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SPERMOGRAMS ANALYSIS AS A MANDATORY PART OF THE CANINE SEMEN CRYOPRESERVATION PROTOCOLS

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Abstract

Quality of fresh semen is a crucial element for the succes of assisted reproduction techniques in all species. 57 ejaculates were collected manually from 25 dogs. Motility and concentration (CASA SpermVision®, Minitübe, Germany, morphology and acrosome status (Spermac® stain) were determined. Semen analysis showed a strong relationship between male's weight and size (testes dimensions) and the volume of the sperm rich fraction, but did not identify a correlation between concentration and male's size. Motility parameters, morphology and acrosome status for the ejaculates included in our study were within the accepted standard values. Age did not significantly influence the volume or the morphology of canine semen.

Key words*:spermogram, canine, cryopreservation*

INTRODUCTION

Quality of fresh semen in the male dog is one of the crucial elements for the succes of assisted reproduction techniques. Semen analysis (spermogram) has to steps: macroscopical (volume, colour, smell and pH of semen) and microscopical exam (concentration, motility, viability and morphology of sermatozoa).

Semen parameters vary betwen individuals and even for the same male, being influenced by numerous endogenous (endocrin, genetic factors etc.) and exogenous factors (food, stress, the reproduction regimen etc.) (de Souza et al., 2007; Goericke-Pesch and Failing, 2013; Rijsselaere et al., 2004).

To confirm infertility in a male, semen parameters should be reevaluated 2-3 times at 1-2 weeks time intervals. On the other hand, a correct estimation of *in vitro* potential of a male dog is not always possible, a good spermogram not being an absolute equivalent of individual fertility (Payan-Carreira et al., 2011).

The goal of the paper was to establish and verify the quality of canine semen based on spermograms in order to cryopreserve the collected semen.

MATERIALS AND METHODS

Research was carried out in the Clinic of the Faculty of Veterinary Medicine of Bucharest on 57 ejaculates collected from 25 dogs. The age of the male dogs varried between 1,5 and 9,5 years, with a mean of 4,8 years.

Before the collection of semen, the males were examined clinically (general physical exam and genital examination) and serologically (Brucella canis testing). The time interval between two collections for a dog was of minimum 3 days. A male was not collected multiple times in the same day.

Semen was collected manually, in the absence of a bitch in estrus, according to the method described by Kutzler (Kutzler, 2005). 3 of the 25 dogs did not respond initially to the digital manipulation. In order to stimulate them, we used swabs collected from bitches in estrus and stored in a freezer.

The sperm rich fraction was kept at 37^{o}C all the time in order to avoid the termic shock. Each of the three fractions of the ejaculate were evaluated macroscopically and microscopically. The macroscopic exam determined the volume and the colour of the fraction. The microsocopic exam of the sperm rich fraction established the concentration and

motility of semen (computer assisted sperm analyzer, SpermVision®, (Minitübe, Germany) (Schafer-Somi and Aurich, 2007).
The following motility parameters were assessed with CASA:
1) Curvilinear velocity (VCL, µm/s), the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon per unit of time.
2) Linear velocity (VSL, µm/s), the straight trajectory of the spermatozoa per unit of time.
3) Mean velocity (VAP, µm/s), the mean trajectory of the spermatozoa per unit of time.
4) Mean coefficient (STR, %), which indicates the linearity of the mean trajectory and is defined as (VSL/VAP) x 100.
5) Linear coefficient (LIN, %), the ratio of the straight displacement in the sum of elementary displacements during the time of the measurement and it is defined as (VSL/VCL) x 100.
6) Wobble coefficient (WOB, %), which indicates the oscillation of the curvilinear trajectory upon the mean trajectory and is defined as (VAP/VCL) x 100.
7) Frequency of head displacement = beat cross frequency (BCF, Hz), the number of lateral oscillatory movements of the sperm head around the mean trajectory.
8) Amplitude of lateral head displacement (ALH, µm), which is the mean width of sperm head oscillation.
9) Distance curved line (DCL, µm), the actual distance that the sperm cell moved during the analysis period.
10) Distance straight line (DSL, µm), the distance from the point in which the cell was first found in the analysis to the location of the cell at the last frame of the analysis in a straight line.
11) Distance average path (DAP, µm), the measured distance using a smoothed line as a reference.
12) Average orientation change (AOC, degrees), the average number of degrees that the head of the sperm moved from left to right during the analysis.

Morphology and acrosome status were evaluated using the Spermac® stain kit (Stain Enterprises, Onderstepoort, South Africa) (Goericke-Pesch and Failing, 2013). A drop of semen was placed on a glass slide and a thin smear was prepared and air-dried for 5 min on a warm plate at 37°C. The slide was then fixed for 5 min and washed with distilled water 5-6 times. Excess water was removed with a piece of absorbent paper and the slide was placed into stain solution A for 1 min. This procedure was repeated for solutions B and C. Finally, the slide was air dried. 200 spermatozoa were evaluated for abnormal acrosome, head, mid-piece and tail forms under a light microscope at x1000 magnification. Under the microscope, the acrosome is dark green, the nucleus is stained red, the equatorial region is pale green and the midpiece and tail are green. Morphological abnormalities were classified as primary and secondary (Johnston et al., 2001).
Statistical analysis was done using the IBM SPSS – ver. 19 for Windows (IBM, New York, SUA) – paired student T test and one way ANOVA. In some cases, in order to establish the degree of correlation between data groups, Pearson coefficient (R) was calculated. On the dot-plot graphics, the linear trendline (r^2) is represented in order to estimate the evolution of the two data sets.
Results are presented as mean values ± standard deviation. The statistical signifance level was $P < 0,05$.

RESULTS AND DISCUSSIONS

Semen was collected from 25 male dogs of different breeds (fig. 1), resulting a total of 57 ejaculates.

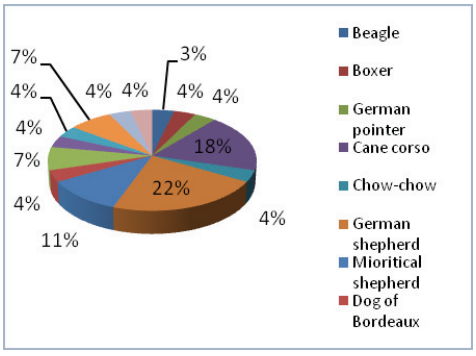

Figure 1. Breed distribution of the dogs used for the collection of semen.

The colour of the ejaculate was characteristic for each fraction: the first fraction was

clear,while the sperm rich fraction was milky-white, opaque to watery-white. The milky-white aspect of the ejaculate was correlated to a higher semen concentration. The third fraction of the ejaculate (prostatic fraction) was clear, transparent for 53 of the 57 ejaculates collected. 4 ejaculates presented a red colour for the prostatic fraction. The microscopical exam showed the presence of a high number of red blood cells, phenomenon correlated with the presence of benign prostatic hyperplasia.
The volume of the 3 fractions of the ejaculate was measured immediately after collection.

Table 1. Mean volume of the ejaculates collected from the 25 dogs included in this study.

Nr.	Breed	Age (years)	Sperm rich fraction (ml)	Prostatic fraction (ml)
	Beagle	4	0.8	5.2
	Boxer	3	2.0	7.8
	German pointer	3.5	1.3	7.5
	Cane corso	4	4.2	8.2
	Cane corso	3.5	3.8	7.9
	Cane corso	3	4.9	9.3
	Chow-chow	5	1.4	4.5
	German shepherd	3	2.5	5.5
	German shepherd	9	1.9	6.4
	German shepherd	8	2.2	7.1
	German shepherd	6	2.0	5.8
	German shepherd	5.3	2.7	8.9
	German shepherd	2.5	3	8.6
	Romanian shepherd	6	4	9.8
	Romanian shepherd	4	4.2	8.7
	Romanian shepherd	5.5	5	8.5
	Dog of Bordeaux	4	2.1	4.2
	Grand dane	9.5	3.9	10.3
	Grand dane	2.8	4.1	9.9
	Golden retriever	1.5	2.6	4.5
	Greyhound	3.5	1.7	3.0
	Labrador	5.5	1.7	4.7
	Labrador	6.8	2	6.7
	Samoyede	7	1.9	5.4
	Terra Nova	4.6	4.3	3.4
Mean		4.82	2.81	6.87
Standard deviation		2.05	1.22	2.15

Analyzing the relationship between the sperm rich fraction of the ejaculate and the male's breed, we found a strong correlation between the two (r^2 = 0,9021), more precisely a direct proportionality between the volume of the sperm rich fraction and the breed's weight (fig. 2). Even since 1983, Olar et al. had signaled the existance of a relationship between the size of the testes and the volume of the sperm rich fraction (Olar et al., 1983). Even though the volume of the ejaculate is not an indicator of the quality of canine semen, it represents a preliminary step in calculating the total number of spermatozoa in the sample.

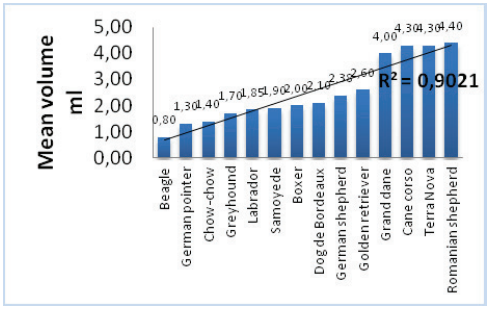

Figure 2. Correlation between breed and the volume of the sperm rich fraction.

The age did not significantly influence the volume of the sperm rich fraction, the liniar correlation between the two being almost 0 (r2 = 0,009) (fig. 3).

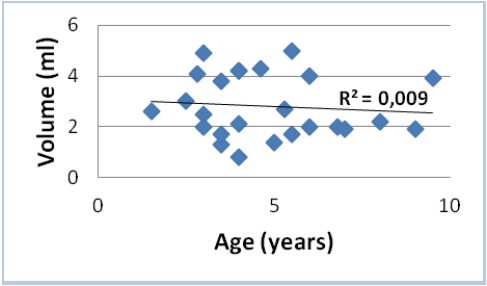

Fig. 3. Correlation between the age of the male and the volume of the sperm rich fraction.

Semen concentration and the total number of spermatozoa were determined automatically with the SpermVision® CASA. Semen concentration was determined for the sperm rich fraction and the total number of spermatozoa was established by multipling

the volume of the sperm rich fraction with its volume. The concentration of the ejaculate is very important for the assisted reproduction technoques, representing the base for the calculus of insemination doses. Fresh semen concentration varied between 202,2 x 10^6 and 1750,3 x 10^6 spermatozoa/ml. We did not identify a correlation between semen concentration and the age or weight of the male dogs (fig. 4).

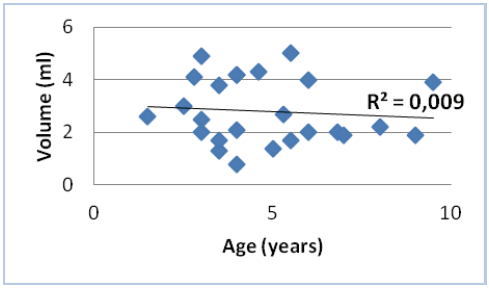

Figure 4. Correlation between the age of the male and the volume of the sperm rich fraction.

Motility of semen is a crucial element for fertility and represents one of the essential elements for establishing the quality of an ejaculate. Mean value for motility in our study was 90,16% (minimum 75,15% and maximum 98,87%) and for progressive motility was 85,14% (minimum 70,38% and maximum 96,22%). This values of motility are according to the standard of the American Society of Theriogenology.

SpermVision® allowed the determination of a complex set of values for canine semen motility, the most important being the velocity parameters (VCL, VSL, VAP). The sperm that deviate with less than 10% from the liniar trajectory are considered to have a straight motility. Excepting the liniar trajectory, the rest of movements are considered non-progressive motility. Since some motility parameters were associated with *in vitro* fertility (VAP, VSL, BCF), we analyzed this values for the fresh semen collected (Silva et al., 2006).

There is a strong correlation between progressive motility, VSL and VAP, this being a good indicator for fertility. For some of the males, VAP has net bigger values than progressive motility going over 160 µm/sec. Although this may look like a good factor for fertility, it can also be a negative factor, a high velocity leading to a rapid depletion of the energetic resources of the sperm cells.

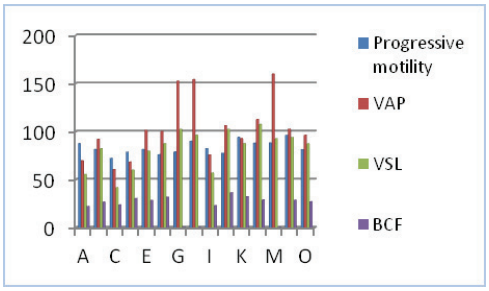

Figure 5. Relationship between progressive motility and certain velocity parameters.

Semen morphology and acrosome status were evaluated using the Spermac stain and the percentages of normal morphology were in the limits of the standard for all the ejaculates. The main types of abnormal forms were detached head, coiled tails, proximal and distal droplet.

Acrosome's integrity is a must for *in vivo* fertility. The percentage of semen with acrosome reaction was 2,37%.

For canine semen it is considered that a percentage lower than 60% of normal semen will negatively influence fertility (Oettle, 1993).

For human semen it has been showen that age has a negative effect on semen morphology, this decline being obvious after 42 years of age (Pasqualotto et al., 2005). In dogs this relationship was not identified, data confirmed also by our study were the correlation between the age of the dogs and semen morphology is almost null (r^2 = 0,0073) (fig. 6).

Other studies showed a positive correlation between normal morphology, progressive and total motility and acrosome intact for canine semen (Agarwal et al., 2003; Ellington et al., 1993; Veznik et al., 2003). For our study, the correlations between semen morphology and motility (fig. 7) and between motility and acrosome status (fig. 8) were weak (r^2 = 0,265, respectivly r^2 = 0,1486), data being in partial agreement with the above mentioned studies.

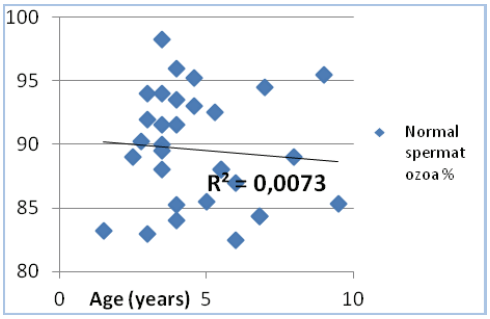

Figure 6. Correlation between the age of the dogs and the percentage of sperm cells with normal morphology.

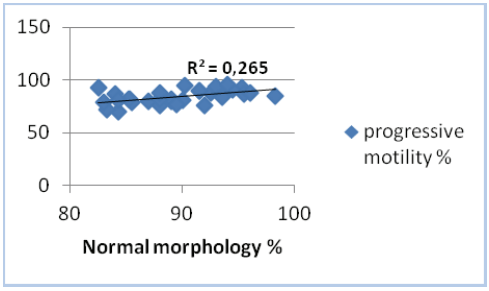

Figure 7. Correlation between intact acrosomes and total motility of sperm.

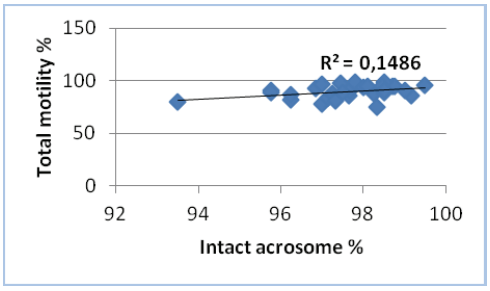

Figure 8. Correlation between intact acrosomes and total motility of sperm.

CONCLUSIONS

Canine semen collection can be easily achieved by manual collection, without needing any special equipment.

Semen analysis showed a strong relationship between male's weight and size (testes dimensions) and the volume of the sperm rich fraction, but did not identify a correlation between concentration and male's size.

Opposed to human medicine, age did not significantly influence the volume or the morphology of canine semen.

Motility parameters, morphology and acrosome status for the ejaculates included in our study were within the accepted standard values.

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