Acta fytotechn zootechn, 24, 2021(4): 293–296
http://www.acta.fapz.uniag.sk

**Short Communication**

# Evaluation of stallion sperm concentration by two different methods and its influence on sperm motility assessment

Katarina Souskova*, Radek Filipcik, Zuzana Reckova
*Mendel University in Brno, Department of Animal Breeding, Czech Republic*

Article Details: Received: 2020-12-18 | Accepted: 2021-05-03 | Available online: 2021-12-31

https://doi.org/10.15414/afz.2021.24.04.293-296



The aim of this study was to compare two methods for determining the concentration of sperm in stallion ejaculate and to evaluate the influence of different levels of concentration on the determination of sperm motility using an objective method. The total number of evaluated samples was 123. For the experiment, 14 stallions of different breeds housed in the Tlumačov Provincial Stud Farm were used. Sperm concentration was assessed by the hemocytometric method using a Bürker chamber and the automatic method using the Sperm Class Analyzer®. The SCA system was used to evaluate sperm motility. The samples were divided into 6 groups with sperm concentrations of 50, 100, 150, 200, 250 and 300·$10^6$ sperm $ml^{-1}$. The results were statistically evaluated by Tukey's HSD test. There were statistically significant differences in the determination of sperm concentrations between the hemocytometric method and the SCA system in samples with concentration exceeding 100·$10^6$ sperm $ml^{-1}$. Sperm motility increased in samples with higher concentration, however the effect of sperm concentration on motility parameters has not been statistically significant. The results of this study indicate that samples with sperm concentration higher than 100·$10^6$ sperm $ml^{-1}$ reduce the accuracy of the SCA system evaluation and these samples need to be diluted to concentration ≤100·$10^6$ sperm $ml^{-1}$ for more accurate evaluation.

**Keywords:** sperm concentration, sperm motility, hemocytometer, SCA

## 1 Introduction

Artificial insemination is a widely used biotechnical method of reproduction in animal breeding (Aurich, 2012; Rečková and Filipčík, 2020). A quality insemination dose is required for successful insemination (Heckenbichler et al., 2011).

The basic evaluation of ejaculate involves determining sperm concentration and motility. Sperm motility is required for its penetration into the oocyte (Amann, 1989; Věžník et al., 2004; Varner et al., 2008). Motility can be assessed subjectively or objectively. The subjective method is based on estimating the percentage of motile sperm (Jequier and Ukombe, 1983; Malmgren, 1997; Broekhuijse et al., 2011). This method of assessment must be performed by a trained technician using appropriate equipment. However, even with these conditions, this method has several disadvantages. These disadvantages include that the subjective evaluation does not include individual sperm evaluation and results may vary between technicians (Jaquier and Ukombe 1983). Objective methods provide more accurate results that can be compared between reproduction centres (Amann and Weberski, 2014). Sperm concentration is an important parameter of ejaculate quality (Věžník et al., 2004) that can be determined by several methods, such as hemocytometric, photometric and CASA system evaluation. The hemocytometric method is considered the "gold standard" but it is time-consuming (Sokol et al., 2000; Prathalingam et al., 2006). The CASA system (Computer-assisted sperm analysis) is an automated method that allows the evaluation of sperm morphology, concentration and motility (Věžník et al., 2004; Lu et al., 2013). This system recognizes motile and immotile sperm and analyses the trajectory of each movement. The advantage of the CASA system is the objectivity of the results, the possibility of comparing data between

***Corresponding Author:** Katarina Souskova, Mendel University in Brno, Zemedelska 1, 613 00, Brno, Czech republic; e-mail: katarina.souskova@mendelu.cz

different institutions, the evaluation of the movement of individual sperm together with the evaluation of other seminal traits (Verstengen et al,. 2002; Mortimer et al., 2015). Nevertheless, results may be affected by multiple trajectory crossings and sperm collisions (Spiropoulos, 2001; Verstengen et al., 2002). The results may also vary depending on the chamber that is used (Ginsburg and Armant, 1990; Lannou et al., 1992; Spiropoulos, 2001; Verstengen et al., 2002). Concentration assessment may be inaccurate for highly viscous and/or heterogenous samples (WHO, 1999).

The aim of this study was to evaluate sperm concentration using two methods: the hemocytometric method and the method using the Sperm Class Analyzer® and to determine sperm motility in the samples with different concentrations.

## 2 Material and methods

For the experiment we used 14 stallions of Hanoverian, Oldenburger, Holsteiner, Zangersheide, Shagya-Arabian, Thoroughbred, Hucul, Czech Warmblood and Kladruber breeds aged 4 to 20 years. The stallions were housed in the Tlumačov s.p.o Provincial Stud Farm. Ejaculates were collected at the Reproduction Centre of the stud farm. Ejaculates were collected into an artificial vagina and post-sperm fraction was removed. The minimum gel-free ejaculate volume was 10 ml, the minimum sperm concentration was $100{\cdot}10^6$ sperm $ml^{-1}$ and the minimum sperm motility was 60%. Sperm concentration of native ejaculates ranged from 113.70 to $531.70{\cdot}10^6$ sperm $ml^{-1}$. Gel-free volume ranged from 16 to 125 ml. The ejaculates were diluted at a ratio of 1 : 1 with a skimmed milk-based extender that is prepared at the Reproduction Centre. The total number of collections was 123. The samples were then transported under optimal conditions to the Laboratory of Livestock Reproduction of Mendel University for the assessment. The samples were stored at 4 °C.

Sperm concentration was determined by the hemocytometric method using a Bürker chamber. Based on the concentration determined by the hemocytometric method, the ejaculates were divided and diluted with saline into samples with a concentration of 50, 100, 150, 200, 250 and $300{\cdot}10^6$ sperm $ml^{-1}$. Sperm concentration and motility were determined using the Sperm Class Analyzer®. A minimum of 500 sperm in at least 5 fields of view were evaluated using a Leja chamber.

The total number of evaluated samples was 123. Data were evaluated using STATISTICA 12.0 software (StatSoft CR s.r.o., Praha, Czech Republic) and Tukey's HSD test. The data were expressed as means ± standard error of the mean. Differences were considered statistically significant at $p \leq 0.01$.

## 3 Results and discussion

Results determined by the SCA system (Table 1) show the average values of sperm motility and progressive motility in samples with different concentrations from 50 to $300{\cdot}10^6$ sperm $ml^{-1}$. The lowest values of sperm motility (48.45%) and progressive motility (22%) were in the least concentrated samples. The second lowest values of motility parameters were contained in samples with a concentration of $100{\cdot}10^6$ sperm $ml^{-1}$. In more concentrated samples there were higher values of motility parameters. The highest results were found in samples with a concentration of $200{\cdot}10^6$ sperm $ml^{-1}$ and reached 67.16% of motile and 32.10% of progressively motile sperm. There were no statistically significant differences in the motility and progressive motility of samples with different concentrations.

Differences in values of sperm concentration determined by the SCA system and hemocytometric method (Table 2) were statistically significant in samples with a concentration above $100{\cdot}10^6$ sperm $ml^{-1}$. In samples with the lowest concentration hemocytometrically ($50{\cdot}10^6$ sperm $ml^{-1}$) determined, there is the lowest difference

**Table 1** Motility parameters (means ± SEM) of stallion semen samples ($n$ = 123) determined by the Sperm Class Analyzer® in samples with a concentration of 50–$300{\cdot}10^6$ sperm $ml^{-1}$ determined by the hemocytometric method

| Sperm concentration – hemocytometric method ($10^6$ sperm $ml^{-1}$) | Sperm motility – SCA system (%) | Sperm progressive motility – SCA system (%) |
|---|---|---|
| 50 ($n$ = 31) | 48.45 ±5.10 | 22.00 ±4.67 |
| 100 ($n$ = 27) | 51.12 ±6.08 | 25.44 ±5.48 |
| 150 ($n$ = 21) | 59.76 ±6.21 | 28.41 ±6.32 |
| 200 ($n$ = 17) | 67.16 ±6.19 | 32.10 ±7.16 |
| 250 ($n$ = 14) | 63.15 ±6.51 | 26.97 ±7.22 |
| 300 ($n$ = 13) | 62.72 ±7.65 | 29.71 ±7.50 |

**Table 2** Sperm concentration (means ± SEM) of stallion semen samples ($n$ = 123) determined by the Sperm Class Analyzer® in samples with a concentration of 50–300·10$^6$ sperm ml$^{-1}$ determined by the hemocytometric method and differences between these two methods

| Sperm concentration – hemocytometric method (10$^6$ sperm ml$^{-1}$) | Sperm concentration – SCA system (10$^6$ sperm ml$^{-1}$) | Difference in sperm concentration between the hemocytometric method and the SCA system (10$^6$ sperm ml$^{-1}$) |
|---|---|---|
| 50 ($n$ = 31) | 46.14 ±2.78 | 3.86 ±2.78 |
| 100 ($n$ = 27) | 79.22 ±5.48 | 20.78 ±5.48 |
| 150 ($n$ = 21) | 96.21 ±5.39 | 53.79 ±5.39** |
| 200 ($n$ = 17) | 129.29 ±9.25 | 70.71 ±9.25** |
| 250 ($n$ = 14) | 145.38 ±9.21 | 104.62 ±9.21** |
| 300 ($n$ = 13) | 172.27 ±12.80 | 127.73 ±12.80** |

** differences are significant for p ≤0.01

**Table 3** Advantages and disadvantages of hemocytometric and CASA system methods of sperm concentration evaluation

| Hemocytometric method | | CASA system | |
|---|---|---|---|
| advantages | disadvantages | advantages | disadvantages |
| measurement accuracy<br>affordability | time effort<br>laboriousness | automatization<br>evaluation of several parameters simultaneously | measurement inaccuracy of heterogenous samples<br>expensiveness |

in the measured values between methods (3.86·10$^6$ sperm ml$^{-1}$). With increasing sperm concentration, the difference in the measured values between observed methods increases. In the samples with a sperm concentration of 50 and 100·10$^6$ sperm ml$^{-1}$, there were no statistically significant differences in the measured values. In the samples with a concentration of 150, 200, 250 and 300·10$^6$ sperm ml$^{-1}$, there were statistically highly significant differences in the measured values of sperm concentration using a Bürker chamber and the SCA system.

An accurate determination of sperm concentration and motility is important for artificial insemination. Both methods, hemocytometric and SCA, have their advantages and disadvantages. Our results show high differences in sperm concentration between the hemocytometric method and the SCA system in more concentrated samples. Verstegen et al. (2002) state that the determination of sperm concentration by computer-assisted sperm analysis is a problem in all studied species. According to WHO (1999), the assessment of sperm concentration may be inaccurate in heterogenous samples. Less diluted samples tend to be more heterogenous due to the formation of clumps. Our results show that in samples with a concentration above 100·10$^6$ sperm ml$^{-1}$, the values obtained by the SCA system were significantly underestimated when compared to the hemocytometer. Our findings confirm Mortimer et al. (1995), who state that in samples with a concentration higher than 100·10$^6$ sperm ml$^{-1}$, the number of sperm is underestimated precisely because of the formation of clumps that are too large for the system to recognize. Kuster (2005) states that errors of sperm concentration determined by CASA systems can be caused by the Segre-Silberberg effect that occurs in chambers with a depth of 20 µm, usually used for CASA system evaluation. This effect causes the concentration in a readable area of the slide to be lower than the actual concentration. This effect is insignificant to the hemocytometer because of the greater depth of the chamber. Knuth and Nieschland (1988) recommend diluting samples above the 100·10$^6$ sperm ml$^{-1}$ limit to achieve a more accurate assessment.

## 4 Conclusions

The results of the evaluation of the sperm concentration using the hemocytometric method and the SCA system show that with increasing concentration, the difference in values between the measured methods increases. Samples with a concentration of ≤100·10$^6$ sperm ml$^{-1}$ had smaller differences in the measured values. Based on these results, it can be stated that for a more accurate assessment of sperm concentration using the SCA system, it is necessary to dilute samples to a concentration of ≤100·10$^6$ sperm ml$^{-1}$. Sperm motility and progressive motility increased in samples with higher concentrations; however, these results were not statistically significant. Further research aimed at the influence of sperm

concentration on sperm motility assessment is needed including subjective motility evaluation.

**Acknowledgments**

Research was financially supported by grant no. AF-IGA2020-IP066.

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