Functional evaluation of dog ejaculates with priority given to the aspect of acrosome integrity

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ABSTRACT: 38 ejaculates of dogs of various breeds from 2 to 6 years old were examined in a short-term 120 min survival test. The examination focused on the functional parameters of sperm (motility, percentage of live spermatozoa, acrosomal integrity) and their morphology. The levels of relationship between individual criteria of sperm analysis were demonstrated. Statistically highly significant correlations between motility, ratio of live spermatozoa, percentage of intact acrosomes and percentage of spermatozoa with altered superficial structures were proven. Our findings proved the sensitivity of superficial structures, primarily plasmatic membranes, and the higher resistance of structures with an organelle basis. Strict morphological sperm analysis provides important information about the rise in qualitative alterations of ejaculates caused by changes in superficial structures during the course of a short-term survival test.

Keywords: dog; ejaculate; sperm analysis; membrane and acrosome integrity

The majority of authors who have devoted themselves to the matter of the fertility of dogs emphasise the primary importance of the high-quality analysis of ejaculates in dogs with an unknown level of reproductive functions and dogs with fertility disorders, but also the examination of ejaculates prior to their preservation (Deibel *et al*., 1976; Chong *et al*., 1983; Dunphy, 1989; Iguer-Ouada and Verstegen, 2001a,b).

Attention has been devoted to the high-quality evaluation of dog ejaculates in particular in relation to the results of their long-term conservation (Pena *et al*., 1999; Pena and Linde-Forsberg, 2000a,b; Szasz *et al*., 2000). Rigau *et al*. (2001) stress the importance of sperm motility as an indicator of the quality of dog ejaculates. A number of newer studies value above all the contributions made by automatised methods like CASA (computer aided sperm analysis) or SQA (sperm quality analyser). The criteria for assessing the quality level of dog semen are given by Iguer-Ouada and Verstegen (2001a,b). As in other species, the importance of the morphological examination of sperm in dogs is emphasised and above all its dynamic representation in a resistance test (Oettle, 1993; Schafer *et al*., 1996; Nothling *et al*., 1997; Dahlbom *et al*., 1997; Koehler *et al*., 1998; Root Kustritz *et al*., 1998; Sirivaidypong *et al*., 1999). Most authors focus this examination on assessing the results of the long-term conservation of dog semen and consider these findings as conclusive as far as its fertilisation capabilities are concerned. The classic morphology of sperm is accompanied in a number of studies by a view regarding the proportion of pathologically changed sperm in ejaculates at a frequency of up to 20%. Our experience, but also the views of Christiansen (1984) or Root Kustritz *et al.* (1998) shift this value to 30%. It is, however, desirable that primary malformations do not exceed 10%. The present study evaluates the functional indicators of sperm, represented particularly by their motility, membrane integrity and acrosome integrity, in connection with morphological normosperm or the proportion of pathological sperm in ejaculates. The morphological assessment of semen remains constantly at the forefront of attention, especially

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from the perspective of determining the functional level of the sexual organs of the male. The use of 'strict' morphological analysis and especially the evaluation of changes in the surface structures of sperm during short-term survival tests permits a more precise diagnosis to be made of disorders in the functions of sexual organs caused both by them directly becoming diseased or under the influence of disorders in the male's internal environment.

MATERIAL AND METHODS

The examination incorporated 38 ejaculates from dogs of different breeds aged 2 to 6 years old, obtained when they are examined in veterinary surgeries.

Collection of ejaculates and methods of evaluation:

Semen was obtained by manual manipulation in glass collectors warmed to 37°C.

- The volume of ejaculate was measured in calibrated containers to the nearest 0.1 ml.
- The motility of sperm and the percentage of motile sperm was determined by microscopic examination of the native ejaculate and by comparing the number of motile and non-motile sperm. The objective determination of the number of motile sperm was carried out by evaluating the trajectories of motile sperm recorded using the LUCIA system of image analysis.

  The recording of sperm movement was conducted on a slide covered with a cover slip magnified 240× and recorded at 10 sequences per 2.4 seconds. 10 recordings were made from each ejaculate. In devising this method, we used the method of spermiophotogrammetry we previously developed as a starting point (Veznik, 1992).

  Recording analysis: The total number of sperm on the surface described was determined. After projecting the recording of the movement, motile and non-motile sperm were counted. By determining and labelling the trajectories, those sperm from among the motile sperm with a progressive direct movement were marked out.
- The speed of sperm movement was routinely determined using the propulsivity method according to Baker *et al*. (1957).
- Fluorochrome primulin (0.1% solution), propidium iodide (500 nM solution) and Sperm Viability Kit (100 nM SYBR 14 and 12 μM propidium iodide fy Molecular Probes, Inc., Eugene, OR, USA) were used to determine live and dead sperm. The smears were assessed in the NIKON Labophot 2 fluorescent microscope magnified 1 000×.
- The integrity of the plasmatic membrane was determined using the Sperm Viability Kit supravital staining and a morphological resistance test during a short-term survival test and staining using the method according to Hancock or Farelly.
- The hypoosmotic test (HOS-test) according to Jeyendran *et al.* (1984, 1992) was used to determine the membrane integrity of the plasmatic membrane, the membrane on the tail and the consequent morphological changes in the tail for documenting live and dead sperm. The semen was incubated for 30 min in a solution of sodium citrate with 1.35% fructose at 150 mosmoles. The results of the HOS test were placed into four categories: negative eosin + curled tail, negative eosin + straight tail, positive eosin + curled tail and positive eosin + straight tail.
- The survival tests were carried out in laboratory temperature conditions (22°C), the ejaculate diluted to a concentration of 100 000 sp/mm^3, a physiological solution buffered with a phosphate buffer at a pH of 7.2 was used as a diluting medium. The interval of laboratory analysis was after 120 min, or 240 and 360 minutes.
- The short-term survival test. Apart from functional indicators, in which the initial value (IV) and the values after 120 min (2V) were found, changes in the surface structures of the sperm were also determined at the start-time and after 120 min of the survival test (Veznik, 1970; Veznik and Svecova, 1992).
- The acrosome immunofluorescence test for evaluating the integrity of the acrosome using the monoclonal antibody Ds-1 (IgG) against intraacrosomal proteins in dog sperm (set according to Geussova *et al*. (1997) Acrosome IF-test, EXBIO Prague).
- Evaluating the binding activity of *Pisum sativum* agglutinin (FITC-PSA in a concentration of 0.01% in a physiological solution at a pH 6.8) in the area of the internal part of the outer leaf of the acrosome (Sirivaidyapong *et al*., 2000).
- Morphological examination based on the multiparametric recording system and determining the index of teratosperm (Menkveld and Kruger, 1995). The SASMO programme was used for evaluation (Veznik *et al*., 2000; 2001).
- The STAT Plus programme was used for processing data (Matouskova *et al*., 1992).

Table 1. Average values and SD of indicators of dog semen level (n = 38)

Category	Values at start time		Values after 120 min	
	mean	SD	mean	SD
Motile sperm (%)	74.04	11.46	55.0	21.9
Live sperm (%)	81.07	9.29	78.57	9.18
Velocity of moving sperm ((μm/s)	40.5	21.36	31.6	29.34
AIF (%)	89.9	5.4	86.6	7.2
Normal sperm (%)	57.5	12.8	47.3	14.9
Pathological sperm (%)	42.5	12.85	52.7	14.08
Surface changes on sperm (%)	31.1	13.9	41.5	15.07
Developmental changes in sperm (%)	9.6	2.64	9.36	2.64

AIF = immunofluorescent test of acrosome integrity

RESULTS

Comparison of the results of determining the motility of sperm by both methods i.e. using the microscopic count and evaluating the trajectories brought information about their suitability. The average value of sperm motility obtained through the microscopic assessment of 38 ejaculates was 74.04% (SD = 11.46), while by determining the trajectories an average of 73.76% (SD = 10.43) was attained. Both methods of evaluating motility were compared and showed a statistically highly significant linear regression (P = 0.01) with a correlation coefficient of 0.7857.

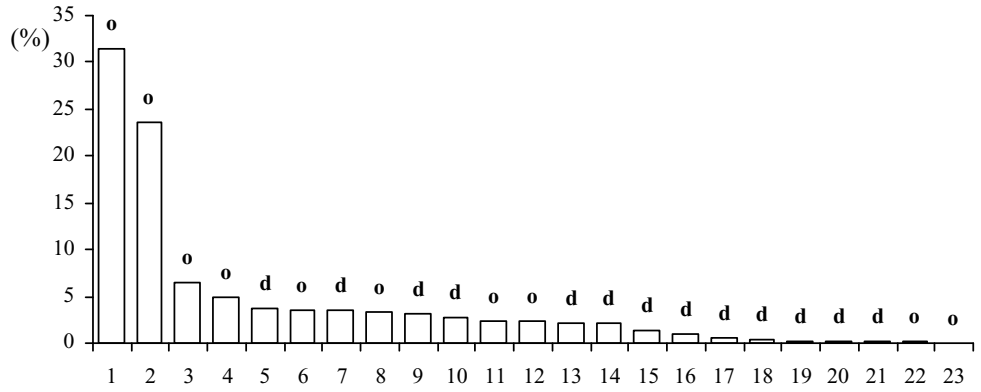
The determination of live and dead sperm using individual supravital staining methods were set against one another and a correspondence between results was found, indicating the usability of all methods and their interchangeability. Comparison of the values of live sperm with the supravital staining methods used showed correspondence and no statistically significant differences were found using the Scheffe method.

The average values of the spermatoanalytical criteria of semen obtained by examination at the start-time and over the 120 min short-term survival test, indicating the qualitative variety of the complex, especially from the high decisive deviations in motility indicators, speed of sperm movement and the high number of pathologically changed sperm, above all surface changes (Table 1).

Changes in the values of individual criteria during the 120 min survival test are a sign of the sensitivity of the indicator and may be ascribed diagnostic weight. The growth of changes on the surface structures of sperm made up 33.4%, which is the largest difference in comparison with the initial values of individual criteria. The fall in values in individual indicators of ejaculates attained in order of importance 27.9% in the speed of sperm movement, motility fell by 27.7%, normosperm fell by 17.7%, the indicators of membrane integrity fell by 3.5% in both the plasmatic membrane and in the area of the acrosome.

The morphological evaluation of sperm using the system of multiparametric recording of changes proved the contribution of the abnormalities obtained over developmental ones at a ratio of 76.15% to 23.85%. The teratosperm index was set for the sample monitored at 1.27, SD = 0.087. In the enumeration of the frequency of findings made on pathologically changed sperm, secondary changes represent 76% (Figure 1), twelfth position in the overview.

The overview of the statistical significance of the relationships between individual criteria for the qualitative evaluation of dog ejaculate (Table 2) indicates a high correlation coefficient and the statistical significance of the percentage of live and motile sperm to the percentage of abnormalities in the surface structures on the sperm. The integrity of the acrosome in relation to pathological sperm



1 swelling of acrosome
2 bending of tail
3 without acrosome
4 curling of tail
5 changed base of head
6 residue of cytoplasm
7 narrowed elongated head
8 coiling of tail
9 dag defect
10 thickened connecting part
11 abnormal position of tail insertion´
12 release of acrosome
13 microcefalia
14 macrocefalia
15 pear-shaped head
16 doubling of tail
17 persistent acroblast
18 ridged head – crista
19 condensation of acrosome
20 doubled head
21 partial absence of mitochondrial spiral
22 vaciolisation of acrosome
23 breaking of tail

o = changes obtained
d = developmental changes

23.85% findings of developmental chandes
76.15% findings of changes obtained

Figure 1. Frequency of average pathological findings (n = 38)

as a whole and to the surface changes in sperm showed a low correlation coefficient without statistical significance. Sperm with positive evidence of zymogen in the acrosomal area showed a swelling of the plasmatic membrane in the same part of the sperm (Figure 2).

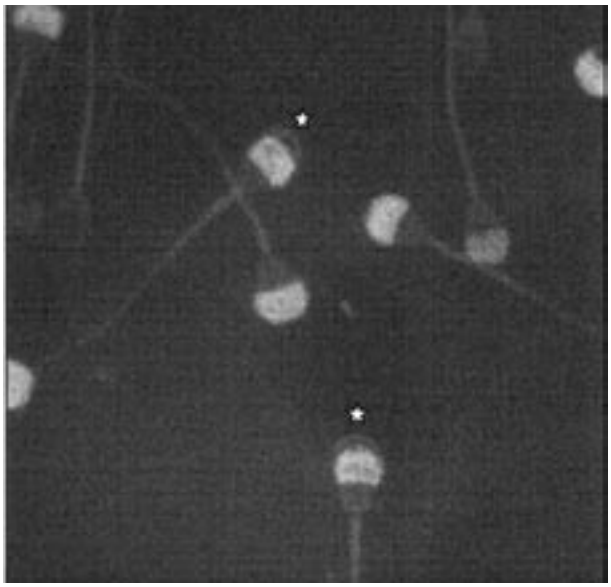


Figure 2. Immunofluorescent evidence of the integrity of the acrosome. The asterists indicate spermatozoa with plasma membrane swelling in the acrosomal region

Using the Acrosome IF-test (AIF) set, the integrity of the acrosome at the initial value of the dynamic test was proven on average in 89.9% of sperm (SD = 5.4). After 120 min this value fell to 86.6% (SD = 7.2). The differences in the averages of positively reacting sperm in the AIF-test and the numbers of live sperm were statistically highly significant (P = 0.01). The differences between the AIF averages and motile sperm were similarly statistically significant. The results show that the trends in the changes in the values of these criteria point to a correspondence, which is shown by the statistically highly significant correlation coefficients between the motility of sperm, the number of live sperm and the positivity of the AIF-test (Table 2). The acrosomal integrity was also checked using the lectine *Pisum sativum*. The results obtained were compared with the AIF-test and a statistically highly significant correspondence (P = 0.01) was shown in the correlation coefficient of 0.9370. While the methods of identifying the acrosomal integrity show a morphological persistence on the organelle level, the structural changes of the surface membrane are directly linked to the functional level of sperm and their motility.

Evaluating the integrity of the plasmatic membrane of the sperm using the effect of hypoosmotic

Table 2. Statistical significance of relationships between individual criteria of qualitative evaluation of canine ejaculates

Category	Motile sperm (%)	Live sperm (%)	AIF positive sperm (%)	Pathology of sperm in total (%)	Surface changes of sperm (%)
Motile sperm		xxx	xxx	xxx	xxx
%		0.8090	0.6609	–0.6309	–0.6988
Live sperm	xxx		xxx	xxx	xxx
%	0.8090		0.7062	–0.7339	–0.8300
AIF positive sperm	xxx	xxx		0	0
%	0.6609	0.7062		–0.3728	0.3757
Pathology of sperm in total	xxx	xxx	0		xxx
%	–0.6309	–0.7339	–0.3728		0.5125
Surface changes of sperm	xxx	xxx	0	xxx	
%	–0.6988	–0.8300	0.3757	0.5125	

AIF = immunofluorescent test of acrosome integrity
xxx = highly statistically significant $P = 0.01$
0 = without statistical significance value of correlation coefficient

Table 3. Average values of categories of hypoosmotic sperm test (HOS) ($n = 20$)

HOS-test	Eosin positive		Total of curled tails	Eosin negative curled tail	Live sperm	Motile sperm
	curled tail	straight tail				
	A	B	C	D	E	F
Mean	21.9%	9.29%	90.7%	72.6%	79.9%	72.9%
SD	18.36	4.66	4.66	12.27	8.75	16.58

C : E – 0.5875, $P = 0.05$; C : F – 0.6050, $P = 0.05$; D : E – 0.7820, $P = 0.01$; D : F – 0.5344, $P = 0.05$

Table 4. Monitoring of survival rate and sperm motile over a 360-minute perion ($n = 8$)

Movement of sperm	VH	2H	4H	6H
Mean	72.5%	60.0%	45.8%	35.0%
SD	12.16	13.84	18.12	22.73
		⊢———	0.8827, $P = 0.05$	———⊣
Live sperm	VH	2H	4H	6H
Mean	78.2%	76.6%	72.9%	70.9%
SD	9.75	10.01	9.85	9.91
		⊢———	0.8397 without statistical signifikance	———⊣

VH = values at start time; 2H = values after 120 min; 4H = values after 240 min; 6H = values after 360 min

influence of the environment showed the correspondence of the category of eosin in negative sperm with a curled tail to the percentage of live sperm with a highly significant correlation coefficient 0.7820 ($P = 0.01$) and a correlation coefficient statistically significant for the percentage of motile sperm (0.5344, $P = 0.05$). The category of eosin in positive sperm with a straight tail and a coiled tail creates a significant prediction value supplementing curled tails in the sum total, which is statistically significant in correlation with both the percentage of live sperm and the percentage of motile sperm (0.5875, $P = 0.05$ and 0.6050, $P = 0.05$). The contribution of eosin-positive sperm with a straight tail and the sum of sperm with a pathologically coiled tail according to morphological analysis is entirely in keeping with the number of dead sperm (20.5% to 23.3%) (Table 3).

The predictive importance of the examination using the 120 min survival test is evident from the correspondence of the fall in motility of sperm during the 120 min test with their survival rate and motility after 6 hours. This correspondence is statistically significant (0.8827, $P = 0.05$) (Table 4).

DISCUSSION

In this study we have focused on the assessment of the diagnostic level of laboratory analyses permitting routine use. In connection with the morphological criteria, attention was devoted to the integrity of the plasmatic membrane and the acrosomal complex (Geussova *et al*., 1997; Pena *et al*., 1999; Guerin *et al*., 1999; Kawakami *et al*., 2000; Sirivaidyapong *et al*., 2000). Our results showed a relatively important resistance of values pointing to the integrity of the plasmatic membrane and the integrity of the acrosome. On the other hand, a striking increase was shown in changes of the surface structures, especially the plasmatic membranes in the short-term sperm survival test. These changes were represented above all by a growth of changes typical of changes in the permeability of the plasmatic membrane in the area of the acrosome.

The assessment of the integrity of the acrosome, whether immunofluorescently using antibodies against intraacrosomal proteins or by connecting PSA lectin, also shows the resistance of this organelle component of sperm in contrast with the membrane covering and functional cell indicators. The positivity of both reactions, both in the initial value and after 120 min, correspondingly shows a fall in intact acrosomes, but in strikingly lower values than are shown by functional shortcomings of the sperm. The quantification of the values of the averages of these indicators shows statistically significant differences, which suggests the higher resistance of organelle membranes. Ashworth *et al.* (1995) warn that the connection of PSA lectin to the acrosomal matrix of sperm increases the number of dead cells. Pena *et al.* (1999) discuss this view and ascribe the toxicity for sperm to the concentration of lectin used. In our work we conducted the connection of PSA lectin with the acrosomal area both supravitally and post-fixationally. The results of the connection in both cases corresponded after 15 min incubation both in number and the character of the reaction. During supravital incubation we showed the significant inhibition of the motility of sperm as early as at 5 minutes.

The integrity of the plasmatic membrane as an important factor for the normal metabolism and function of sperm has been monitored by many authors in different animal species using a reduced osmolarity test. Jeyendran *et al*. (1992) found in dogs that the changes provoked by the hypoosmotic environment could be used for predicting sperm quality. Our findings also show the usability of this test for interpreting the membrane resistance of sperm. The findings showed the statistical significance of comparing the result of the HOS-test with the categories of live and dead sperm and secondary changes on the surface structures of sperm.

The short-term survival test, which rests on the comparison of values obtained at the start-time of the test and after 120 min has been used by us since 1970. In bulls this test showed a high diagnostic worth when semen cannot be frozen because of a high increase of structural surface changes in sperm. In the current study we were able to show the importance of this test both in criteria assessing the functional level of sperm and in changes in the morphological structure.

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