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Usefulness of hemocytometer as a counting chamber in a computer-assisted sperm analyzer (CASA)

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Abstract

Several methods are used to determine sperm cell concentration, such as the haemocytometer, spectrophotometer, electronic cell counter and computer-assisted semen analysers (CASA). The utility of CASA systems has been limited due to the lack of characterization of individual systems and the absence of standardization among laboratories. The aims of this study were to: 1) validate and establish setup conditions for the CASA system utilizing the haemocytometer as a counting chamber, and 2) compare the different methods used for the determination of sperm cell concentration in bull semen. Two ejaculates were collected and the sperm cell concentration was determined using spectrophotometer and haemocytometer. For the Hamilton-Thorn method, the haemocytometer was used as a counting chamber. Sperm concentration was determined three times per ejaculate sample. A difference ($P < 0.05$) was found between all methods of measuring sperm cell concentration. However, no difference was found between the haemocytometer and CASA system when the haemocytometer was used as a counting chamber ($P > 0.05$) or between the haemocytometer count and the spectrophotometer. Based on the results of this study, we concluded that the haemocytometer can be used in computerized semen analysis systems as a substitute for the commercially available disposable counting chambers, therefore avoiding disadvantageous high costs and slower procedures.

Keywords: bull, CASA, concentration, haemocytometer, sperm.

Introduction

In order to produce uniform insemination doses with a certain number of sperm per dose, the accurate and precise determination of sperm concentration in an ejaculate is important. Inaccurate assessment of sperm concentration in raw semen will result in inadequate sperm concentrations in the insemination doses. In addition, this also implies that some insemination doses contain an excessive number of sperm and that males with high genetic potential are not being used efficiently, reducing the revenue from the bull stud.

Spectrophotometers are probably the most frequently used method by AI stations for the assessment of sperm concentration (Foote, 1972). However, the detection spectrum is limited for such instruments and the accurate quantification of sperm numbers in dilute or concentrated samples is problematic (Fenton *et al*., 1990). For individual raw ejaculates of bull semen, differences in the amount of particles and debris (cytoplasmic droplets, fat droplets, bacteria) can result in an inaccurate determination of the sperm concentration (Woelders, 1991). Electronic particle counters rapidly determine the sperm concentration but they tend to include any debris within the size range of the sperm in the count (Evenson *et al*., 1993).

Electronic cell counters and spectrophotometers are unsuitable for determining sperm concentration when samples are diluted in extenders containing egg yolk or milk (Parks, 1992; Graham, 1994). Haemocytometers are not used by AI stations for routine sperm assessments as this method is slow and time-consuming as multiple measurements of each sample are needed to obtain a precise result.

The advent of various computerized semen analysis systems has also made possible the acquisition of objective measurements of sperm motion and concentration for clinical and research use. However, the utility of these systems has been limited due to the lack of characterization of individual systems and the absence of standardization among laboratories (Knuth *et al*., 1987). The quality of the data obtained is dependent on the setup conditions under which the measurements are made (Mahoney *et al*., 1988). The setup conditions must be optimized for a specific system and animal species and must take into account the illumination system, the magnification of the optical system, the intensity and size of the sperm identified, the number of fields analyzed, and the number of frames and frame rate used for image acquisition.

In order to assess the quality of data, it is important to determine how the results from automated systems correlate with those from simultaneous manual determinations. The use of standard setup conditions

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with these systems will make it possible to rapidly and reliably characterize sperm parameters of interest.

The purpose of this study was to evaluate the use of the haemocytometer as a counting chamber in the Hamilton-Thorn computerized semen analysis system for measuring bull sperm concentration. In addition, to compare some of the different methods available for the determination of sperm cell concentration, a haemocytometer, spectrophotometer, and the Hamilton-Thorn computerized semen analysis system were used.

Materials and Methods

Semen samples

Three 15-month-old Holstein bulls were used in this study. Two ejaculates were collected at 20 min apart using an artificial vagina on a weekly basis for 3 weeks. Two ml semen from each ejaculate sample were used for analyses.

Determination of sperm cell concentration using the spectrophotometer

A spectronic 20 spectrophotometer (Spectronic Instruments, Rochester, NY, USA) was used to determine the sperm concentration in each ejaculate sample. Immediately after semen collection, 100 µl of the raw semen from each ejaculate sample was diluted in 7900 µl sodium citrate (2.9%; w/v) solution to give a final dilution of 1:80 of semen to diluent (v/v) in the spectrophotometer tubes. The tubes were then gently mixed and placed in the spectrophotometer and readings were obtained. Before placing the samples in the spectrophotometer, it was calibrated using blank sodium citrate solution. The semen concentrations were recorded and the diluted samples were saved for further estimations of sperm concentration using the haemocytometer and the computerized system.

Determination of sperm cell concentration using the Hamilton-Thorn computerized semen analysis system utilizing the haemocytometer as a counting chamber

The counting chamber used to determine the concentration of sperm cells in each ejaculate sample was the haemocytometer (American Optical Corporation, Buffalo, NY, USA). The volume of the haemocytometer was 0.1 mm^3 (Sorensen, 1976) and, depending on the dilution ratio each laboratory uses, the concentration was:

concentration = sperm counted × dilution ratio × haemocytometer factor.

This formula provided the concentration in cubic mm; in order to transform this into ml, the count was multiplied by 1000 (Sorensen, 1976).

The haemocytometer factor was the result of the number of squares counted (five large squares) in the haemocytometer divided by the total number of squares in the haemocytometer and the result was multiplied by the chamber volume (Sorensen, 1976).

The area in which the Hamilton-Thorn system was able to detect sperm cells was measured using a stage micrometer (Carl Ziess, Inc., Thornwood, NY, USA). The optical settings were adjusted so that the Hamilton-Thorn system would detect the maximum number of sperm cells in the counting chamber (haemocytometer).

Collection of a series of images of the sample was set at 30 frames, and the image acquisition rate was 30 frames per sec. The magnification was set at 10X and the illumination was dark field. The field was manually selected. The size and contrast gates were set as follows: minimum contrast, 80; minimum size, 5; low-size gate, 0.1; high-size gate, 3.4; low-intensity gate, 0.3; high-intensity gate, 1.7.

In our study, the dilution ratio was 1:80 and by measuring the area in which the machine was able to count sperm cells and the volume of that part of the chamber (0.49 mm × 0.355 mm × 0.1 mm, length × width × depth, respectively; volume = 0.017395 mm^3), the following formula was developed based on the formula used for the haemocytometer count (Sorensen, 1976):

concentration (million/ml) = cells counted × dilution ratio (80) × haemocytometer factor (57.4878).

The sperm cell concentration was estimated using the Hamilton-Thorn HTM-2000 automated semen analyzer with version 12 software (Hamilton-Thorn Research, Beverly, MA, USA), where four fields were manually chosen on the haemocytometer. Two simultaneous measurements of sperm cell concentration were recorded. The first concentration was obtained from the computer system according to its settings and the second concentration was calculated and adjusted according to the haemocytometer factor as described earlier, where only the sperm cells that were counted using the computer system were used in the calculations.

Determination of sperm cell concentration using the haemocytometer

Diluted semen samples were transferred into a water bath at 60°C to immobilize the sperm cells for counting. A haemocytometer (American Optical Corporation, Buffalo, NY, USA) was used to count the sperm cells in each ejaculate sample. Each ejaculate sample was counted four times and the sperm concentrations were recorded.

Statistical analysis

Sperm cell concentrations were averaged across the four samples per bull. The data were then analyzed (SAS; Institute Inc., Cary, NC, USA, 1999), using the least squares method (PROC GLM). The

model used was:

$Y_{i(j)k1} = \mu + T_i + \delta_j + \gamma_{k(j)} + \acute{\varepsilon}_{i(j)ks}$

where

$Y_{i(j)k1}$ = sperm concentration of each bull

μ = overall mean sperm concentration

T_i = fixed effect of method I (I =1 to 5) used to count sperm cells

δ_j = random effect of bull ID j (j =1 to 5)

$\gamma_{k(j)}$ = random effect of ejaculate k (k = 1 to 2)

$\acute{\varepsilon}_{i(j)ks}$ = random residual error

Results

There was a difference ($P < 0.05$) between the Hamilton-Thorn computer system and the haemocytometer for measuring sperm cell concentration. However, no difference ($P > 0.05$) was found between the haemocytometer and the Hamilton-Thorn computer system when we used the haemocytometer as a counting chamber (Table 1). In addition, there was no difference ($P > 0.05$) between the haemocytometer count and the spectrophotometer.

Table 1. Different methods for determining the concentration of bull sperm (mean ± SE).

Method	n[1]	Mean (x10^6 spermatozoa/ml)	SE
Haemocytometer	18	565.21[a]	69.34
HThorn (Haemo)[2]	18	463.73[a]	55.24
Spectrophotometer	18	651.46[a]	84.99

[1]n: 3 bulls x 2 ejaculates x 3 weeks = 18 samples. [2]HThorn (Haemo): concentration obtained using the Hamilton-Thorn system plus the haemocytometer and haemocytometer settings. [a]No significant difference using Duncan's new multiple range test.

Discussion

The determination of sperm concentration in a sample of semen is extremely important as this parameter is used to determine the volume of a sample needed in order to contain the correct number of cells in each insemination dose (Foote, 1972; Christensen, 2001). Using the Hamilton-Thorn system would be beneficial to AI stations, but the machine requires counting chambers particularly designed to fit the machine settings. These counting chambers are disposable and cannot be used again. Their high cost makes their use by different AI stations or research laboratories unfeasible. Also, the same ejaculate sample must be analyzed many times, especially for semen freezing, in order to make sure that the dilutions are correct and that each straw has the minimum number of sperm cells required for optimal fertility, which is not advantageous from an economic point of view.

Each AI station or research laboratory is equipped with a haemocytometer which is used to calibrate the other instruments for sperm counting in order to make sure that the sperm concentration is correct. In this study, we used the haemocytometer as a counting chamber for the Hamilton-Thorn computer system and we modified the settings on the machine to fit the new counting chamber. However, the concentrations obtained using the haemocytometer, given that the software of the computer system made the calculations, were significantly different from those of the microscopic counting method using the haemocytometer. In this study, we used the haemocytometer as a counting chamber, but the computer system counted the sperm cells within a pre-determined volume and magnification. The concentration was calculated using this number in the formula that we developed for the haemocytometer. No significant difference was found between the haemocytometer counts and the counts obtained from the Hamilton-Thorn computer system. Therefore, the next step will require software especially designed for the haemocytometer so the machine will perform the count automatically.

When comparing the haemocytometer to the spectrophotometer, no significant difference was found, suggesting that the spectrophotometer might be used in AI stations as a fast and reliable tool for counting sperm. However, attention and care should be given to the calibration and regular checking of the machine (Foote, 1972) due to differences in the amount of particles and debris (cytoplasmic droplets, fat droplets, bacteria) in raw ejaculate samples that can result in an inaccurate determination of sperm concentration (Woelders, 1991).

In this study, a significant difference was found between the haemocytometer and the computer system (Hamilton-Thorn), but although utilizing the haemocytometer, the concentration was based on the computer software not designed to use with the haemocytometer. Christensen (2001) compared sperm counts obtained by an electronic counter, a spectrophotometer and flow cytometry against the haemocytometer. He found that the sperm count by flow cytometry was highly correlated with the haemocytometer count, indicating that no significant difference existed between the two methods. In our study, we did not find a significant difference between the haemocytometer count and the spectrophotometer count.

In this study, we were able to use the haemocytometer as a counting chamber instead of the commercially available counting chambers for the Hamilton-Thorn computer semen analysis system within certain settings that would be applicable to other computerized or automated semen analysis systems.

However, further studies are required to investigate the results of this study and make it applicable to all animal species.

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References

Christensen P. 2001. Danish semen analysis: fertility vs. quality tests. *In*: Proceedings of the 19th Technical Conference Artificial Insemination and Reproduction, 2001, Milwaukee, WI. Columbia, MO: NAAB. pp. 96-101.

Evenson D, Parks J, Kaproth M, Jost K. 1993. Rapid determination of sperm cell concentration in bovine semen by flow cytometry. *J Dairy Sci*, 76:86-94.

Fenton S, Ax R, Cowan C, Coyle T, Gilbert G, Lenz R. 1990. Validation and application of an assay for deoxyribonucleic acid to estimate concentrations of bull sperm. *J Dairy Sci*, 73:3118-3125.

Foote RH. 1972. How to measure sperm cell concentration by turbidity (optical density). *In*: Proceedings of the 4th Technical Conference Artificial Insemination and Reproduction, 1972, Milwaukee, WI. Columbia, MO: NAAB. pp. 57-61.

Graham J. 1994. In vitro assays of bull fertility. *In*: Proceedings of the 15th Technical Conference Artificial Insemination and Reproduction, 1994, Milwaukee, WI. Columbia, MO: NAAB. pp. 74-81.

Knuth U, Yeung C, Nieschlag E. 1987. Computerized semen analysis: objective measurement of semen characteristics biased by subjective parameter setting. *Fertil Steril*, 48:118-124.

Mahoney M, Alexander N, Swanson R. 1988. Evaluation of semen parameters by means of automated sperm motion analyzers. *Fertil Steril*, 49:876-884.

Parks J. 1992. Applications of flow cytometry in semen processing and handling. *In*: Proceedings of the 14th Technical Conference Artificial Insemination and Reproduction, 1992, Milwaukee, WI. Columbia, MO: NAAB. pp. 12-17.

Sorensen A. 1976. *Repro Lab: A Laboratory Manual for Animal Reproduction*. Ames, IA: Kendall/Hunt Publishing.

Woelders H. 1991. Overview of in vitro methods for evaluation of semen quality. *Reprod Domest Anim Suppl*, 1:145-164.