCO_3$. Depending on the initial concentration dilution ranged between 1/15 and 1/60 to be able to count about 100 sperm cells per 1 mm^2 square (0.1 µl) in the hemocytometer (Improved Neubauer). The suspensions were loaded by capillary action using a negative displacement pipette, and cells were allowed to settle for 5 minutes at room temperature. Per hemocytometer half, two different 1 mm^2 squares (total of 16 squares of 250 x 250 µm) were counted (every hemocytometer count is from the same dilution).

Table 1: Hamilton Thorne CEROS settings, optimized for boar sperm.

The research report describing the optimization of all settings is available on request.

Setting	Value
Frames Acquired	10
Frame Rate	60 Hz
Minimum Contrast	46
Minimum Size	7
Default Static Cell Size	12 pix
Default Static Cell Intensity	90
VAP Cutoff	20 µm/sec
VSL Cutoff	5 µm/sec
Straightness Threshold	45%
Prog. Min VAP	45 µm/sec
Static Head Size	0.65 – 4.90
Static Head Intensity	0.50 – 2.50
Static Elongation	0 – 87%
Chamber Depth	Average chamber height of the used batch, as provided by the Quality Control Certificate (19.6 – 19.8 µm)

3. Flow cytometry (FACS)

50 µl of the sperm suspensions as used for the hemocytometer counting were brought into 900 µl of DNA staining solution (LDS-751 Quinaldin in PBS/0.1% BSA) together with 50 µl of beads suspension (1029/µl, Flow-Count Fluorospheres, Beckman Coulter).

15.000 events were analyzed by the FACS (Calibur, Becton Dickinson). Additional information regarding the gating of the FACS results is in progress and will be added in a later stage. The signal of LDS positive events (R2) was related to the beads signal (R1), and the formula (R2/R1) x 1029 x dilution gives the sperm concentration.

For each method, average, range, and standard deviation were calculated. The number of analyzed chambers, or hemocytometer fields is indicated in Tables.

Motility assessments of boar sperm in the Leja 4-chamber slide

Boar sperm (4 different randomly obtained samples) was diluted in X-CELL to a concentration of $50x10^6$/ml, was loaded into a pre-heated slide, and was kept at 37°C during the experiment. At 1, 3, 6, and 10 minutes after loading the motility and progressive motility (using the HT CEROS) were determined in the center of the chamber (4 fields) and at the right (2 fields) and left edges (2 fields) of the chamber (seen from the entry port).

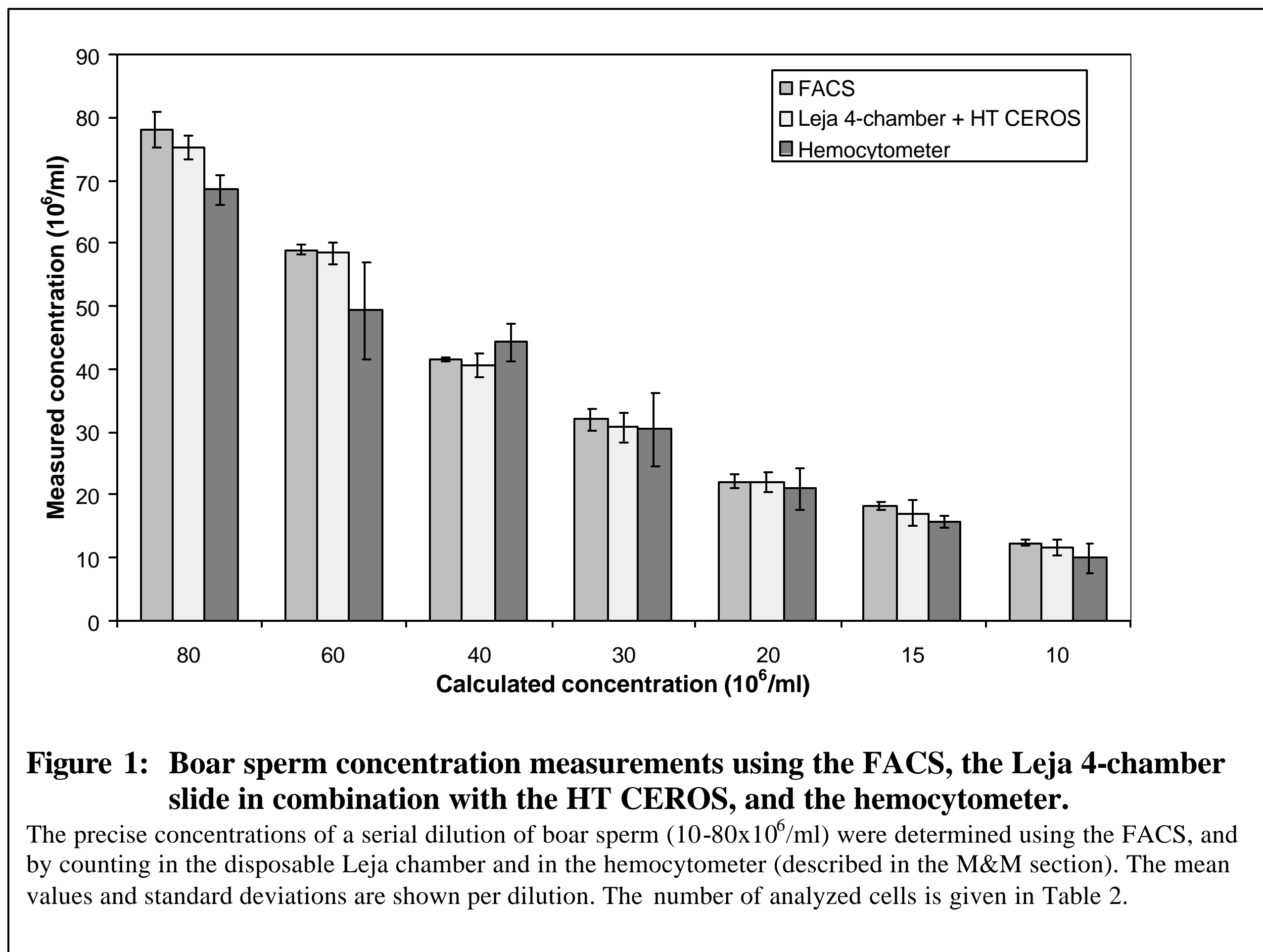

Figure 1: Boar sperm concentration measurements using the FACS, the Leja 4-chamber slide in combination with the HT CEROS, and the hemocytometer.

The precise concentrations of a serial dilution of boar sperm ($10\text{-}80x10^6$/ml) were determined using the FACS, and by counting in the disposable Leja chamber and in the hemocytometer (described in the M&M section). The mean values and standard deviations are shown per dilution. The number of analyzed cells is given in Table 2.

RESULTS

Comparison of the concentrations of serial dilutions of boar sperm, as determined by the FACS, the Leja 4-chamber slide/HT CEROS system, and the hemocytometer

Boar sperm was diluted to concentrations of 10, 15, 20, 30, 40, 60 and $80x10^6$ cells/ml. The precise concentrations were determined as described in the M&M section, and the final results are depicted in Figure 1. In this Figure, the average concentrations as measured with the three methods are given together with the standard deviations (SD).

The results show that the three different methods of concentration determination yield very similar results, especially when the standard deviations are taken into account. As can be seen, at all concentrations, the average concentration as measured in the Leja 4-chamber slide in combination with the HT CEROS is closer to the FACS outcome than the hemocytometer concentration is. Furthermore, at all concentrations except at $15x10^6$/ml, the standard deviations belonging to the Leja slide measurements are lower than the SD of the hemocytometer countings.

In counting statistics, the absolute number of counted cells is very important and informative. Therefore, information regarding this absolute number of counted cells and the number of analyzed chambers and hemocytometer squares is given in Table 2. From this Table it becomes apparent why the SD belonging to the hemocytometer is higher than to the Leja 4-chamber slide. The number of analyzed cells by the computer system is much larger than can be counted in a reasonable time span using the hemocytometer.

The deviation of hemocytometer countings as compared to the Leja 4-chamber/computer outcome was further investigated by counting a similar number of total cells with the two methods, and than comparing the means, the ranges, and standard deviations. A boar sperm sample of $37x10^6$/ml was used for this experiment and the results are shown in Table 3. It can be concluded that the mean concentration as measured by the Leja/CEROS combination equals the FACS ($37x10^6$/ml), whereas the hemocytometer gives a slightly higher concentration ($40x10^6$/ml). Furthermore, both the range and SD are smaller of the Leja/CEROS measurements when compared to those of the hemocytometer; the range for the Leja/CEROS combination is $14.7x10^6$/ml, and for the hemocytometer $24x10^6$/ml.

These results indicate that the Leja 4-chamber slide and the HT CEROS computer are very repeatable and precise counting methods, and are more reliable than the hemocytometer.

Table 2: Number of counted cells (belonging to Figure 1).

Per concentration the number of counted cells per chamber (of a Leja slide) and per 0.1 µl (hemocytometer, 1 mm^2 square) is given, as well as the number of analyzed chambers or counted squares.

	Leja 4-chamber + HT CEROS		Hemocytometer	
Con.	No. chambers	cells/chamber	No. 1 mm^2 squares	cells/square
80	5	494	4	114
60	6	381	4	82
40	6	267	4	148
30	7	200	4	102
20	8	141	4	140
15	8	114	4	106
10	12	78	4	67

Table 3: Comparison between the hemocytometer and the Leja 4-chamber/HT CEROS combination, after counting a similar number of total cells.

Boar sperm was diluted to a concentration of $37x10^6$/ml, and was analyzed as described in the M&M section. The mean concentration, range and standard deviation are given, and the number of analyzed Leja-chambers or hemocytometer squares. Together with the mean number of counted cells per chamber or square, the total number of counted cells was calculated.

	Leja 4-chamber + CEROS	Hemocytometer	FACS
Dilution	no	1/20	
Mean concentration (10^6/ml)	37.1	40	37
Concentration range (10^6/ml)	31.4 – 46.1	29.2 – 53.2	36.3 – 37.7
SD	4.6	6.7	0.99
Counted cells per chamber/square	257	200	
Analyzed chambers/squares	12	15	
Total number of counted cells	3084	3000	

Motility assessments of boar sperm in the Leja 4-chamber slide

As mentioned in the Introduction, boar sperm is very sensitive for toxic components. We therefore tested for what time period the sperm cells retain full motility at 37°C within the Leja slide. For this purpose four different boar sperm samples (diluted to $50x10^6$/ml) were loaded into the chamber and motility measurements were done at 1, 3, 6, and 10 minutes in the center of the chamber, as well as near the edges, where the concentration of toxic components (from glue and ink) is supposed to be highest.

As can be concluded from Figure 2, no decline in motility parameters is apparent within the tested time period. We therefore conclude that the Leja slides do not exert toxic effects on boar sperm within 10 minutes after loading.

At 15 to 20 minutes after loading, a decline in motility parameters is observed especially near the edges (results not shown). Due to evaporation, the samples are drying out at these moments and therefore, no accurate concentration and motility measurements can and will be performed at these moments.

DISCUSSION AND CONCLUSION

In this study the accuracy of the Leja 4-chamber slide and the hemocytometer on boar sperm concentration determination was investigated. A third method was introduced, flow cytometry by the FACS. This method was very useful in this investigation as it provided an independent reference with a small deviation. In daily practice, this method is probably not useful. First, because of the costs and complexity of the equipment, second, all samples have to be diluted twice, and third, a separate assessment of motility is necessary.

Figure 1 and Table 3 visualize the comparison, showing a good agreement between the three methods. In general, the averages as determined in the Leja slide are closer to the FACS than the hemocytometer outcomes. Furthermore, the SD and ranges of the Leja slide/computer system measurements are smaller than of the hemocytometer results. These data indicate that concentration determination using the Leja slide/HT system is more accurate and reliable than using the hemocytometer.

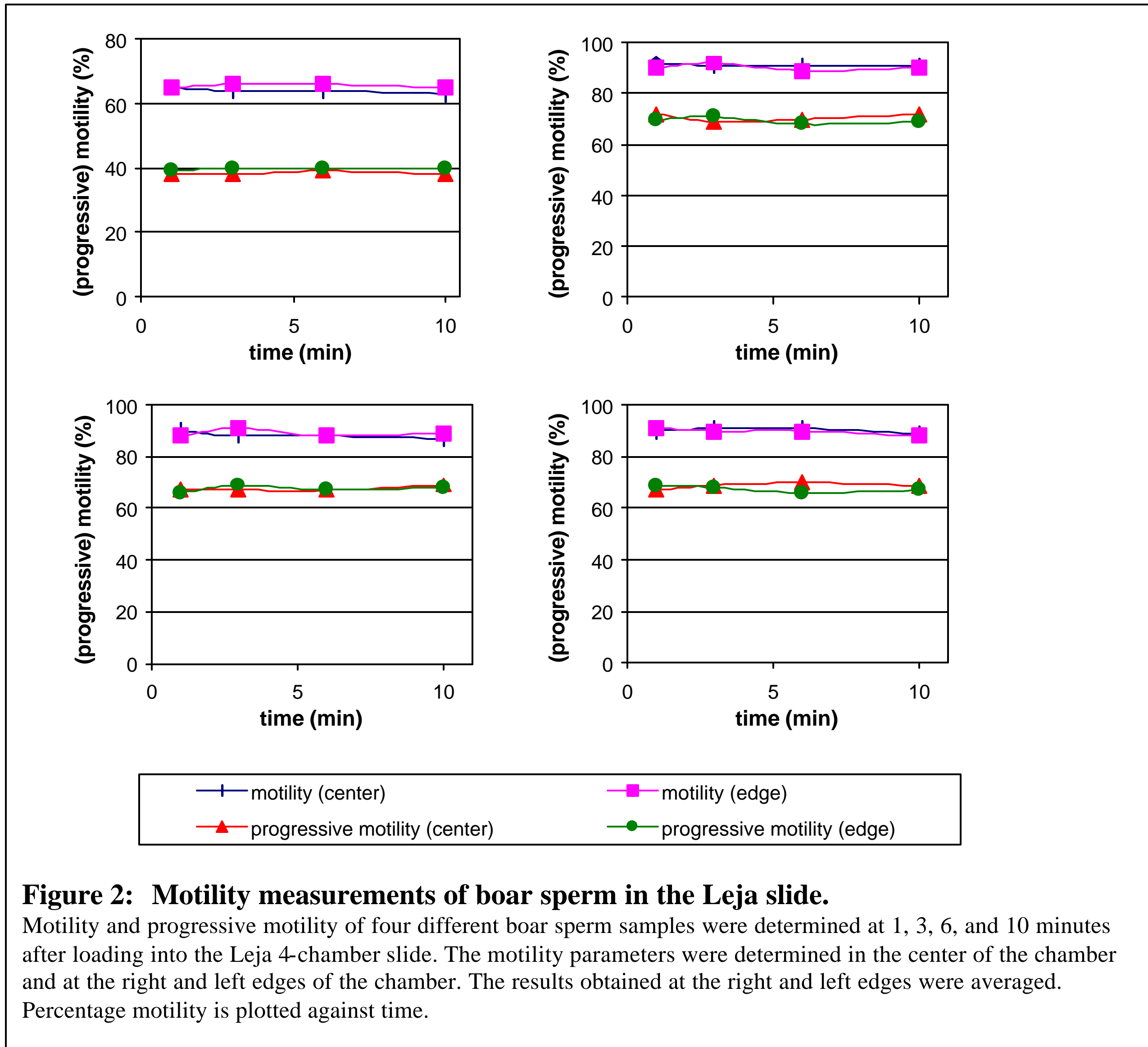

Figure 2: Motility measurements of boar sperm in the Leja slide.
Motility and progressive motility of four different boar sperm samples were determined at 1, 3, 6, and 10 minutes after loading into the Leja 4-chamber slide. The motility parameters were determined in the center of the chamber and at the right and left edges of the chamber. The results obtained at the right and left edges were averaged. Percentage motility is plotted against time.

There are also some practical advantages to favor the Leja slide. First, it does not need to be cleaned and assembled, and second, concentration and motility parameters can be determined in the same chamber. Third, in combination with the HT system, large numbers of sperm cells can be analyzed within a short time span, whereas counting numbers of cells for a statistically correct answer in the hemocytometer is very time consuming. Furthermore, samples need to be diluted prior to hemocytometer analysis, introducing a potential error.

An often raised disadvantage of disposable slides is their toxicity. This especially becomes apparent using the very sensitive boar sperm. The experiments in Figure 2 show that boar sperm stays fully motile for at least 10 minutes in the Leja chamber. Under normal operating conditions, analysis is complete within 5 minutes, and therefore, the Leja slide appears to be non-toxic.

In many institutes, the hemocytometer is used as the “Golden Standard” for counting. The results presented here, together with practical advantages indicate that the performance of the Leja slide in combination with the HT system surpasses the hemocytometer. It therefore maybe necessary to reconsider usage of the hemocytometer as the standard reference in sperm counting.

Abbreviations

HT	Hamilton Thorne
SD	Standard Deviation
FACS	Fluorescence Activated Cell Sorter