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Influence of Breed and Individual Variation in the Quality of Frozen Canine Semen

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Abstract

The present work aims to report the individual and racial difference found in the freezing of canine semen, as well as to analyze the correlation of the parameters of *in vitro* evaluation of semen before and after freezing. For this, 36 ejaculates were collected of 12 dogs of the Beagle, schnauzer, Doberman and Boxer breeds, by the digital manipulation method and frozen in Tris-Citrate medium containing 6% of glycerol. Semen was evaluated, before and after freezing, on sperm movement through vigor, motility and spermatic index, and on the integrity and viability of the spermatic membrane by hypoosmotic and supravital staining tests. Sperm longevity was estimated in the thawed semen through the thermoresistance test. Individual variation was observed on spermatic index in the thawed semen, but not in the fresh. The parameters of spermatic membrane integrity and viability presented individual variation in both. When the breed variable is considered was not observed significant differences on parameters in the fresh semen, but in the thawed semen significant variations in the integrity and viability of the sperm membrane were observed. Schnauzers had the lowest sperm longevity after thawing. As Doberman and Boxer breeds presented the best freezing results in the in vitro evaluation. Except for the supravital staining test, the parameters studied showed significant correlations between the data collected in the fresh semen and those observed in the thawed semen

Keywords: Canine semen; Individual and racial variation; *In vitro* evaluation

Introduction

The specialized breeding of dogs has, a long time, been taking on significant dimensions with increasingly technological demands. A Recent demographic research reveals the growing economic and social importance that pets have been occupying in our society, and therefore, by moving a large trade of animals, products and services. Specifically in Brazil, data published in the National Health Plan by the Brazilian Institute of Geography and Statistics (IBGE) indicate a current estimated population of 52.2 million of domiciled dogs [1]. The data show that, in Brazil, there are more dogs than children, since the National Household Sample Survey [2] indicates that, at the same time there were 44.9 million children up to 14 years old. In addition, the potential use of these animals as experimental models for endangered wild carnivore species has also driven the quest for technological knowledge in assisted reproduction [3-5].

Evolutionary analyzes based on the comparison of mitochondrial DNA suggest that the dog diverged from the wolf (Canis lupus) more than 15,000 years ago [6], although the establishment as a domestic dog is a result of a small number of individuals domesticated [7,8] and despite this, from an intense selection, especially in the last 300 years, it was observed the creation of more than 400 distinct breeds, being dog, the most morphologicaly variable between the domestic species [6,9]. Although initially canine breeds have been developed from a specific aptitude (hunting, guarding, grazing, etc.), they are currently being selected, mainly due to esthetic parameters, from extremely endogenous genetic selection [10]. Leroy et al. observed that the genetic variability of populations of several canine breeds presents genetic variability in marked decline, and in some breeds, endogamy compromises the future maintenance of the population, with a large proportion of individuals to present a coefficient of inbreeding greater than 6.25%, corresponding to the mating of animals with at least two grandparents in common [11].

Since the type of genetic selection used by the vast majority of dog breeders is solely for aesthetic parameters of the racial patterns, it is possible to maintain and disseminate potential diseases and hereditary deleterious traits, with many canine breeds dealing with genetically specific diseases inherited and directly correlated, long ago, with inbreeding in these breeds [12]. In a large 15-year study, Bellumori et al. observed that in a range of 24 different genetic disorders studied, about 10 including cardiomyopathy, dysplasias, cataract, and hypothyroidism were associated with purebred dogs, and only one (ruptured cruciate ligament) was more common in without-breed animals [13]. According to Urfer, the size of the litter is inversely proportional to the degree of inbreeding [14].

The arbitrary selection of reproductive traits has been possible in part due to the fact that, in most dog breeds, a natural breeding system or artificial insemination with fresh or cooled semen is used, where the males are poorly demanded [15]. However, with the increasing applicability in the use of semen freezing, where there is more need for good semen qualitaty, dogs that had been considered as good reproducers started to present characteristics of low freezing and cryopreservation of the semen.

The freezeability of canine semen is defined as the ability of spermatozoa to survive freezing [16,17] and are directly implicated in variations in the lipid composition of the sperm membrane and in the sensitivity of the sperm to the toxic effects of the cryoprotectant [18-20]. Parameters such as sperm motility, membrane integrity, and morphological normality have been correlated with fertility in several

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studies [21-24], however, when the evaluation seeks to determine the individual freeze of the sample, low correlations have been observed between *in vitro* evaluation of semen before and after freezing [17,25]. Most of the works developed in canine species that evaluate the quality of semen before and after freezing, use semen of different animals grouped in pools, so that it is not possible to compare an individual quality of the semen before and after freezing [26-30].

The present work aimed to evaluate the racial influence on the freezing of canine semen, as well as to analyze the correlation of the parameters of *in vitro* evaluation of the semen before and after the freezing.

Materials and Methods

Twelve, clinically healthy, Beagle (n=3), Schnauzer (n=3), Boxer (n=3) and Doberman (n=3) adult male dogs were used. The animals used belonged to brasilian outstanding breeders, specialized in the commercialization and competitive participation in dog shows of national and international scope. The animals selected for the experimentation were certified reproducers and considered of high racial standard. All procedures used followed the protocol of ethical conduct established by the Ethics Committee for Animal Use of the Federal University of Viçosa (CEUA-UFV).

The semen was collected in a single aliquot containing the spermatic fraction by the digital manipulation method in graduated centrifuge tubes coupled to a plastic funnel, the set being heated before collection and the centrifuge tube kept inside a container containing water at 38°C. Three collections were carried out in each animal, totaling 36 collections.

Immediately after collection the semen was kept in a water bath at 38°C. An aliquot of 20 µL was evaluated for vigor (movement intensity classified from 0 to 5) and sperm motility (percentage of sperm motile classified from 0 to 100%), under a slide, previously heated in magnification of 100 and 400X under direct optical microscopy. The values obtained were used to compose the spermatic index (SI) using the formula: SI=[M+(V × 20)]/2, where M=sperm motility and V=sperm vigor (Morais et al.).

The integrity and viability of the spermatic membrane were evaluated by hyposmotic and supravital staining. The hyposmotic test is based on the rolling of the spermatozoon tail with whole membrane exposed to a hyposmotic medium, so a sample of 20 µL of semen was incubated at 38°C for half an hour in 0.5 mL of fructose and citrate solution sodium to 60 mosmol. Then 100 cells were observed under optical microscopy with a 400-fold increase. From the value obtained, the percentage of rolled tails in fresh semen, previously evaluated, was discounted. For the supravital staining test, 20 µL of semen were added to 40 µL of pre-heated eosin-nigrosine dye and evaluated under smear on slide and immediately air-dried, at 400X magnification. The stained spermatozoa are counted as unfeasible.

The semen was centrifuged at 300 g for ten minutes, the supernatant being discarded and the pellet resuspended in Tris-citrate base diluent medium containing 6% glycerol to give a final concentration of 100 × 10^6 spermatozoa/ml, from accounting in a hematimetric chamber.

The semen was then filled into 0.25 ml straws and then packed into pre-heated capped test tubes. The test tubes were placed into a glass vessel containing 650 mL of water at 38°C. The glass container in turn was hermetically sealed and submerged in 7 L of water and ice contained inside styrofoam box. The volumes of either, warm water and ice were pre-adjusted so that the resulting cooling curve reached 4°C over a period of 1 hour. After cooling the semen was kept in the styrofoam box for another 1 hour in equilibrium at 4°C before freezing. The freezing was carried out in a styrofoam box containing liquid nitrogen, by placing the straws at 10 cm from the surface of the nitrogen for 10 minutes.

After one week the straws were thawed by immersing in water at 38°C for 1 minute. Then the semen was transferred to 1.5 ml eppendorf flasks kept incubated in a water bath at 38°C for analysis of vigor, motility and membrane integrity as described for fresh semen. Sperm longevity was estimated in the thawed semen through the thermoresistance test. For this, the semen was incubated in a water bath at 38°C for 2 hours and during this period its vigor and motility were evaluated at 15 minute intervals. The vigor and motility data obtained during the thermoresistance test are presented as spermatic index.

The data evaluated were described for the average and respective standard deviation. For the comparison of means, the mean confidence interval with 5% margin of error was calculated through the statistical function of the Excel program Windows XP. Correlation data were calculated based on Pearson's correlation.

Results and Discussion

Although all canine breeds have common ancestors, during their development and even today, extremely endogenous genetic selections are performed in order to fix desirable phenotypic or behavioral characteristics [6,10,11]. Thus, the genetic proximity and the degree of inbreeding observed among dogs of the same breed may influence the *in vitro* quality of the thawed semen through the fixation of individual characteristics associated to increase or decrease of the survival of the spermatozoa to the freezing process. The genetics of a breed is generally determined by the genetics of its actual founders, who are individuals belonging to the total population of founders but who have made the greatest contribution to the formation of the present population through a large number of descendants [11]. The dogs evaluated in the present work are exemplary highly representative of the morphological pattern of the respective breeds, being representatives of the genetics of their breeds since both they and their direct ancestors contributed to the formation of the current squad with a large number of progenies.

One of the major obstacles to the improvement of the semen cryopreservation technique in dogs is the difficulty in estimating semen fertility after thawing. *In vivo* tests, although decisive in determining semen fertility, require a large number of animals per evaluated treatment and are subject to variations other than specifically semen quality such as: individual female fertility, detection of optimal timing of insemination, the method of insemination, the dose and inseminating volume, among others [31]. Analyzes made *in vitro* in turn are quite practical and allow the simultaneous evaluation of a large number of treatments, however, to be conclusive, they need prior confirmation of their correlation with the fertility rate through *in vivo* tests.

Several studies have correlated the parameters of *in vitro* seminal evaluation with each other and with the fertility rates [21-24,32]. The sperm movement is an easy parameter to measure and at the same time, represents a good indicator of sperm function, since the movement is a manifestation of the structural and functional components of the sperm, highly correlated with the fertility rate, morphological normality and sperm membrane integrity [21-24]. On the other hand, changes in the integrity and viability of the spermatic membrane, evaluated through the hyposmotic test and supravital staining test, are ultimately

	Supavital Stanning Fresh semen	Hyposmotic Test Fresh semen	Spermatic index Fresh semen	Supavital Stanning Thawed semen	Hyposmotic Test Thawed semen	Spermatic Index Thawed semen (T^0)	Spermatic index Thawed semen (T^1)	Spermatic index Thawed semen (T^2)	Spermatic index Thawed semen (T^3)
Mean Beagle	6.78 ± 2.67^a	86.73 ± 7.24^a	91.11 ± 4.37^a	69.7 ± 8.84^a	14.60 ± 18.48^a	46.79 ± 17.41^a	33.57 ± 15.58^{ab}	23.93 ± 11.09^b	12.57 ± 11.62^b
Mean Schnauzer	6.50 ± 2.17^a	88.22 ± 9.70^a	90.83 ± 4.17^a	72.00 ± 13.76^{ab}	11.80 ± 18.89^a	42.86 ± 15.67^a	13.93 ± 16.14^a	6.14 ± 9.73^a	2.93 ± 7.17^a
Mean Doberman	12.39 ± 8.62^a	89.91 ± 5.94^a	86.11 ± 9.26^a	63.67 ± 4.89^{ab}	41.76 ± 8.26^b	50.83 ± 13.74^a	43.33 ± 6.87^b	28.75 ± 9.97^b	15.83 ± 18.30^b
Mean Boxer	10.78 ± 7.27^a	94.38 ± 1.03^a	93.89 ± 1.57^a	53.00 ± 14.80^b	23.89 ± 21.57^a	59.72 ± 10.17^a	38.06 ± 16.57^{ab}	30.50 ± 14.23^b	17.94 ± 16.53^b

Data presented in Mean+Standard Deviation; Different letters in the same column represent significantly different means ($p<0.05$)

Table 1: Mean of percentage of reactive cells in the hyposmotic test and percentage of cells stained in the supravital staining test, in fresh and thawed semen and sperm index in fresh semen, and in thawed semen at thawing time (T^0), and 15 (T^1), 30 (T^2) and 45 (T^3) minutes after, in Beagle, Schnauzer, Doberman and Boxer dogs.

	SI^F	SS^F	HO^F	SS^T	HO^D	SI^0	SI^1	SI^2	SI^3
SI^F	1	-0.68 (0.0)	0.619 (0.0)	-0.351 (0.049)	0.422 (0.02)	0.458 (0.008)	0.36 (0.043)	0.501 (0.004)	0.294 (0.102)
SS^F		1	-0.267 (0.23)	0.214 (0.339)	-0.464 (0.034)	-0.119 (0.598)	-0.192 (0.392)	-0.409 (0.059)	0.173 (0.441)
HO^F			1	-0.473 (0.006)	0.404 (0.027)	0.438 (0.012)	0.0489 (0.005)	0.416 (0.018)	0.327 (0.068)
SS^T				1	-0.473 (0.008)	-0.468 (0.007)	-0.617 (0.0)	-0.48 (0.005)	0.42 (0.017)
HO^D					1	0.305 (0.101)	0.684 (0.0)	0.53 (0.003)	0.459 (0.011)
SI^0						1	0.649 (0.0)	0.687 (0.0)	0.481 (0.005)
SI^1							1	0.837 (0.0)	0.661 (0.0)
SI^2								1	0.771 (0.0)
SI^3									1

Data presented as Pearson's Correlation, (significance level); SI^F -spermatic index in fresh semen; SI^0 -semen at thawing; and 15, 30 and 45 minutes after (SI^1, SI^2 and SI^3); SS^F -percentage of cells stained by supravital staining in fresh semen; SS^T - percentage of cells stained by supravital staining in thawed sêmen; HO^F -percentage of reactive cells to the hyposmotic test in fresh sêmen; HO^D - percentage of reactive cells to the hyposmotic test in thawed sêmen.

Table 2: Correlation between the values of sperm index, percentage of reactive cells to hyposmotic test and percentage of cells stained by supravital staining evaluated in fresh and thawed semen.

a reflection of structural damages to the membrane and metabolic alterations caused by spermatozoa cooling, by reducing their motility and their ability to bind to the ovum [33-35].

In the present work, a significant variation was observed between the individual and racial means of spermatic membrane integrity and viability and the spermatic index in the samples submitted to freezing (Table 1). In the evaluation of fresh semen, significant individual variation was observed only in the integrity and viability of the sperm membrane, and when comparing the data grouped in the studied breeds, no significant variation was observed. In the same way, Batista et al. observed statistically significant differences in vigor and motility only between samples of thawed semen, but not in fresh semen in Mastiff dogs [17].

Some authors attribute these individual variations in the quality of the thawed semen, the differences in lipid composition of the sperm membrane and the sensitivity of the sperm to the toxic effects of glycerol [18-20]. Thus, the ability of spermatozoa to survive the freezing process can be linked to individual characteristics, which are not fully elucidated by the *in vitro* evaluation of fresh semen.

The toxicity of glycerol seems to be associated with changes in the viscosity of the cytoplasm that possibly inhibit metabolic processes involving solutes diffusion [34]. Hammerstedt et al. point out that variations in the toxicity of glycerol can be attributed to different degrees of influence of this on the natural viscosity of the cytoplasm [18]. Moreover, the proportion of cholesterol and saturated fatty acid present in the spermatic membrane, determines a greater or lesser tendency to undergo conformational changes when exposed to low temperatures, which can lead to altered membrane permeability and decreased sperm motility [33,34,36]. However, some seminal features, such as the percentage of cells exhibiting morphological abnormalities, are highly correlated with low fertility rates in dogs [22,24].

Santos et al. describe the occurrence of high rates of morphological abnormalities in the semen of four individuals Schnauzer dogs resulting in loss of fertility, the authors attribute this to the high degree of inbreeding in these animals [37]. In the evaluation of the racial means in the present study, although there was no difference in sperm index values immediately after thawing, animals of the breed Schnauzer had the lowest values during the whole thermoresistance test. However, if we evaluate the difference between the mean values of the sperm

index after thawing and 45 minutes after, we observed a very similar drop percentage among the different studied breeds. The 45-minute interval is taken as the timing between the thawing of the semen and its insemination in most procedures. Thus, in order to be used in artificial inseminations, the low performance in the test of semen resistance of Schnauzer dogs could be compensated by the increase of the inseminating dose in order to obtain, at the moment of insemination, a number of mobile spermatozoa close to wanted.

In the individual evaluation, particularly low values are also observed in Schnauzer dogs, regarding the integrity and viability tests of the spermatic membrane. In the racial analysis, the variation of reactive spermatozoa to the hyposmotic test in the thawed semen, shows a great superiority in the values obtained in the Doberman breed (Table 1).

The evaluation of the movement of thawed spermatozoa at different time intervals through the thermoresistance test is considered as a good predictor of sperm fertility in several species, and low survival rates in the thermoresistance test were associated with low fertility [16]. However canine semen has the particular characteristic of low performance in the resistance test compared to other species, and this feature is not necessarily associated with low fertility, because in some studies, semen samples with relatively low performance in this test had normal fertility rates in dogs [38].

Structural membrane damage and metabolic changes in the cell during cooling can irreversibly deform sperm motility [33,34]. Thus, both the hyposmotic test and the supravital staining test with eosin-nigrosin were positively correlated with sperm motility [39-41]. The hyposmotic test was also correlated with the percentage of morphologically normal spermatozoa in ejaculation and the fertility rate in stallions [32].

Although Nöthling et al. and Batista et al. observed that fresh evaluated *in vitro* seminal quality has a low correlation with post-thawing performance [17,25], in the present work, sperm movement through sperm index in fresh semen is significantly correlated with sperm membrane integrity and viability tests and thermoresistance test after thawing (Table 2). The results observed in the hyposmotic test performed in fresh semen were also significantly correlated with supravital staining tests and thermoresistance test (Table 2). The supravital stanning test performed on fresh semen was significantly correlated only with the hyposmotic test in the thawed semen.

Conclusion

Based on the *in vitro* evaluation of the semen of dogs breeeds in the present work, although the quality of fresh semen does not present significant variation among the breeds studied, the integrity and viability of the sperm membrane and longevity of the thawed semen varies significantly both between individuals and between breeds. Thus, the ability of the canine spermatozoon to survive the freezing process is individually variable and this variation may present some genetically inherited components that manifest as a characteristic of seminal freezing of the breeds. We can also conclude that the evaluation of motility and sperm membrane integrity in samples of fresh semen are significantly correlated with the integrity and viability of the sperm membrane and the longevity of thawed spermatozoa.

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