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Original article

Canine post-thaw sperm quality can be predicted by using CASA, and classification and regression tree (CART)-analysis

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Abstract

In individual dogs, despite good quality of raw sperm, some parameters are significantly changed after thawing, which cannot be predicted. We therefore investigated whether motility parameters objectively obtained by CASA, membrane integrity (MI), cell morphology or a combination are suitable to improve the prediction of bad post-thaw quality. For this purpose 250 sperm analysis protocols from 141 healthy stud dogs, all patients introduced for sperm cryopreservation, were evaluated and a Classification and Regression Tree (CART) -analysis performed. The sperm was routinely collected, analysed, and frozen by using a modified Uppsala system. After thawing, data were routinely examined by using CASA, fluorescent microscopy for membrane integrity (MI) and Hancock's fixation for evaluation of cell morphology. Samples were sorted by post-thaw progressive motility (P) in good (P > / = 50%, n=135) and bad freezers (P<50%, n=115). Among bad freezers, 73.9% showed in addition post-thaw total morphological abberations of >40% and/or MI <50%.

Bad freezers were significantly older than good freezers ($p<0.05$). Progressive motility (P), velocity curvilinear (VCL), mean coefficient (STR), and linear coefficient (LIN) were potential predictors for post-thaw sperm quality since specifity was best (85.8%) and sensitivity (75.4 %) and accuracy (80.4 %) good. For these objectively measured raw sperm parameters, cut-off values were calculated allowing prediction of bad post-thaw results with high accuracy: P = 83.1 % VCL = 161.3 μm/sec, STR = 0.83 %, and LIN = 0.48 %. Raw sperm samples with values below these cut off values will have below average post-thaw quality with a probability of 85.8%. We conclude that VCL, P, STR and LIN are potential predictors of the outcome of sperm cryopreservation, when combined.

Key words: dogs, spermatozoa, crypreservation, quality prediction, CART-analysis

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Introduction

Cryopreservation of dog spermatozoa is a routine procedure and the dog belongs to a species with relatively high resistance to cold and osmotic stress. This is due to the composition of canine spermatozoa membranes with high cholesterol: phospholipid ratio, similar to humans (Drobnis et al. 1993). Nevertheless, the quality of thawed sperm will be highly variable among dogs and ejaculates (Linde-Forsberg 1991, Linde-Forsberg et al. 1999). This variation depends, among others, on pre-freeze sperm characteristics, the extender, as well as the freezing and thawing protocol (Ström et al. 1997, Pena and Linde-Forsberg 2000, Nizanski et al. 2001, Schäfer-Somi et al. 2006, Bencharif et al. 2010, Rodenas et al. 2014, Brito et al. 2017, Sanchez-Galabuig et al. 2017). Furthermore, in individual dogs, despite good quality of raw sperm, motility, morphology and acrosomes were significantly changed after thawing (Nöthling et al 1997, Eilts 2005, Nöthling and Shuttleworth 2005).

This "dog effect" could not be explained so far. In the canine ejaculate, the existence of spermatozoa subpopulations with different characteristics has been proven, some subpopulations showing differing DNA integrity (Nunez-Martinez et al. 2007), others increased percentages of capacitation like changes (Ortega-Ferrusola et al. 2017) and some outstanding motility and velocity after thawing, with high resistance to capacitating conditions and osmotic stress (Nunez-Martinez et al. 2007, Pena et al. 2012). Furthermore the composition of the seminal plasma must be considered. Microvesicles, like prostasomes, were found to affect the sperm motility during cold and osmotic stress which is believed to be related to their zinc binding ability (Mogielnicka-Brzozowska et al. 2015, Zelli et al. 2017). In a recent study it could be demonstrated that native seminal plasma cholesterol concentration was lower in dogs with poor sperm quality after freezing-thawing in comparison to dogs with acceptable post-thaw quality (Schäfer-Somi and Palme 2016). Our ongoing studies investigate the presence and function of ABC transporters for the cholesterol transport during spermatozoa membranes in fresh and frozen-thawed sperm (Palme et al. 2014, Schäfer-Somi and Palme 2016).

However, in most laboratories, these examinations are not part of the routine diagnostics. It has been stated in a former study (Nöthling et al. 1997), that morphology of spermatozoa and percentage of progressively motile sperm in raw sperm has little value in predicting the progressive motility after thawing in frozen dog sperm. However, in the cited study, motility was evaluated subjectively and not by CASA; we therefore aimed to investigate, whether improved, objective raw sperm analysis could help to identify the predictive variables and whether cut-off values can be calculated by using Classification and Regression Tree (CART) -analysis. CART-analysis is a non-parametric regression method that by using Receiver Operating Characteristic (ROC) curves associated with the classification tree-based and logistic regression modeling allows to calculate cut-off values for chosen variables (Strobl et al. 2009); in our case for potential predictors of decreased post-thaw quality with P <50%, morphological abnormalities >40% and/or MI <50% (Schäfer-Somi and Palme 2016). Prediction of P alone does not allow prediction of the fertilizing potential of frozen thawed sperm; however, it is one of the most important parameters related to fertility. The total number of progressively motile sperm was found to be related to success rates after insemination (Nöthling et al. 1997). In a previous study we used P, morphological abberations and membrane integrity for classification of sperm donors into good and bad freezers according to post-thaw sperm motility (Schäfer-Somi and Palme 2016). Several investigations have been performed to identify sperm samples prone to bad post-thaw quality and some methods provided acceptable results such as computer-assisted analysis of sperm morphometry (Nunez-Martinez et al. 2007) or volume measurement of sperm heads (Petrunkina et al. 2004). However, these methods require special equipment. The aim of the present study was to retrospectively evaluate a large pool of sperm samples and routinely used CASA parameters including membrane integrity. By using regression ROC analysis, cut-off values should be calculated and by CART–analysis the best combination of parameters for prediction of bad post-thaw quality evaluated; we aimed to calculate a posttest probability in which the decision whether to freeze a sample or not will be based.

Materials and Methods

Animals

All 250 sperm samples were collected from 141 healthy stud dogs, aged 76±2 months (x±SEM), with a range of 15-165 months, and presented at the platform for artificial insemination and embryo transfer, Vetmeduni Vienna (A) for routine sperm freezing. Most were stud dogs that already had produced litters, however in some dogs, fertility was not proven. The dogs belonged to 55 different breeds: American Staffordshire Terrier (n=9), Beagles (n=9) and Labrador Retrievers (7) were most frequently presented, all other breeds were represented by 1-5 different stud dogs (American Akita n=1, American Staffordshire terrier n=9, Appenzell

Mountain Dog n=2, Australian Labradoodle n=1, Australian Shepherd Dog n=5, Austrian Black and Tan Hound n=1, Beagle n=9, Belgian Herding Dog n=1, Berger Blanc Suisse n=2, Berger de Beauce n=3, Black Russian Terrier n=1, Bobtail n=5, Border Collie n=3, Boston Terrier n=2, Boxer n=3, Brasilian Terrier n=2, Bullmastiff n=3, Bullterrier n=3, Chesapeake Bay Retriever n=1, Cocker Spaniel n=1, Dachshund n=5, Dobermann n=5, English Setter n=4, English Staffordshire Terrier n=3, English Toy Terrier n=1, Fila Brasileiro n=1, Flat coated Retriever n=1, French Bulldog n=5, German shepherd n=3, German Shorthaired Pointer n=1, Giant Schnauzer n=1, Golden Retriever n=4, Great Dane n=2, Greater Swiss Mountain Dog n=1, Groenendael n=1, Hollandse Herderhond n=1, Irish Setter n=3, Irish Wolfhound n=2, Jack Russel Terrier n=4, Labradoodle n=1, Labrador Retriever n=7, Malinois n=2, Mastiff n=3, Miniature Schnauzer n=1, New Foundland Dog n=3, Nova Scotia Duck Tolling Retriever n=2, Pudel n=1, Rhodesian Ridgeback n=3, Saluki n=1, Sheltie n=2, Siberian Husky n=3, Skye Terrier n=2, Small Münsterländer n=1, Terrier Brasileiro n=2, Tervueren n=1, Tibet Spaniel n=1). Each dog gave on average 1.8 ejaculates with a range of 1 to 6.

Sperm collection and evaluation

All sperm samples (n=250) were collected by digital manipulation, after teasing with either a bitch in oestrus or a swab with secretions of a bitch in oestrus. Most dogs were used to the procedure of sperm collection, some not; therefore, the collection was always done in a separate, quiet room by an experienced veterinarian.

The sperm-rich (SRF) fraction was collected separately and used for further quality assessment and sperm freezing.

Sperm freezing and thawing

A modified Uppsala system was used for sperm freezing (Schäfer-Somi and Palme 2016). Briefly, the sperm-rich fraction of each sample was diluted 1: 1 with a TRIS, citric acid, fructose-based extender containing 20% of egg yolk and 3% glycerol (Extender I, Peter and Linde-Forsberg 2003). The diluted sample was equilibrated for 1h at 5°C, then diluted 1: 1 (v: v) with the freezing extender containing 7% instead of 3% glycerol and in addition 1% Equex STM paste (Extender II, Peter and Linde-Forsberg 2003), filled into 0.5 ml straws and frozen as described by (Schäfer-Somi et al. 2006) in a computer-controlled rate freezer (IceCube 14 M;Sylab, Purkersdorf, Austria).

Sperm analysis

Since sperm samples from patient dogs introduced for sperm preservation were used and no experimental animals, all sperm analyses followed a routine scheme. Further sperm characteristics like seminal plasma composition or sperm subpopulations were not investigated during the routine procedure.

In case of urine admixture or if sperm concentration was < / = 100 million sperm/ml or if the second fraction could not be separated accurately (n= 104), samples were centrifuged prior to freezing at 750 g for 5 min (Rijsselaere et al. 2002). The sperm pellet was processed as the undiluted sample. All frozen sperm samples were thawed in a water bath at 70°C for 8 sec.

In all samples, progressive motility (P) as well as membrane integrity (MI) were analysed by standardized computer-assisted sperm analysis (CASA, Sperm Vision®, Minitube, Tiefenbach, Germany) after dilution to 100 x10^6 spermatozoa /ml with a TRIS-citric acid-fructose buffer, as described in detail by (Schäfer-Somi et al. 2006). The membrane integrity was assessed by using SYBR-14/PI double staining (Schäfer-Somi and Aurich 2007). Briefly, 100 μl of sperm were placed in a vial with 2 μl of SYBR-14/PI and incubated for 10 min at room temperature in darkness. One droplet was placed on a glass slide, covered with a glass coverslip and evaluated via fluorescence microscopy at magnification x 400 (Olympus AX70, Olympus Optical Co., Ltd., Japan; U-MWB filter block, BP420-480 excitation filter, BA515 suppressor filter, dichromatic mirror: DM500). Evaluation was performed at magnification of 400× using the fluorescence microscope that is accessory part of SpermVision, with phase contrast objectives (Olympus AX70, Olympus Optical Co., Ltd., Japan; U-MWB filter block, BP420-480 excitation filter, BA515 suppressor filter, dichromatic mirror: DM500).

The heads of viable spermatozoa show bright green colour, damaged membranes are stained red, both colours are recognized automatically by a video camera. Each sample was evaluated once, but at least 500 sperms were evaluated per sample and the mean calculated by the software provided by SpermVision. If staining was non-satisfactory, staining and measurement were repeated. Results are given as percentages of spermatozoa with intact (green) membranes and could be controlled by counting the green heads per field as visible on

the screen. Spermatozoa concentration in raw sperm was determined with the Nucleocounter SP-100 (ChemoMetec A/S, Allerod, Denmark) according to the manufacturer's instructions. The percentage of spermatozoa with normal morphology inclusive

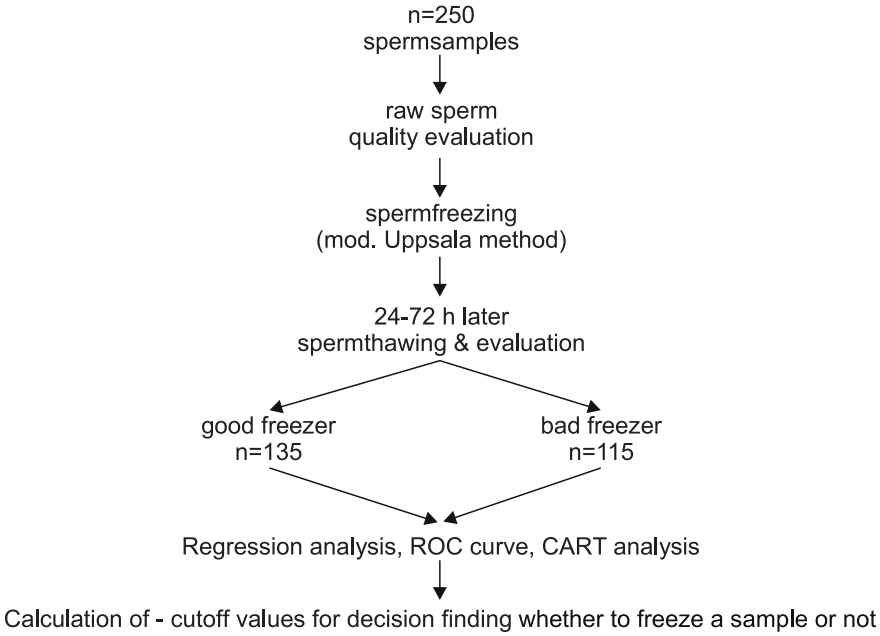

Fig. 1. Experimental scheme.
All samples were examined and processed equally; allocation to the group of good and bad freezers was done according to post-thaw sperm quality; bad freezers showed P <50%, viability <50% and/or >40% morphological abberations.

acrosome integrity was determined after fixation in Hancock's solution (Schäfer and Holzmann 2000) under a phase contrast microscope (x 1000; oil immersion), at least 200 cells were examined per sample.

Briefly 250 raw sperm samples were analysed by using CASA, fluorescent microscopy and Hancock's solution for assessment of morphological aberration. All samples were diluted with the cooling medium and equilibrated for 1h. Samples were then diluted with freezing medium and immediately filled in 0.5 ml straws, and machine-frozen. After thawing, all samples were analysed again and grouped into bad and good freezer samples according to post-thaw quality: progressive motility (<50% or > / = 50%), percentage of morphological abberations (< 40% or >/=40%) and/or membrane integrity (<50% or > / = 50%). Data pools from both groups underwent statistical calculation (Fig. 1).

Statistical analyses

IBM SPSS statistics version 24 (SPSS Ltd, Hong Kong) was used. Normal distribution of data was examined by using the Kolmogorov-Smirnov Test. Comparison between groups of good and bad freezers was done by using Student's t-test. In case of not normally distributed data, the Mann-Whitney U-Test was used. Correlation between selected parameters was calculated using Pearson's correlation. A regression analysis was calculated for sperm parameters that differed significantly between good and bad freezers before sperm freezing: velocity curvilinear (VCL), progressive motility (P), linearity (LIN), straightness (STR) and morphological abberations of sperm cells. A Receiver Operating Characteristic (ROC)-curve was generated and the coordinates examined for cut-off values providing the best accuracy, specifity and sensitivity for prediction of good and bad freezers. To identify the impact of different parameters and their combination, the CART-analysis was performed. Several models were computed providing different accuracy and sensitivity or specificity. All decision trees were pruned to provide an efficient decision algorithm. All data are given as average values and standard error (x ± SEM). A p value of $p<0.05$ was considered statistically significant.

Results

Effect of age and sperm concentration on motility, membrane integrity, and morphology

In raw sperm (all 250 samples), the age of dogs correlated negatively with VAP ($p<0.05$), DCL ($p<0.05$), VCL ($p<0.01$) and membrane integrity (MI, $p<0.01$), and positively with morphology ($p<0.01$); in thawed sperm similarly and in addition negatively with progressive motility (P, $p<0.01$).

Sperm concentration of raw sperm samples correlated negatively with P ($p<0.05$), after thawing with P ($p<0.01$), VAP ($p<0.05$), VCL ($p<0.01$) and MI ($p<0.01$).

Table 1. Raw sperm parameters.

	P % X ± SEM (n)	VCL μm/sec X ± SEM (n)	STR % X ± SEM (n)	LIN % X ± SEM (n)	Membrane integrity % X ± SEM (n)	Morphological abnormalities % X ± SEM (n)	Acrosome % X ± SEM (n)	Neck % X ± SEM (n)
Bad freezer native	69.7[a] ± 1.6 (108)	140.9[a] ± 3.3 (108)	0.82[a] ± 0.0 (108)	0.47[a] ± 0.0 (108)	82.2[a] ± 1.2 (108)	49.7[a] ± 2.0 (103)	5.7[a]± 0.7 (115)	16.6[a] ± 1.5 (115)
Good freezer native	83.5[b] ± 0.9 (120)	170.6[b] ± 2.6 (112)	0.85[b] ± 0.0 (112)	0.52[b] ± 0.0 (112)	90.7[b] ± 0.4 (115)	29.8[b] ± 1.4 (124)	4.4[a] ± 0.3 (135)	5.7[b] ± 0.5 (135)

In Table 1 raw sperm quality of good and bad freezers is shown. Differences in numbers are due to a loss of data during retrospective evaluation.
P = progressive motility, VCL = velocity curvilinear, STR = straightness, LIN = linearity, Morphological abnormalities=percentage of morphologically abnormal sperm, acrosome= acrosome damages, neck = neck abnormalities.
[a,b] Parameters with different indices in one row differ significantly, $p<0.01$.

Table 2. Sperm quality after thawing.

	P % X ± SEM (n)	VCL μm/sec X ± SEM (n)	STR % X ± SEM (n)	LIN % X ± SEM (n)	Membrane integrity % X ± SEM (n)	Morphological abnormalities % X ± SEM (n)	Acrosomes % X ± SEM (n)	Neck % X ± SEM (n)
Bad freezer thawed	28.2[a] ± 1.3 (115)	105.0[a] ± 2.8 (108)	0.81[a] ± 0.0 (108)	0.46[a] ± 0.0 (108)	47.8[a] ± 1.5 (106)	50.7[a] ± 2.1 (106)	17.2[a] ± 1.3 (115)	11.5[a] ± 1.1 (115)
Good freezer thawed	63.6[b] ± 0.7 (113)	124.5[b] ± 2.8 (111)	0.86[b] ± 0.0 (111)	0.54[b] ± 0.0 (111)	68.3[b] ± 1.0 (108)	35.4[b] ± 1.1 (116)	13.5[a] ± 0.7 (135)	4.8[b] ± 0.5 (135)

Most relevant sperm parameters after thawing. Differences in numbers are due to a loss of data during retrospective evaluation.
P= progressive motility, VCL= velocity curvilinear, STR= straightness, LIN= linearity, Morphological abnormalities = percentage of morphologically abnormal sperm, acrosome= acrosome damages, neck= neck abnormalities.
[a,b] Parameters with different indices in one row differ significantly, $p<0.01$.

Comparison between good and bad freezers

Sperm concentration and pH in raw sperm did not differ between good and bad freezers (446.6±27.7 and 382.2±73.5 x 10^6/ml, $p>0.05$; 6.0±0.02 and 6.1±0.02, $p>0.05$, respectively). The volume of the main secretion was significantly higher in bad freezers (2.8±0.4 vs 1.8±0.1, $p<0.05$). The average age of bad freezers was 80.9±3.6 m (n=115), with 47.8% of dogs being old according to Tesi et al (2018; > 84 m). The average age of of good freezers was 72.0 ± 2.9 m (n=135), with 36.2% of dogs being old; the difference was significant when tested one-sided ($p<0.05$).

In Table 1, selected raw sperm parameters of both groups are shown. All parameters were in the range of normal except the total percentage of morphological abnormalities in bad freezers, which averaged 49.7%.

In raw sperm, P, VCL, STR, LIN and MI were significantly better in good freezers (all $p<0.01$), whereas the total percentage of morphological abnormalities and neck abnormalities was significantly lower ($p<0.01$) than in bad freezers. The percentage of acrosome damages in raw sperm did not differ between groups ($p>0.05$).

Thawing significantly decreased P, DAP, DCL, DSL, VAP, VCL, VSL, ALH and MI, and increased the percentage of morphological abnormalities and acrosome damages in both groups. All parameters were significantly better in good than in bad freezers, except the percentage of acrosome damages (Table 2).

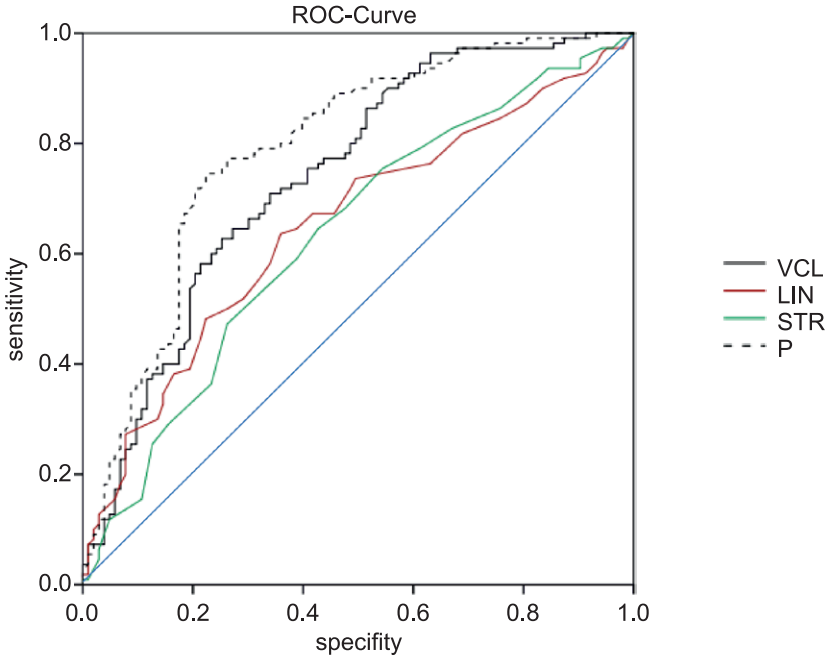

Fig. 2. ROC curve for selected parameters.
Progressive motility (P), velocity curvilinear (VCL), mean coefficient (STR), and linear coefficient (LIN).
To estimate a post-test probability in which the decision whether to freeze a sample or not will be based, a ROC curve was created with selected parameters; this curve considered sensitivity (the probability that a sperm sample is correctly classified as good freezer sample) and specifity (the probability that a sperm sample is correctly classified as bad freezer sample). By using the coordinates, cut-off values were calculated providing optimal sensitivity and specifity. The area under the curve was 0.741 for VCL, 0.651 for LIN, 0.633 for STR and 0.791 for P.

Table 3. Results of different CART-analysis models for the prediction of bad post thaw quality.

Model	Normalized importance (%)					Performance (%)		
	P	VCL	LIN	STR	total	sens	spec	acc
1	94.6	100	26.4	29.2	88.9	89.1	75.2	82.6
2	100	79.3	40.9	34.5	-	75.4	85.8	80.4
3	100	76.2	-	-	-	77.0	74.3	75.7
4	100	-	-	-	85,2	73.2	87.5	79.9
5	82.5	52.7	34.7	24.1	100	90.1	72.5	83.8

acc = accuracy, LIN = linearity, P = progressive motility, sens = sensitivity, spec = specifity, STR = straightness, total = total percentage of morphologically abnormal sperm, VCL = velocity curvilinear.
For each parameter which was added to the model, the normalized importance (relative to the most important parameter) within the classification process is shown. For each model the overall-performance given as sensitivity, specificity and accuracy is shown.

Regression analyses and ROC curve

Regression analyses and ROC curve (Fig. 2) with selected data from raw sperm revealed the following cut off values for the prediction of bad sperm quality after thawing: P = 83.1 % VCL = 161.31 μm/sec, STR = 0.835 %, LIN = 0.485 %.

CART-analysis

The most complex decision tree taking VCL as first classifier and considering all parameters (model 1, Table 3) resulted in the highest accuracy of 82.6% with a sensitivity (percentage of correctly classified good freezers after thawing) of 89.1% and a specificity (percentage of correctly classified bad freezers after thawing) of 75.2%. In this model, the whole information inclusive morphology was taken into account. Specifity was even better, with P as first classifier and when the total percentage of morphologically abnormal sperm was not included (model 2, Table 3); but this involved a loss of sensitivity and accuracy. The highest specifity was obtained with P and total percentage of morphologically abnormal sperm (model 4, Table 3). However, this model showed lowest sensitivity and accuracy.

Discussion

A representative number of canine thawed sperm samples from donors with good native sperm quality was analysed for post-thaw quality. Samples with bad post-thaw quality (P<50, morphologically abnormal sperm > 40% and/or MI <50%) were retrospectively compared to those from good freezers and a ROC curve analysis for the prediction of bad post-thaw quality performed with the native sperm parameters. This has not been done before with such homogenous, comparable and large groups. Even though data from 55 breeds were available, the number of animals per breed was not sufficient for statistical evaluation of a breed effect. However, only 2 dogs belonged to a breed that was described to tend to bad post-thaw sperm quality, namely the Irish Wolfhound (Dahlbom et al. 1995). Breed differences mainly concern the sperm concentrations due to different size of the testicles (Johnston et al. 2001), even though in some breeds on average lower sperm motility and more sperm abnormalities were measured (Stockner and Bardwick 1991). It has not been investigated whether this is a genetic problem such as the result of inbreeding; however, bad freezers may occur in any breed (Nöthling et al. 1997, Eilts 2005, Nöthling and Shuttleworth 2005).

All samples were collected, analyzed and frozen-thawed with the same methods. Sperm concentration post-thaw was in the range described to be without effect on post-thaw quality (Okano et al. 2004). Dogs were on average middle aged, but in the group of bad freezers, more dogs were "old" at the time of sperm collection; recently, Tesi et al. (2018) assigned dogs to the group of old animals when the age was > 84 months. This is important since in our and previous studies, the age was negatively correlated with the percentage of normal spermatozoa (Rijsselaere et al. 2007), which probably explains the high percentage of morphologically abnormal cells in raw sperm of bad freezers. In one study, age also correlated negatively (Hendrikse and Antonisse 1984) with sperm concentration in 2025 ejaculates, which was not the case in our study, but might become relevant when more and older dogs are evaluated. In the here-assessed population, a negative impact of age on the bad post-thaw sperm quality of some samples cannot be excluded.

Several authors assessed correlations between sperm concentration, and computer-calculated motility and P in raw sperm (Günzel-Apel et al. 1993, Iguer-Ouada and Verstegen 2001, Rijsselaere et al. 2003). In our study we assessed the same in raw and thawed sperm; however, this had no impact on post-thaw sperm quality.

The VCL values of bad freezers in native sperm were significantly lower than in samples of good freezers. The native VCL values of good freezers were in the range measured in fertile dogs, whereas those of bad freezers resembled VCL values measured in subfertile dogs (Rijsselaere et al. 2007); VCL thus seems to be a useful parameter to predict bad post-thaw quality, which was proven by regression analysis and CART-analysis. However, since sensitivity and specifity for the single parameter were not optimal, a CART-analysis was performed and a decision tree created, allowing to choose the best combination of parameters for best prediction.

With CART-analysis, several decision trees can be calculated which differ slightly in the relative importance of the factors (classifiers) included, but they have similar performance with an accuracy between 76% and 82%. To select the best model, the importance of sensitivity or specificity has to be considered by taking into account the costs of false positives (wrongly classified as good freezer) or false negatives (wrongly classified as bad freezer). The complex models provided best accuracy with good sensitivity and specifity, while the simplest model only considering P and morphological abnormalities provided best specifity. However, both accuracy and specifity are considered important in cases where the question is to freeze or not to freeze, to prevent expensive freezing with bad outcome.

We retrospectively evaluated data from dogs presented for routine sperm freezing. Therefore special analyses like measurement of sperm head swelling (Petrunkina et al. 2004) or functional assays like the hypoosmotic swelling test (Karger et al. 2016) were not performed, neither in native nor in thawed sperm samples. The predictive value of these assays is controversial. It is well known that individual differences concerning membrane and DNA resistancy towards osmotic and cold stress exist and are in part due to cell subpopulations with variable sensibility (Petrunkina et al. 2004, Nunez-Martinez et al. 2007, Pena et al. 2012). However, at present there is no test available providing reliable results for the prediction of post-thaw quality. The present results shall provide a possibility to quickly decide with the best accuracy whether a sperm sample should be cryopreserved or not.

Conclusions

It is concluded that, within the here evaluated dog population, age might have predisposed some dogs to be bad freezers. To correctly predict bad freezers, which assumes high specifity and accuracy, VCL, P, STR and LIN are most useful, when combined. Even though the cut-off values provided by ROC analysis will vary

between laboratories, the present study reveals that such validations can be helpful.

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