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The Efficacy of *In Vitro* Sperm Tests in Predicting Pregnancy Success after Artificial Insemination in the Bitch

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Abstract

In many species, including the dog (Canis familiaris), semen analysis protocols has improved in recent years. *In vitro* assessment of semen quality provides important information; however, the ultimate parameter for determining semen quality is pregnancy outcome. The objective of the present study was to investigate the efficiency of *in vitro* sperm tests in predicting seminal quality in dogs by correlating those results with pregnancy success after artificial insemination (AI). Forty-eight semen samples refrigerated for 24, 48 or 72 hours were examined for sperm motility and progressive motility, membrane integrity, and zona pellucida-binding capacity before being used to artificially inseminate bitches. Results indicated positive correlations between pregnancy success and sperm motility (0.77), vigor (0.72), membrane integrity (0.72) and zona pellucida-binding (0.81). Both pregnancy success and the sperm characteristics declined with increased semen storage time. In conclusion, the semen characteristics evaluated in this study can be used to predict the conception potential of semen samples in the dog.

Keywords: Sperm tests; Semen refrigerated; Pregnancy; Artificial insemination; Canine

Introduction

Male fertility assessment, based on *in vitro* assays, has been extensively studied in many species, including cattle, pigs, small ruminants, humans and dogs [1-5]. Semen quality is indicated by sperm motility and vigor, membrane integrity, acrosome integrity, morphology, sperm cell DNA quality and ability to bind and fertilize oocytes. However, pregnancy rates are still the best indicators of fertilization capacity, but the relationship between semen characteristics and fertilization potential remains controversial [6]. Microscopy has been the standard technique for assessing sperm motility and vigor; however its subjectivity might lead to inaccurate results, thus, alternative methods to evaluate semen quality have been proposed. *In vitro* and *in vivo* tests to assess fertilization capacity of canine semen have been standardized to increase precision. Those methods allow for evaluation of several functional characteristics of sperm, such as binding, penetration, and fertilization of the oocyte [7]. Sperm motility refers to the proportion of motile spermatozoa in one ejaculate, while swimming vigor describes sperm movement intensity. These two parameters are used in conjunction to determine the quality and viability of canine semen. Other studies have suggested that progressive motility and sperm vigor are directly correlated to normal spermatozoa morphology, which in turn is positively correlated to fertilization success [8].

Sperm membrane integrity provides the protection required for adequate cell metabolism. The hyperosmolar swelling test analyzes the sperm membrane assuming it is intact; however, in hypotonic solution, it swells as a result of fluid inflow, resulting in folded tails [9]. It was only in the 1990s that hyperosmolarity testing was included in routine examinations of canine semen to accurately determine sperm membrane integrity [10,11]. Several authors have demonstrated that this technique produces equally satisfactory results when sperm samples are incubated in hypotonic solution or distilled water for 5 min [12]. Sperm-zona binding is critical for fertilization, and, therefore, it serves as an indicator of the fertilization potential of spermatozoa. Zona-binding tests aim to predict the fertilization ability of sperm, which is expected to be directly related to the fertilization potential of the male breeder. Results from this test are expected to be similar to those from *in vivo* fecundity tests given that they are capable of detecting sperm cell lesions at the molecular level, unlike conventional semen analysis tests [13]. Studies on humans and stallions have shown a positive correlation between zona-binding and male fertility [14], indicating that this test is possibly one of the most accurate *in vitro* techniques for semen analysis, given that it simulates an *in vivo* situation. Because many of the parameters evaluated by *in vitro* assays are not associated with the actual fertilization ability of sperm, pregnancy remains the most reliable parameter for testing different semen handling and storage procedures [15]. However, *in vivo* fertility tests are difficult to conduct in canids. This is because bitches have a long estrus cycle, and a large number of females would be necessary to assess semen quality and conception potential [16].

In a study on the correlation between *in vitro* and *in vivo* tests for canine semen evaluation, Olar observed that determining fertilization ability immediately after thawing is more important than maintaining motility after incubation [17]. Froman et al. investigated *in vitro* fertilization capacity of fresh and thawed canine sperm and concluded that sperm freezing compromised penetration capacity in mice oocytes [18]. This explains the low fertilization rates in *in vivo* assays using thawed semen. Because oocyte recovery from bitches is usually poor, the use of oocytes from laboratory animals has become a common practice for heterologous fertilization tests in dogs. As an alternative, homologous *in vitro* fertilization tests can be performed using ovaries obtained from ovariohysterectomy surgeries in clinical practice. As

reported by Hay et al., dog oocytes collected after ovariohysterectomy procedures should be used immediately after surgery or storage to evaluate thawed semen from the domestic dog and other canids, such as the grey wolf (Canis lupus) and red wolf (Canis rufus) [19]. Those authors suggest that semen samples maintaining motility and progressive motility after thawing achieve satisfactory levels of penetration in homologous oocytes, whereas reduced motility and higher incidences of acrosome lesions in thawed canine sperm are associated with low homologous oocyte penetration [19]. Mastromonaco et al. observed that homologous oocytes must be fresh in order to be used in *in vitro* fertilization tests [20]. Those authors concluded that storage in hypertonic saline solution compromises the zona pellucida of canine oocytes, reducing sperm penetration capacity during incubation, thereby affecting semen quality assessment tests. They also observed that integrity of cumulus cells is essential for good interaction between the canine oocyte and spermatozoa.

The objectives of the present study were to:

- Investigate the correlation between canine semen characterists, assessed by *in vitro* tests, and pregnancy success of bitches inseminated with the samples analyzed *in vitro*.
- Compare the fertility semen samples used after being freshly diluted or cooled for 24, 48 or 72 hours.

Materials and methods

Animals

Six adult male dogs were used as semen donors. The breeds were English Bulldog, French Bulldog, Pug, Poodle, Yorkshire Terrier and Chowchow. Individuals were healthy, aged between 1.6 and 4.6 years and were proven breeders.

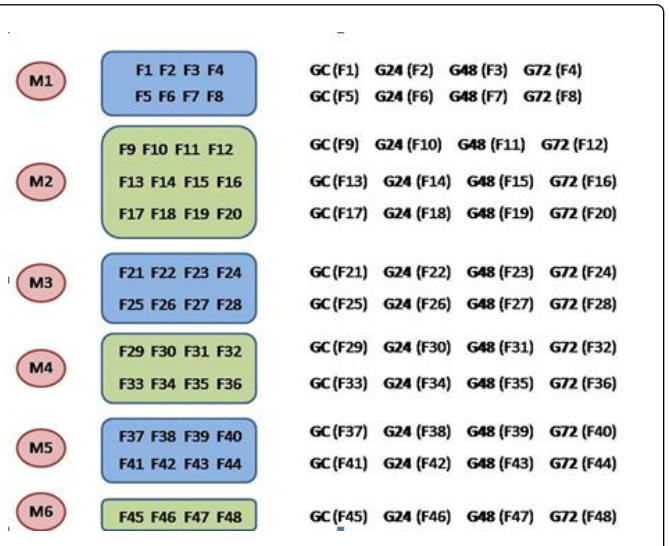

Figure 1: Experimental design. Treatment groups were divided according to breed. M=male dogs (n=6); F=female dogs (n=48); CG=control group; G=24h-cooled semen; G=48h-cooled semen; and G=72h-cooled semen.

Each semen sample was used to inseminate a group of 4 females of the same breed. Bitches from each breed group were divided into 4 treatments, consisting of insemination with fresh semen or semen cooled for 24, 48 or 72 hours [Figure 1]. The 48 bitches selected for insemination were 2.3 to 4.9 years old, healthy and had been pregnant at least once, with a minimum interval of one estrus cycle between the last gestation and the experiment. Male and female dogs received and water ad libitum during the study. All animals were vaccinated and dewormed at least 30 days before the beginning of the study.

Semen Analysis

Manual stimulation of the penis was performed to collect the sperm-rich fraction of semen. Three parameters were evaluated:

Sperm motility and vigor

10 µL of semen were placed onto a pre-warmed slide (37°C), covered with a cover slip and examined under phase contrast microscopy (Nikon, Eclipse 80i) at 10X and 20X magnification. Sperm motility was subjectively assessed and classified according to the proportion (percentage) of motile sperm in the sample, ranging from 0 (all cells stationary) to 100 (all cells moving). Sperm vigor, as indicated by the intensity of progressive motility, was classified according to a scale of 0 (no movement) to 5 (rectilinear movement) [21].

Sperm concentration

Semen was diluted at a 1:20 ratio (50 µL of ejaculate to 950 µL distilled water) and co-incubated at room temperature for 5 min (Hishinuma and Sekine 2003). The solution was placed in a Neubauer hemocytometer chamber and, after sedimentation, sperm cells were counted under a phase contrast microscope (Nikon, Eclipse 80i) at 40X magnification [21]. Cell count was expressed in sperm per mL.

Membrane Integrity

Membrane integrity was analyzed using the hyperosmolarity swelling test. Semen was diluted in distilled water (1:19 v/v) and incubated at 37°C for 5 min. One drop of the sample was placed on a slide and covered with a cover slip. The slide was examined under a phase contrast microscope at 40X magnification (Nikon, Eclipse 80i). Of the 200 spermatozoa counted on each slide, those with coiled tails were identified as having a functional intact membrane [12].

Zona-binding test

Oocytes were recovered from ovaries obtained after routine ovariohysterectomy at the Veterinary Hospital (HV/UENF). Immediately after surgery, the ovaries were immersed in 250 mL of saline pre-warmed at 38°C and taken to the laboratory. Oocytes were evaluated under a magnifier and selected based on morphology, washed and transferred to a petri dish containing 95µL-drops of Talp FIV medium. Each drop contained 10 oocytes and was covered with mineral oil (Sigma®) and kept in at the incubator (38°C, 5% CO_2) for 1h. To each drop, 100µl of semen (at 2×10^6 spermatozoa/mL of culture medium) were added and incubated (38°C, 5% CO_2) for 18 hours. After incubation, oocytes were examined under a phase contrast microscope (Nikon, Eclipse 80i) at 10X magnification in order to count the number of spermatozoa bound to the pellucid zone. A score from 0 to 3 was used to evaluate binding: 0 = no binding; 1 = 5 to 25 bindings, 2 = 26 to 50 bindings, 3 = more than 51 bindings [22].

Semen cooling

The ejaculate from each dog was diluted in a medium containing skimmed milk and glucose and divided into 2 aliquots of 2 mL [23]. Semen dilution was calculated using the formula V1C1=V2C2, where: V1 is the aliquot volume; C1 is the initial concentration of fresh semen; and V2 is the final volume of the diluted aliquot diluted to a final concentration C2 of 200 × 10^6 spermatozoa/mL [24]. The volume of the diluent was V2 – V1 [25]. One aliquot was used for the laboratory tests and the other to inseminate the bitches according to treatments (fresh semen or semen cooled for 24, 48 or 72 hours). Semen was cooled in a vertical refrigerator at 4C.

Estrus cyclicity and pregnancy monitoring

Insemination time was defined by the beginning of the estrus cycle, which was identified via vaginal cytology and assessment of serum progesterone concentrations. Estrus was determined by vaginal cytology when keratinization of more than 80% of epithelial cells (superficial nucleate and anucleate cells) was observed and by serum progesterone concentrations equal to or greater than 6.0 ng/ mL were [26].

Pregnancy detection was performed via ultrasonography 21 days after diestrus, using a mindray 2200DPvet device, with multi-frequency convex and linear probes. The females were examined again at 35 and 55 days of gestation.

Pregnant females were monitored during labour and, when necessary, a C-section was performed. All litters were monitored until weaning at approximately 30 days of birth.

Artificial Insemination

Artificial insemination (AI) procedures were conducted at the reproductive and outpatient clinic at the Universidade Estadual do Norte Fluminense. A single AI was performed for each animal [27], using a bovine uterine infusion pipette, cut in half and attached to a 5mL plastic syringe. The female's hind legs were maintained in an elevated position for approximately 10 min and semen was deposited in the vaginal vault. Treatment groups were as follows: diluted fresh semen (control group, CG); semen cooled for 24 (G = 24); 48 (G = 48); and 72 h (G = 72). All samples were evaluated immediately prior to AI [Figure 1].

Statistical analysis

Statistical tests were conducted using SAS 2002 software at a significance level of 0.05. Results obtained from each *in vitro* laboratory test were compared with pregnancy results after AI with corresponding semen samples. ANOVA was applied to compare sperm motility and vigor, as well as membrane integrity among treatment groups. Chi-square test was used to compare scores from zona-binding tests.

Results

Both the results from *in vitro* tests (sperm motility and vigor, and membrane integrity) and pregnancy success decreased with increased semen storage time. Of the 48 bitches inseminated, 34 became pregnant. Mean values for motility, sperm vigor, and percentage of intact membrane of the semen samples whose use resulted in pregnancies were 72%, 3 and 68% respectively. Semen samples that did not result in pregnancies had an average of 21% motile sperm, sperm vigor score of 1 and 84% of spermatozoa with damaged membranes [Table 1].

Pregnancy	(N)	Motility (%)	Vigor (s)	Integrity (%)
Non-pregnant	14	21.79 (± 10.30) [a]	1.21 (± 0.58) [a]	16.21 (± 7.39) [a]
Pregnant	34	72.1 (± 21.61) [b]	3.38 (± 1.07) [b]	68.03 (± 26.81) [b]
Different letters in a column indicate a significant difference (P<0.05)				

Table 1: Sperm motility, vigor and membrane integrity of semen samples used in AI procedures and the pregnancy outcome (mean ± SD).

A positive correlation was observed between sperm-zona binding scores and pregnancy success (p<0.05). Zona-binding scores (1 to 3) accounted for successful fertilization of 70.82% of bitches, whereas the 14 females inseminated with semen displaying zona-binding scores of 0 did not become pregnant [Table 2].

Zona-binding score	Pregnant bitches	Non-pregnant bitches
0	0 (0%) [a]	14 (29.16%)
1	10 (20.83%) [b]	0 (0%)
2	8 (16.66%) [b]	0 (0%)
3	16 (33.33%) [c]	0 (0%)
Different letters in a column indicate significant difference (p < 0.05).		

Table 2: Pregnancy rate according to zona-binding scores of semen samples used in artificial insemination procedures.

Pregnancy rate was strongly associated with sperm motility and vigor, as well as membrane integrity and zona-binding capacity (p < 0.05), irrespective of semen freshness and storage time. Pregnancy success was correlated to the results of *in vitro* tests. Pregnancy success and the parameters of *in vitro* analysis of semen (motility, vigor, membrane integrity, and zona-binding capacity) declined with increasing storage time [Table 3].

Statistics	Motility	Vigor	Integrity	Zona-binding	Pregnancy rate
Motility	-	-	-	-	-
Vigor	0.96**	-	-	-	-
Integrity	0.98**	0.94**	-	-	-

Zona-binding	0.96**	0.89**	0.96**	-	-
Pregnancy	0.77**	0.72**	0.72**	0.81**	-
**Correlation values indicate highly positive about the variables ($p < 0.05$)					

Table 3: Correlation between semen sample characteristics and pregnancy rate of inseminated bitches.

Discussion

The difficulty in conducting studies to assess the relationship between *in vitro* and *in vivo* results is particularly true for companion animals such as the dog because resulting pregnancies and offspring may be undesirable. Studies in other species have indicated a correlation between pregnancy success via AI and semen quality assessed by *in vitro* tests [28-30]. Likewise, the findings in the present study indicate a correlation between the results from *in vitro* tests of semen quality and pregnancy success.

Similarly to the findings of the present study, motility and vigor of stallion sperm assessed by computerized analysis (CASA) and pregnancy rate in mares is strongly associated with results of *in vitro* tests of semen quality. Greater sperm abnormalities reduce sperm movement, lowering pregnancy rates in the first breeding season [29]. Although CASA procedures have proved to be reliable and are successfully applied in animal reproduction centers, our study employed subjective assessment of sperm motility and vigor, since both AI procedures and *in vitro* semen evaluation were performed simultaneously. Moreover, semen quality results obtained by subjective and CASA methods are significantly correlated [5].

Nevertheless, cold storage compromised sperm motility, vigor and pregnancy rate, showing a reduction in semen quality over time. Previous research in dogs did not compare *in vivo* and *in vitro* results; however, in agreement with the present findings, they reported that sperm motility and vigor (assessed *in vitro*) declined with increasing storage time [25]. Cooling lowers sperm metabolism, but unlike freezing, it does not cause quiescence. Thus, reduced fertility as a result of storage time is likely due to factors such as sperm metabolite accumulation, which may alter osmolarity and pH of the diluent, compromising sperm functionality and motility. Moreover, energy depletion may contribute to the negative consequences of prolonged storage time. These hypotheses were in agreement with Verstegen et al, who observed that cooled sperm stored for up to 96 h exhibits considerable recovery of progressive motility when the diluent was replaced daily [31].

In the present study, membrane integrity scores of sperm were correlated to pregnancy outcomes. As previously reported, membrane damage is associated with low fertilization ability [32], likely because it compromises oocyte recognition and spermatozoa binding to the pellucid zone. Sperm must function adequately in order to achieve satisfactory interaction with the oocyte. Sperm membranes are known to be rich in polyunsaturated fatty acids and thus, very susceptible to lipid peroxidation, which causes partial or total functionality loss [33, 34].

In this study, oocytes used in zona-binding tests were homologous and fresh, obtained after ovary removal from the donor bitches. This binding test is a reliable method for assessment of sperm quality and has the advantage of being faster than *in vitro* fertilization tests, which require long incubation periods. Reports from the early 2000s suggest that zona-binding tests in previously prepared oocytes can be used to evaluate different sperm treatment methods, such as the effects of semen cooling on fertilization ability of canine spermatozoa [35]. Results obtained in zona-binding assays confirmed the effectiveness of this test, demonstrating that binding indexes are associated with pregnancy outcomes. Similar results were observed in cattle, where zona-binding and spermatic penetration tests performed using homologous oocytes were considered strong indicators of *in vivo* fertility [30].

The results presented in this study indicate that sperm motility and vigor, membrane integrity and zona-binding scores are related to pregnancy success in the domestic dog. *In vitro* tests can, therefore, be used to predict the fertilization potential of canine semen.

Conclusion

The process of refrigeration for 24, 48 and 72 hours interfere directly in index positive pregnancies in bitches inseminated with refrigerated samples, decreasing the number of pregnant bitches after 48 hours of cooling. Tests conducted *in vitro* (motility and sperm vigor, membrane functionality and connection test oocyte) have a positive correlation with the rate of pregnant bitches inseminated.

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