World Rabbit Sci. 2007, 15: 115 - 119

VALIDATION OF A SPECTROPHOTOMETRIC METHOD USED FOR THE MEASUREMENT OF SPERMATOZOA CONCENTRATION IN RABBIT SEMEN

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Abstract: In rabbits, the number of inseminated spermatozoa plays an important role in the fertility rate obtained after artificial insemination. However, methods for the correct determination of spermatozoa number are scarce. The aim of this study was to develop and validate a spectrophotometric method for the measurement of spermatozoa concentration corrected by the seminal particle/spermatozoa ratio. For this purpose 360 ejaculates were collected from 36 rabbit bucks. The semen samples were divided into three groups based on their particle/spermatozoa ratio subjectively determined by microscopic viewing. The spermatozoa concentration of each semen sample was evaluated by spectrophotometer and these results were compared with those obtained by using a haemotocytometer. The results of the standard spectrophotometric method were correlated with the results of the haemotocytometer count of spermatozoa (r=0.76; $P<0.01$). When the optical density was corrected by considering the P/S ratio, the correlation values greatly improved (r=0.94; $P<0.01$). The simplicity, rapidity and low cost of this method make it suitable for the routine measurement of spermatozoa concentration of heterospermic pools in rabbit artificial insemination centres.

Key words: Rabbit, spermatozoa, seminal particles, spectrophotometer

INTRODUCTION

Artificial insemination (AI) in rabbit is widespread throughout Europe. The success of this reproductive technique relies mainly on the status of does at the moment of AI (lactation, receptivity, body and health conditions). However, the number of spermatozoa inseminated and the spermatozoa characteristics are also important factors (Castellini and Lattaioli, 1999; Brun *et al*., 2002, Theau-Clément *et al.,* 2003) and should be accurately evaluated.

While the "golden standard" method for assessing spermatozoa number is the haemocytometer, it is time-consuming and tedious, and, therefore, not suitable for large numbers of specimens. Recently, Theau-Clément and Faliéres (2005), found a good correlation between the NucleoCounter SP100® and the haemocytometer counts but the equipment and materials are expensive. Another technique, CASA (Computer-Assisted Semen Analyzer) is mainly used to evaluate of spermatozoa kinetic traits (Theau-Clément et *al*., 1996a, Lattaioli and Castellini, 1998), but it is inaccurate for determination of spermatozoa concentration in semen (Pizzi *et al*. 1993; Theau-Clément *et al.* 1996b; Mahmoud *et al.*, 1997; Liu *et al*., 1991; Christensen *et al.*, 2005). Consequently, a faster, more precise and cheaper analytic technique should be developed for routine use in rabbit AI centres.

Spermatozoa concentration in several animal species is usually evaluated by spectrophotometer (SP). Unfortunately, rabbit semen contains varying amounts of seminal particles, which makes

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Received *September 2006* - Accepted *March2007*.

spectrophotometric analysis of spermatozoa unreliable (Farrel *et al.*, 1992). Although similar particles are known to be secreted by different accessory sex glands in different mammalian species and to play an important role in sperm motility, the origin of these particles and their biological composition and function in rabbit are still unclear (Minelli *et al.*, 2001). Several studies have shown that the concentration of the particles in semen may be higher than that of the spermatozoa and the ratio varies depending on the collection rhythm and the health status of the rabbit bucks. A biological variation has also been observed with regard to the number of these particles produced by individual rabbits (Zaniboni *et al.*, 2004; Castellini *et al.*, 2006).

The main objective of this investigation was to develop a robust, cost-effective method for the rapid determination of spermatozoa number in rabbit semen by taking into account the particle/spermatozoa ratio. This method was then compared with the golden standard and classic spectrophotometer assay methods.

MATERIALS AND METHODS

Sample collection and handling

Semen samples were collected from 36 rabbit bucks of different genetic strains (New Zealand White and hybrids) and different ages (12-36 months) by means of an artificial vagina (IMV l'Aigle, France). The collection period varied from 4 to 21 days during which a mean of 10 collections were performed on each rabbit to give a total of 360 ejaculates. All semen samples were diluted (1/100) with phosphate buffer saline (PBS) and analysed in duplicate within two hours after collection using all three methods.

Assay methods

Haemocytometer Assay: spermatozoa and non-spermatozoa particles were counted by haemocytometer (Burker 100 mm deep-BT, Brand, Wertheim, Germany) according to the guidelines for semen handling (Boiti *et al.,* 2005).

Standard Spectrophotometer Assay (SP): Hitachi Spectrophotometer (Model U-2000), set at 520 nm was used to measure the optical density (OD) of each semen sample.

Proposed Spectrophotometer Assay (PSP): prior to the spectrophotometer assay, each sample was assigned to one of three groups based on their particle/spermatozoa (P/S) ratio, which was subjectively determined by using an Olympus CH2 microscope (magnification 400×). The first group (A) contained semen samples with a P/S ratio between 0-1.9, the second group (B) contained samples that had a P/S ratio between 2 to 3.9 and the third group (C) contained samples with a P/S ratio≥4 (Figure 1). A standard calibration curve was created for each group by dilution of the respective pooled semen sample (pool A, pool B and pool C) with a known spermatozoa concentration.

Statistical analysis

To assay the repeatability of the SP methods and haemocytometer count, two aliquots of each semen sample were assayed separately at 520 nm and the Pearson correlation coefficient between the two sets of data was calculated.

For the standard method, the concentration was predicted by a linear regression of the haemocytometer count on the OD. For the proposed SP method, an analysis of variance was used with the fixed effect of the subjective P/S ratio (class) and OD as a covariate. The accuracy of the SP methods was evaluated by Pearson correlation coefficient and linear regression *vs*. their haemocytometer count.

All statistical analyses were carried out using the Stata Statistical Software (StataCorp, 2005).

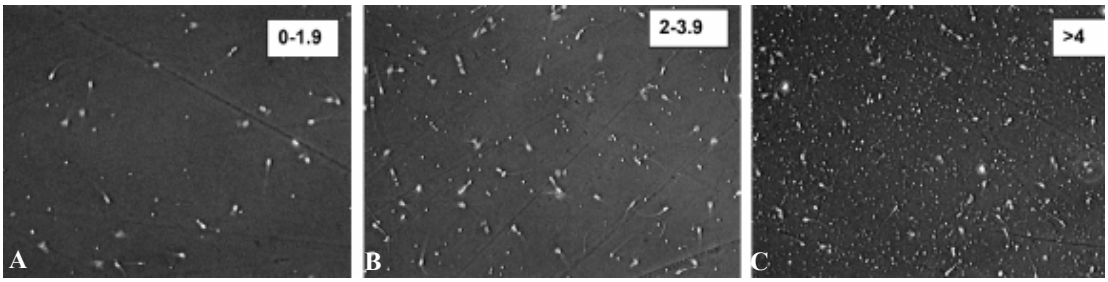

Figure 1: Images of semen samples classified in the three groups (A, B and C) according to their particle/ spermatozoa ratio. Group A is characterized by a P/S ratio 0-1.9, group B by a P/S ratio 2-3.9 and group C by a P/S ratio≥4 .

RESULTS AND DISCUSSION

In the present study, a simple method measuring the spermatozoa concentration in rabbit semen has been proposed and validated with respect to repeatability and accuracy. The repeatability results are illustrated in Figure 2. The correlation coefficient between the two data sets was r=0.99 (P<0.01) for the haemocytometer assay, r=0.97 (P<0.01) for the proposed SP method and r=0.75 (P<0.01) for the standard SP.

The semen samples were divided into three groups according to their P/S ratio. A preliminary subjective evaluation was determined by microscopic viewing. The main characteristics of each group are reported in Table 1. The A group, which contained 50% of the ejaculates, had the highest spermatozoa number and OD and the lowest objective P/S ratio, while the opposite results were obtained for the C group. The values for the B group, despite the high particle concentration, were intermediate for all the variables. The percentage of error in semen sample classification was low only for a and B groups, but was increased 50% for the C.

To assay the comparability of the proposed method and standard SP method with the haemocytometer assay, the data of each SP method were plotted against the results obtained by the reference method, and the best line of fit for the data was determined by linear regression analysis (Figure 3). The results of the correlation and linear regression analyses between the haemocytometer method and the standard or proposed SP method are shown in Table 2. The correlation between the proposed method and the reference one (r=0.94, P<0.01) was higher than the correlation between the standard

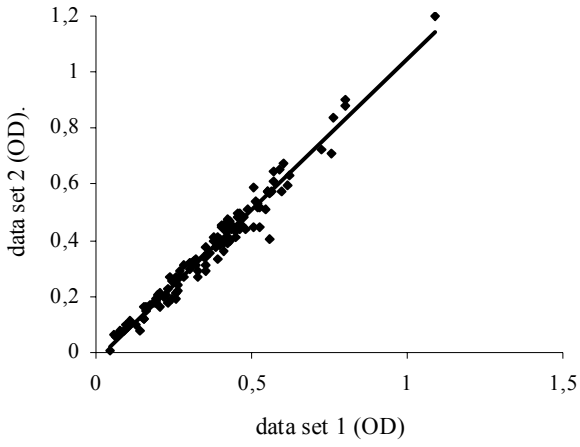

Figure 2: Repeatability of the proposed SP method based on duplicate analyses (data set 1 and data set 2) of 360 semen samples (r=0.97, P< 0.01).

Table 1: Main characteristics of the three groups of semen samples (Lsmeans±SEM).

Group	A	B	C	Overall mean
No. (%)	171 (47.5%)	95 (26.4%)	95 (26.1%)	360
Particles n×10^6	401.8±26.3	529.8±27.6	559.7±28.2	482.3±8.3
Spermatozoa n×10^6	306.9±5.4^c	198.8±4.7^b	90.55±6.7^a	218.0±2.5
P/S ratio	1.33±0.44^a	2.66±0.58^b	8.67±0.58^c	3.63±0.60
Optical Density	0.39±0.01^b	0.37±0.01^{ab}	0.32±0.0^{1a}	0.37±0.01
Error in classification, %	3.46	4.12	8.42	4.65

P/S ratio: particle/spermatozoa ratio

SP method and the reference method (r=0.76, P<0.01). The lower correlation found for the standard SP method was probably due to the presence of interfering substances, like the refringent non-spermatozoa particles. In fact, when the P/S ratio was taken into account, the correlation was greatly improved. On the other hand, the standard SP method could be suitable for determining spermatozoa number if only pooled semen samples are used. Pooling semen samples reduces the variation related to the P/S ratio. However, the standard SP method should not be used to select single rabbit bucks.

The assessment of P/S ratio could be very useful for correcting the overestimation of spermatozoa number in samples that contain a high number of particles and give high OD values. To obtain a maximum fertility rate, doses 1-5 times higher than the effectively required dose are currently used (Castellini and Lattaioli, 1999). This procedure could be improved if the P/S ratios of the semen samples are correctly determined and the samples are then processed accordingly.

In conclusion, the SP method in conjunction with a preliminary qualitative microscopic assessment of the P/S ratio gives accurate and reproducible results which are comparable to results obtained with the golden standard method. These positive results combined with the short analysis time and simplicity of the procedure make this method a useful tool for determining spermatozoa concentration in rabbit heterospermic pools.

Acknowledgment: Research funded by PRIN 2005 prot. 2005070702_004. We are grateful to Sister Mary Traynor for her helpful linguistic suggestions.

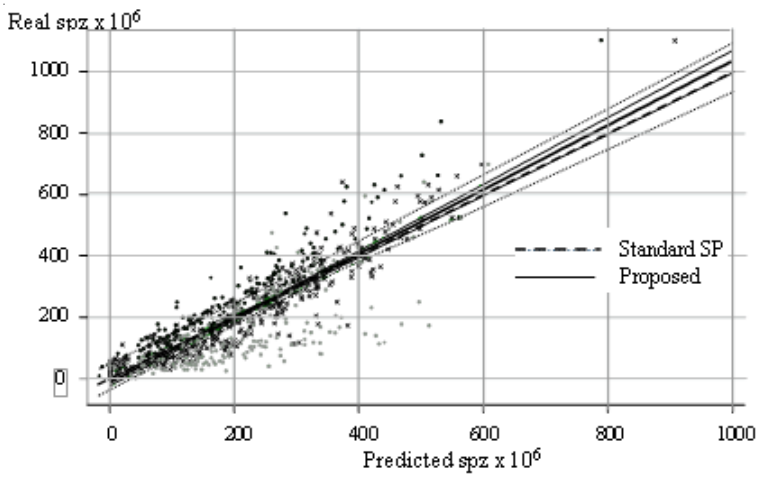

Figure 3: Comparison of the standard SP and the proposed SP methods with the haemocytometer assay for determining spermatozoa number in semen samples (number of spermatozoa x10^6; prediction and 95% confidence interval).

Table 2: Correlation and linear regression analyses of results obtained with the standard SP and proposed SP method, each *vs*. the haemocytometer assay.

	Linear regression		Correlation coefficient	Samples within + 20%
	Intercept (±SE)	Slope (±SE)		
standard SP *vs*. haemocytometer	−4.79±11.15	1.00±0.04**	0.76**	26
proposed SP *vs*. haemocytometer	−14.52±5.37	1.05±0.02**	0.93**	62

** $P< 0.01$.

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