Sensitivity evaluation of the computer-assisted sperm analysis (CASA) in the determination of frozen-thawed bull semen concentration

Avaliação da sensibilidade da técnica computadorizada de análise (CASA) para a determinação da concentração espermática do sêmen bovino congelado

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Abstract

Sperm concentration is traditionally evaluated by counting cells in a hemocytometric Neubauer chamber, often a highly subjective, time-consuming, and laborious technique prevalent in andrology laboratories around the world. However, the Computer-Assisted Semen Analysis (CASA) represents a more consistent method of evaluating sperm concentration that may provide enhancing efficiencies of sperm count. The purpose of this study is to compare the results of these two methods in the analysis of post-thaw concentration of bovine semen. Four hundred and twenty five batches of semen from different bulls were selected, thawed at 37°C for 30 seconds and then homogenized. Aliquots of 40 µL of semen were diluted in 960 µL of distilled water, fixing the rate at 1:25 dilution for analysis in a Neubauer chamber. Conversely, aliquots of 5 µL for each semen dose were submitted to CASA, considered a minimum of five random fields and 2000 sperm count per analysis. The average concentration of sperm cells was $38.96^{a} \pm 1.28$ in the Neubauer analysis and $35.14^{b} \pm 0.82$ for the CASA, with the correlation coefficient of 0.87 ($P < 0.0001$) and reliability of 0.78 (scale ranging from 0 to 1) between the two methods. In conclusion, the results of two techniques for assessing sperm concentration have similar results. However the CASA methodology would yield greater benefit due to precision, consistency, and reduced disposal issues, particularly for large processing laboratories.

Keywords: Bull semen. CASA. Neubauer. Sperm concentration.

Resumo

Tradicionalmente, a concentração espermática é avaliada por meio da contagem de células em câmara hemocitométrica de Neubauer, técnica laboriosa adotada na rotina dos laboratórios de andrologia. Uma alternativa para essa contagem é a técnica computadorizada de avaliação espermática (CASA), método que pode aumentar a eficiência e acurácia na determinação da concentração de espermatozoides em uma amostra de sêmen. O presente trabalho relata a avaliação da sensibilidade da técnica CASA para o acesso da concentração de espermatozoides bovinos em pós-descongelação. Foram selecionadas 425 doses de sêmen de reprodutores de diferentes raças, descongeladas a 37°C por 30 segundos e homogeneizadas. Alíquotas de 40 µL de sêmen foram transferidas para tubos cônicos de 1,5 mL previamente preenchidos com 960 µL de água destilada, fixando a taxa de diluição em 1:25 para contagem em câmara de Neubauer. Em contrapartida, alíquotas de 5 µL de cada dose de sêmen foram avaliadas com o emprego do sistema CASA considerando o número mínimo de cinco campos aleatórios e 2 mil espermatozoides por análise. A concentração média de células espermáticas foi de $38,96^{a} \pm 1,28$ e $35,14^{b} \pm 0,82$, respectivamente para amostras avaliadas em câmara de Neubauer ou sistema computadorizado, apresentando o coeficiente de correlação de 0,87 ($P < 0.0001$) e concordância de 0,78 (escala de 0 a 1). Conclui-se que as duas técnicas de avaliação da concentração espermática possuem eficiência similar. No entanto, em virtude da precisão, rapidez e por dispensar a diluição prévia das amostras para a contagem, a CASA é uma alternativa para a contagem de células espermáticas em câmara de Neubauer, sobretudo para grandes centrais de produção de sêmen bovino congelado.

Palavras-chave: Sêmen bovino. CASA. Neubauer. Concentração espermática.

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Introduction

Bull semen is a precious commodity and its utilization must be monitored closely to avoid wasteful over-use. One major challenge impacting the practice of artificial insemination (AI) is attaining the most efficient use of this limited resource. The goal is to use the least amount of semen possible to achieve desired fertility rates (FOOTE; KAPROTH, 1997), thereby optimizing the supply of the most rewarding semen with ever-greater numbers of inseminated cows (DEN DAAS et al., 1998) and consequential elevated production/commercial values. Moreover, the efficient use of top-yielding sperm takes on increased importance with the advent of sexed bovine semen, which is characterized by low-performance industrial processing and the packaging of doses containing low sperm concentration (SEIDEL JUNIOR; SCHENCK, 2008; SILVEIRA et al., 2013). Therefore, the accurate determination of sperm count is of great economic and biological importance to any successful AI programs.

Accurate determination of sperm concentration in an ejaculate is a critical component of the spermiogram and, in the case of livestock, necessary for optimizing calculable insemination dose numbers (DOUGLAS-HAMILTON et al., 2005). The invention of hemocytometry marked a major technical advance for physiologists, and has even been cited as the "gold standard" for counting sperm levels (PRATHALINGAM et al., 2006). However, the hemocytometric chambers evaluation is costly and laborious, because the process requires sample dilution prior to testing and the human eye analysis of a very large sample number of immobilized spermatozoa to achieve desired standards of accuracy (MAES et al., 2010).

Due to the human subjectivity inherent in the Neubauer method for determining the sperm in-solution concentration, there is an increased interest in the far more technical and consistent CASA method, which utilizes cameras capable of consistently identifying sperm concentration (DOUGLAS-HAMILTON et al., 2005). Thus, the aim of this study was to evaluate the sensitivity of CASA technique versus the Neubauer method in the determination of frozen-thawed bull sperm concentration.

Materials and Methods

The study was based upon 425 frozen semen batches cryopreserved in defined and undefined (including egg yolk based medias) extenders, from different bulls and various breeds produced in both Brazil and the USA. Under the Neubauer method, all doses were thawed in a water bath at 37°C for 30 seconds, deposited in 1.5 mL conical tubes, homogenized and placed in a dry bath at a constant temperature of 37°C. The solution was diluted at a rate of 1:25, wherein 40 µL aliquots of sperm were transferred to 1.5 mL conical tubes pre-filled with 960 µL of distilled water. Diluted samples were evaluated in duplicate under phase contrast microscopy (x400) in improved hemocytometric Neubauer chamber, proceeding to count the number of sperm heads present on five squares (5 in 25 possible; 5/25) in each of the reticle (N/2) of the chamber (WHO, 2010). The final concentration (C) of spermatozoa in semen samples was determined by de equation: $C = (N/2) \times (5/25) \times \text{dilution factor} \times (1/10) \times 1000$, where "1/10" represents the height of the Neubauer chamber (0.1mm) and "1000" represents the correction factor for the volume expressed in mililiters.

In contrast, using the CASA method aliquots of 5 µL of each raw semen dose were deposited on SpermTrack® chamber (Proiser®, Valencia, Spain) with a height of 20 µm and evaluated using the computer system ISAS® V.1.2. (Proiser®, Valencia, Spain) that performs counting based in individual cells through a high-speed image capture system coupled to a negative phase contrast microscope. The software settings were those recommended by the manufacturer for analysis of bull semen motility and concentration, namely: frames per second: 60 Hz; number of frames: 30; minimum contrast: 50; minimum resolution of cell size: 5 pixels; slow-static cells with average path velocity (VAP) cut-off: 10 µm/s; VAP cut-off: 50 µm/s; straightness (VSL) threshold: 70%; Connectivity: 12; Temperature: 37°C; user defined chamber depth: 20 µm. Every sample constituted a minimum of five random fields and 2000 spermatozoa per analysis.

All of the analyses (Neubauer and CASA groups) were performed by the same experienced and trusted veterinarian.

The data generated were subjected to descriptive statistical analysis for quantitative data and Pearson correlation test and two tailed Student "T" tests were performed using SAS software (SAS Institute Inc., Cary, NC, USA) version 9.1.3 ($p < 0,05$). To evaluate the closeness of agreement between the two independent methods we used Lin (1989) and Bland and Altman (1999) mathematical models. These analyses were performed using the Package MethComp in the R statistical package (R CORE TEAM, 2014) to generate the interclass coefficient.

Results

The average concentration of spermatozoa using the Neubauer chamber was $38.96^{a} \pm 1.29 \times 10^{6}$ sperms/straw, whereas the results obtained from the CASA methodology was $35.14^{b} \pm 0.82$ (Table 1), with a correlation coefficient of 0.87 (Figure 1; $P < 0.0001$).

Table 1 – Mean, standard error, minimum and maximum sperm concentrations and coefficient of variation of frozen-thawed bull semen evaluated by Neubauer hemocytometer chamber or Computer Assisted Semen Analysis (CASA)

	Neubauer Chamber	CASA
Mean sperm concentration ($x10^6$/ml)	38.96	35.14
Standard error ($x10^6$/ml)	1.29	0.82
Minimum sperm concentration ($x10^6$/ml)	2.00	2.00
Maximum sperm concentration ($x10^6$/ml)	162.00	107.00
Coefficient of variation (%)	68.20	48.20

The reliability coefficient was 0.78 on a scale ranging from 0 to 1, where the values close to 0 indicating discrepancy between the two methods used to Viewed the sperm concentration and a score close to 1 indicating that the semen analysis will be identical if performed at Neubauer chamber or CASA (Figure 2). From the 425 data obtained through the sperm concentration evaluated by Neubauer or CASA methods, only 19 are outside the limits of ± 2 SD, demonstrating the agreement between the two sperm concentration tests (Figure 3).

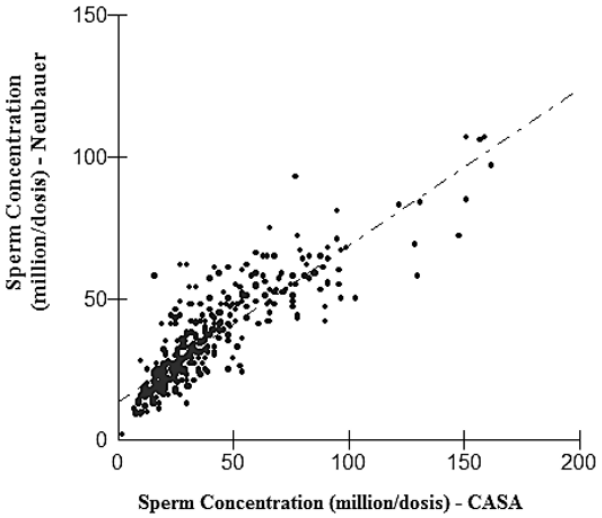

Figure 1 – Plots of agreement between the concentration of bovine semen after thawing evaluated using a Neubauer hemocytometric chamber or Computer-Assisted Semen Analysis (CASA); r = 0.87 ($p < 0.0001$)

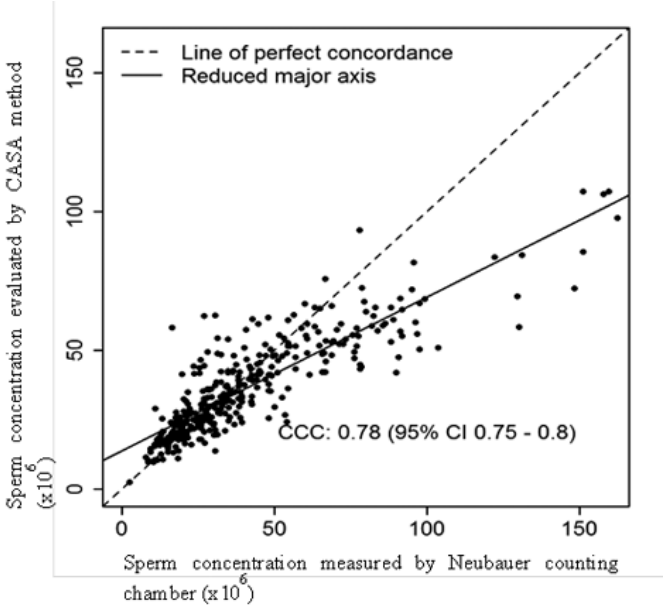

Figure 2 – Scatter Plot of the results of the bull sperm concentration evaluated by Computer Assisted Sperm Analysis (CASA method) or Neubauer counting chamber. CCC = concordance correlation coefficient with 95% CI

Discussion

As demonstrated by some authors and in different studies, the type of analyzing chamber can greatly influence the results of computer-assisted sperm analysis (IBĂNESCU et al., 2016). In order to produce uniform insemination doses with acceptable number of sperm per dose the accurate and precise determination of sperm concentration in an ejaculate is important (ATIQ et al., 2011; ELJARAH et al., 2013). Inaccurate estimations can

lead to misinterpretation of the spermiogram and, in the case of livestock production, can lead to faulty insemination doses, which can adversely affect stud power, fertility, fecundity, and cost effectiveness of breeding programs (DOUGLAS-HAMILTON et al., 2005).

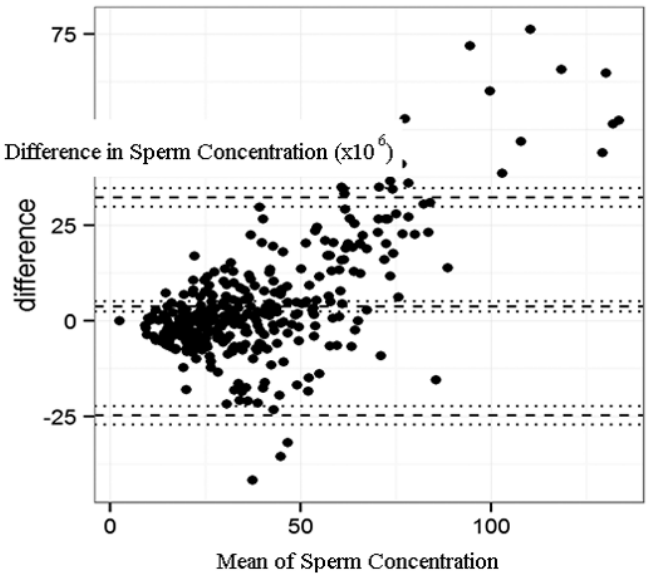

Figure 3 – Scatter Plot of the average differences in sperm concentration measured by Computer Assisted Semen Analysis and Neubauer chamber, according to Bland and Altman (1999). Upper and lower dashed lines shows the mean differences (+25x10^6 or −25x10^6 sperms each with 95% CI, respectively) between the two methods used to measure bull sperm concentration

Sperm concentration is determined by a number of methods, most of which derive their calibration methodology from hemacytometry (KUSTER, 2005). The accuracy of these kinds of cumbersome and time-consuming manual analyses, like the subjective motility sperm evaluation, not only suffers from human bias but also sample preparation and analysis is tedious and slightly haphazard. As a result, relatively few (usually ≤ 200) spermatozoa are being evaluated (GRAHAM; MOCÉ, 2005) and most of the times a limited number of squares (n = 5) are counted for estimation of the concentration of sperm cells.

There is a large amount of variation between technicians and within technicians in the determination of sperm concentration by counting in the haemocytometer, contributing for a low sensitivity of this test. In order to improve these deficiencies inherent in the Neubauer methodology new techniques involving CASA technology are increasingly found in large semen production facilities (MAES et al., 2010; PRATHALINGAM et al., 2006). One of the biggest advantages of computer analysis is the fact of all the CASA systems works similarly, reconstructing the spermatozoon trajectory from instant images, in which the software detects the sperm head (CONTRI et al., 2010). For the concentration, the software utilized the same concept, identifying each sperm in the field, guaranteeing high sensitivity in the analysis and high correlation and concordance with the hemocitometer count, according to this study.

Although there was a high correlation and reliability between the results obtained using the two different testing techniques, lower average sperm concentration ($P < 0.05$) was noted in the samples evaluated by CASA. These reduced values of the sperm concentration are common and agree with results from previous studies involving similar CASA systems. According to Maes et al., (2010) a possible explanation for these results is derived from the presence of clumped spermatozoa, which may be observed in clinical material. The software tolerances inherent in many CASA systems are set up to ignore sperm heads (or blobs) in the images that exceed the pixel size range mandated by the software parameters (5 pixels for bull semen evaluation). Accordingly, these clumped spermatozoa are digitalized as a single image that can exceed the tolerance level, and are consequently ignored by the system.

The numerical difference between the maximum values obtained for the sperm concentration comparing the two methods (Table 1), shows the inclination for the CASA method to understate concentration levels compared to Neubauer count. The discrepancy may be due to software limitations, in which successive images may be overlapping to some extent and thereby preventing an accurate count of the sperm in individual images. Furthermore, Verstegen et al., (2002) observed that in highly concentrated sperm samples, the CASA sperm motion analysis reveals that many fast moving cells are not counted or are undercounted, either due to recurring collisions or to being simply undetected due to a highly compacted and densely-populated field.

According to Davis and Katz (1993) computer analysis shows maximum precision if sample sizes can be limited to lots between 20 and 50 million total sperm. These levels would require prior dilution of semen above these values for an accurate assessment of sperm concentration by CASA. Although there is no consensus in the literature, the same semen extender employed for sperm cryopreservation

or buffered solutions such as 2.96% sodium citrate and DPBs may be employed for dilution prior to the sperm concentration analysis by CASA, improving the accuracy and repeatability of the method.

Another hypothetical explanation for the reduced precision in the CASA for highly concentrated sperm samples could be the physical properties of particles in solution. Particles thus suspended in a laminar flow become concentrated at predictable distances from the walls of the chamber and are subsequently transported to the leading edge of the flow, resulting in a wave of higher concentration at the meniscus (KUSTER, 2005). This phenomenon, referred to as the Segre-Silberberg effect (SS), is due to the decreasing velocity of the fluid near the wall, which results in a high transverse velocity gradient (DOUGLAS-HAMILTON et al., 2005). Consequently, a relatively lower concentration of cells is counted when evaluating the center of the microscopic field (Figure 4), which can also result in an underestimation of sperm concentration by CASA (MAES et al., 2010).

Another factor that may compromise the accuracy of the sperm concentration analysis through CASA corresponds to the extender used for the sperm cryopreservation. The lipid particles found in most extenders could play a deleterious role on sperm movement trajectory evaluated by CASA (CRESPILHO et al., 2012) and possibly in sperm concentration determined by the same method. Because of this, in the present study we did not consider bull sperm samples cryopreserved in milk extenders for the concentration evaluations. According to previous studies, evaluation of bovine semen in lactose-based diluents may be difficult, because of the presence of numerous fat globules which may compromise the identification of sperm cell by CASA (OLIVEIRA et al., 2012).

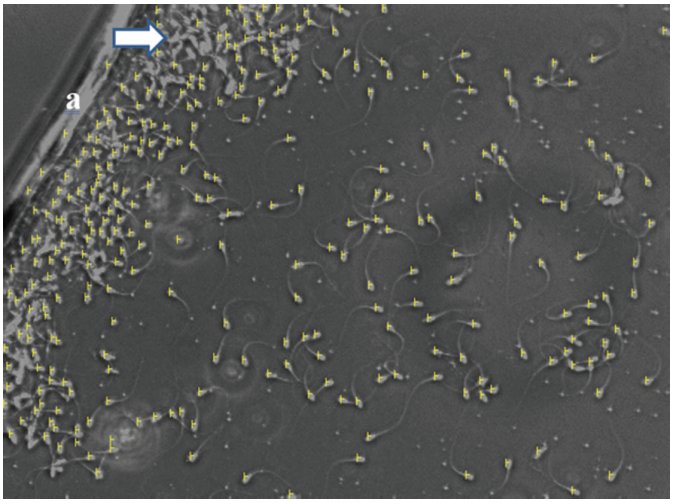

Figure 4 – Presence of clumped spermatozoa near the CASA chamber wall (a), demonstrating the Segre-Silberberg effect (SS). Each sperm cell identified with the yellow mark was recognized like a spermatozoa. Spermatic cells that not received the mark were not considered for the sperm concentration analysis

Conclusion

In conclusion, the CASA system has similar accuracy to that of the Neubauer counting method when frozen-thawed semen is evaluated, although CASA does routinely understate sperm concentration values in densely populated bull semen samples. Nevertheless, due to precision, consistency, practicality, and low time-consumption related to computerized sperm concentration evaluation, these benefits may overcome the possible drawbacks for many andrology laboratories.

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