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COMPARISON OF THREE DIFFERENT METHODS FOR THE ANALYSIS OF RAM SPERM CONCENTRATION

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ABSTRACT

Determination of sperm concentration is a critical component of semen analysis. Traditionally, the haemocytometer has been the standard tool for calibrating other techniques used to estimate sperm concentration, including photometry, Coulter counters, flow cytometry and computer-automated semen analysis (CASA). In the present study, fresh ram sperm samples (n = 7) from the Native Wallachian (NW) Slovak sheep breed were collected from one male by electro-ejaculation (EE) and analysed for sperm concentration using flow cytometer FACS Calibur, CASA Sperm Vision™ and using EVE™ Automatic Cell Counter. Our results showed no significant (P ≥ 0.05) differences in the sperm concentration when analysed by these three methods. Thus, it is possible to use a cell counter to determine the approximate sperm concentration directly at the place of semen collection. This is a very practical finding since instruments such as flow cytometer or CASA are not suitable for transport to the place of semen collection.

Key words: ram sperm; concentration; flow cytometry; CASA; cell counter

INTRODUCTION

Semen analysis is used for the evaluation of ejaculate quality and subsequently the overall male fertility. It must be noted that there is wide inter-laboratory variation in the results, although this has been reduced in recent years by automated systems using electro-optics or computer-assisted analysis. Furthermore, there is marked variation in sperm output on a day-to-day basis.

In the quest for predicting fertility of an individual, improving semen handling, dilution and storage protocols, and understanding the impact of environment, andrologist have changed their approaches to semen analysis. The technologies used today are fast developing and readily implemented in research. Semen is one of few naturally occurring monocellular suspensions, so sperm function analysis by flow cytometry using fluorochromes is an ideal technique for high throughput, objective and accurate analysis. The complementary use of microscopical assessment by CASA where sperm parameters can be objectively assessed is equally important. The objectivity and repeatability of these techniques have driven research on the function, identification of heterogeneity and fertility of ejaculate (Boe-Hansen and Satake, 2019).

A flow cytometric method has been developed for rapid determination of sperm concentration in semen from various mammalian species. All cells containing DNA are stained with SYBR-14 or propidium iodide (PI) and sperm concentration is determined in the relation to an internal standard of fluorescent

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microspheres (beads). Satisfactory staining can be achieved within 2-3 min and the following flow cytometric analysis on the FACSCount AF System rapidly provides the user with a precise and accurate assessment of the sperm concentration (Hansen *et. al.*, 2002).

CASA technology has been used for objective and reproducible evaluation of sperm concentration and motility in different mammalian species (Ax *et al.*, 2000; Kubovičová *et al.*, 2011). It was developed for automated analysis of sperm images. CASA allows rapid, relatively inexpensive, and fairly precise estimation of sperm concentration, although accuracy is marred by several technical issues and variations. The principle of CASA involves visualization and digitization of successive images of sperm using a microscopy setup (hardware) followed by image processing and analysis to identify and count sperm (software). Since the area of the images is known, the volume evaluated and the sperm concentration can be calculated (Brito *et al.*, 2016).

Currently, several types of cell counters working on different principles are used in practice. Primarily the Cell NucleoCounter is an instrument designed for automated evaluation of sperm concentration. Evaluation is quick and requires only a small volume of sample (10 to 100 µL depending on the anticipated concentration). No calibration is required, operation is easy, precise and accurate results can be obtained. Since sperm identification is relatively specific, there is no interference from seminal plasma composition and gel, lubricants, extenders or debris, thus allowing evaluation of a wide range of sample types. For these reasons, use of the NucleoCounter is becoming more common in clinical settings and semen processing centres (Brito *et al.*, 2016).

The aim of our study was to compare the accuracy of sperm count determination by EVE cell counter based on the standard trypan blue technique with traditionally used methods like flow cytometry or CASA assay, and the possibility for its future use in external conditions of Slovak local sheep farms.

MATERIAL AND METHODS

Semen collection

Clinically healthy ram of Native Wallachian (NW) sheep breed aged 2 years was used in this experiment. The ram was housed in external conditions in individual stall, fed with hay bale and oats, water and mineral salt were supplied *ad libitum*. The semen samples were collected once a week by electro-ejaculation from the same ram for the duration of 7 weeks. Before this procedure rectum was cleaned of faeces. A three-electrode probe 1" for ram and boar with diameter of 2.54 cm and length of approximately 16 cm, connected to a power source that allowed voltage and amperage control, was used (MiniTüb Electro-ejaculator; MiniTüb, Tiefenbach, Germany). The EE regime (automatic mode, type of curve 2 – the power output is linearly increased from 0.5 Volt to 7 Volt) consisted of consecutive series of 2 s pulses of similar voltage, each separated by 2 s break. The initial voltage was 0.5 V, which was increased in each series until maximum of 7 V. Upon reaching a voltage of 7 V, impulses remained at this level until the ejaculation was complete. After collection, the semen was transported to the laboratory in thermo box with a water bath at 37 °C.

Sperm concentration measurement

Since no practical existing method allows all sperm in a semen sample to be counted, a subsample is counted to make inferences upon the whole sample. A critical objective is to obtain a representative sample that contains a sufficient number of sperm so that counts can be performed efficiently, the recommended optimal number of sperm to be counted varies according to the counting method. Therefore, the technician must take into account the method to be used and the expected sperm concentration in the sample in order to dilute the sample appropriately prior to evaluation. Dilution rates can range from 1:1,000 for highly concentrated samples (e.g. ram semen) to 1:5 for less concentrated samples (e.g. boar semen) (Brito *et al.*, 2016).

Flow cytometry

Flow cytometry measurement of the sperm count is based on the addition of an internal standard (fluorescent beads with known concentration) to the semen sample. Semen was diluted in a saline (0.9 % NaCl; Braun, Germany) at a ratio of 1:9 (v/v). Diluted semen sample (50 µl) was placed into the tube, subsequently 50 µl of Fluorescent Count Standard (EXBIO Praha, Vestec, Czech Republic) and 500 µl of PBS (Biosera, NUAILLE, France) were added, then the suspension was mixed and analysed by flow cytometer FACS Calibur (BD Biosciences, USA; Figure 1A). At least 10,000 cells were analysed per sample.

CASA

Semen was diluted in a saline (0.9 % NaCl; Braun, Germany) at a ratio of 1:40 (v/v), immediately placed (2 µl) into a Leja Standard Count Analysis Chamber (depth of 20 microns; MiniTüb, Tiefenbach, Germany) and evaluated under a Zeiss AxioScope A1 microscope using the CASA system. For each sample, six microscopic view fields were analysed for the sperm concentration (CON; 1×10^9), linearity, straightness, cross wobble (LIN, STR, WOB) and percentage of total motility (TM; > 5 µm.s^{-1}) and progressively moving spermatozoa (PM; > 20 µm.s^{-1}) as previously described by Kulíková *et al.* (2018) using Sperm VisionTM (MiniTüb, Tiefenbach, Germany; Figure 1B).

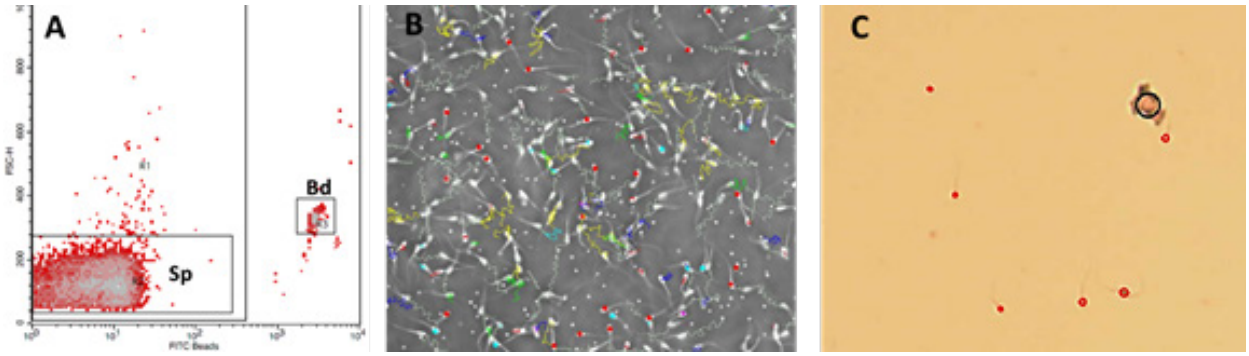
Cell counter

Spermatozoa concentration was assessed at the indicated time-points by Trypan blue staining. Semen was diluted in a distilled water at a ratio of 1:1000 (v/v). Distilled water was used to immobilize sperm since it results in osmotic shock. Diluted semen sample (10 µl) was mixed with (10 µl) of Trypan blue (0.4 %; NanoEnTek, Seoul, Korea), immediately placed (10 µl) into a cell counting slide and analysed by EVETM Automatic Cell Counter (NanoEnTek, Seoul, Korea; Figure 1C). Cell counter is able to measure cell concentrations ranging from 1×10^4 to 1×10^7 cells.mL^{-1} and cells with sizes ranging from 5 µm to 60 µm. The counter was set up for the measurement of ram spermatozoa as follows: diameter 5 – 30 µm and 50 % of circularity.

Each semen sample was measured by each device in 3 replicates. To avoid bias, all dilutions of raw semen were performed by one person.

Statistical analysis

The results of sperm concentration measurement from each method were recalculated to relative values in percentages, where the highest measured value at each measurement was expressed as 100 %. The absolute values from the replicates and the relative values (%) were then statistically processed by a one-way ANOVA test using a SigmaPlot 11.0 software (Systat Software Inc., Germany). Data were expressed



A) dot plot from flow-cytometric analysis indicating sperm population (Sp) and fluorescent beads (Bd); B) image of sperm population analysed by CASA system; C) image of sperm population analysed by cell counter (red circles = spermatozoa, black circle = debris).

Figure 1. Representative figures of sperm concentration measurement by three different methods

as the mean ± standard error of the mean (SEM). Values at $P \leq 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

In our study the ejaculate from the same ram was collected for 7 weeks and evaluated for concentration values by three different methods (flow cytometry, CASA and cell counter, Table 1).

Average values of sperm concentration measured by flow cytometry, CASA and cell counter over the whole period were about 80 %, 70 % and 90 %, respectively. Although the sperm concentration values measured by CASA were nearly 10-20 % lower than as values measured by cell counter and flow cytometry, the differences between the counting techniques were not statistically significant ($P \geq 0.05$). There was considerable variability between individual measurements. Sperm concentration of the tested ram also varied considerably week after week.

Obtaining reliable results requires very accurate dilution. Because of diluent and semen sample volumes are usually low and dilution ratios are relatively large, even minor sampling errors can significantly affect the results. Proper use, maintenance, and calibration of instruments used to prepare dilutions are essential (Brito *et al.*, 2016).

The lowest values of sperm concentrations were measured in our experiments by CASA. There are more than a dozen CASA systems marketed for analysing animal sperm and although these systems are based on similar principles, there are several differences in the hardware and software among systems (Amann and Katz, 2004; Amann and Katz, 2014). As with all methods used for measuring sperm concentration, correct pipetting, dilution and thoroughly mixing of the sample are essential for obtaining reliable results. In addition, semen extender, sperm concentration, frame acquisition rate, presence of non-sperm debris that is recognized as sperm, and type of chamber have affected CASA results (Iguer-ouada and Verstegen 2001;

Table 1. The evaluation of ram sperm concentration by three different methods

Number of sperm evaluation sperm evaluation	Flow cytometry (conc. × 10^9)	CASA (conc. × 10^9)	Cell counter (conc. × 10^9)
1	100 % (2.89 ± 0.04)	42.6 % 1.23 ± 0.11	46.7 % (1.35 ± 0.07)
2	100 % (4.7 ± 0.14)	69.9 % (3.28 ± 0.72)	75.5 % (3.55 ± 0.49)
3	92.2 % (3.41 ± 0.97)	74.9 % (2.77 ± 0.10)	100 % (3.7 ± 0.28)
4	40.7 % (0.57 ± 0.03)	52.9 % (0.74 ± 0.04)	100 % (1.4 ± 0.14)
5	76.8 % (1.19 ± 0.06)	96.8 % (1.5 ± 0.14)	100 % (1.55 ± 0.35)
6	100 % (0.56 ± 0.01)	73.2 % (0.41 ± 0.06)	89.3 % (0.50 ± 0.12)
7	73.3 % (0.99 ± 0.01)	60 % (0.81 ± 0.29)	100 % (1.35 ± 0.01)
Mean	83.29 ± 8.27 %	67.19 ± 6.62 %	87.36 ± 7.62 %

The results are expressed as average ± SEM. The highest measured value at each measurement was expressed as 100 %, the subsequent two values were relatively derived from this value. Differences between groups are not significant (One-Way ANOVA).

Rijsselaere *et al.*, 2003). Presence of non-sperm debris that are identified as sperm and sperm agglutination that renders sperm "too large" to be identified are common problems when CASA is used. Immobilization of sperm is essential when performing manual counts and might also increase the precision and accuracy of CASA results (Bailey *et al.*, 2007). However, use of DNA fluorescent stains for CASA is a more practical alternative (Zinaman *et al.*, 1996). Moreover, the most validated and traditional parameter measured by CASA is motility, which is a fundamental functionality of CASA system (Boe-Hansen and Satake, 2019). Although use of CASA for evaluation of sperm motility has gained enormous popularity in research and clinical labs, its use for evaluation of sperm concentration for clinical or commercial purposes is not recommended by the World Health Organization (WHO, 2010) or the National Association of Animal Breeders (Brito *et al.*, 2012).

Flow cytometry allows rapid, automated counts of large numbers of sperm (i.e. tens of thousands). This ability, combined with the capability to exclude other semen components (e.g. gel, extender, debris) and cell types makes flow cytometry a very precise and accurate method for evaluation of sperm concentration. However, routine use of flow cytometry has been limited by instrumentation cost, need for a skilled operator, and somewhat complex methods of sample preparation and data evaluation. This method has been used primarily for research purposes, validation of other methods and calibration of different instruments, but use by large semen processing centres has increased in recent years (Brito *et al.*, 2016).

The coefficient of variation (CV) from CASA in the most studies were between 6 and 8 %, whereas those for spectrophotometers were between 3 and 6 %. The CV's obtained with the NucleoCounter or flow cytometry were consistently between 3 and 4 % (Brito *et al.*, 2016).

Recent studies have also used the NucleoCounter and flow cytometry as gold standards for accuracy assessment. Despite the small inconsistencies among studies, authors have generally concluded that the NucleoCounter and flow cytometry are accurate methods for estimating sperm concentration. The NucleoCounter and flow cytometry, along with the hemocytometer, are all considered reference methods and are recommended by the National Association of Animal Breeders to be used for calibration and quality assurance of spectrophotometer (Brito *et al.*, 2012).

Anzar *et al.* (2009) found out the sperm concentration determined by haemocytometer was lower than by flow cytometer and NucleoCounter. Thus, flow cytometer and NucleoCounter can be used with equal confidence to estimate sperm concentration and membrane integrity in domestic animals and human semen.

Morrell *et al.* (2010) reported a positive correlation (r = 0.73) between the flow cytometer and automated cell counter when using semen samples with populations of > 40 % membrane-intact sperm. Similarly, in study of Prathalingam *et al.* (2006) the flow cytometry results showed the lowest coefficient of variation (2.3 %), with the plate reader showing the highest coefficient of variation (20.0 %). There was no significant difference between any of the methods used, and none of them consistently over- or underestimated numbers when compared against to each other. It is concluded that flow cytometry showed the highest repeatability of results. However, the precision and accuracy of sperm concentration estimates are determined primarily by technician skills and limitations inherent to the method used, including equipment specifications and setup in case of automated methods (Brito *et al.*, 2016).

In conclusion, the results obtained from the present study indicate that sperm concentration measurement performed using the EVE Automatic Cell Counter is relatively reliable, and can substitute the flow-cytometric or CASA method. Thus, in the future is possible to determine the sperm concentration directly at the place of semen collection under the external conditions.

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