

Theoretical aspects of canine cryopreserved semen evaluation

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Abstract

Evaluation of canine cryopreserved semen has the ultimate goal of determining if an individual frozen ejaculate will have acceptable fertility. This is difficult in that there is no accepted normal fertility for the dog. The fertility of the female also plays a crucial role in estimating the fertility of the male. Poor female fertility can make a fertile male appear less fertile. Variability of animals, breeding technique, breeding timing, and number of cells inseminated make comparisons in canine fertility difficult to truly measure. Many more animals are needed to provide meaningful statistical results than are usually used. Several tests, including motility in bright field and phase contrast microscopy, computer analysis of motility, sperm morphology, sperm membrane integrity, capacitation and sperm function tests have been investigated to predict fertility, however few of these tests have actually been correlated with fertility. More work is needed to create one or more tests that accurately predict fertility of cryopreserved canine semen.

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1. Introduction

The theoretical aspects of evaluating cryopreserved canine semen have been reviewed recently by Martínez [1]; the objective of this manuscript is to provide a more philosophical overview of how cryopreserved canine semen is evaluated. It is intended to be a practical approach for veterinarians using cryopreserved semen to help interpret the true value of

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different evaluation methods in predicting potential fertility. This manuscript considers the ultimate goal in evaluating cryopreserved semen, statistics used in fertility analysis, and problems associated with investigating evaluation of cryopreserved canine semen.

2. Goals of semen evaluation

There is an ultimate goal when canine semen is evaluated, but there are also intermediate goals that semen evaluation attempts to answer during each specific step in semen-processing or use. The ultimate goal is to predict the fertility of an ejaculate when inseminated into a bitch of normal fertility. This is always the goal when evaluating fresh, chilled, or cryopreserved semen. However, intermediate goals of semen evaluation become apparent when semen is processed for cryopreservation. A semen-processing center has intermediate goals, including not only to determine whether the fresh sample has good fertility, but whether it can be successfully cryopreserved. It is of no use to have fresh semen with good fertility that cannot successfully undergo cryopreservation. The ultimate goal of the semen-processing center is to be able to predict the fertility of the frozen-thawed semen; this may be considerably different than the pre-freeze fertility or the ability of the semen to successfully undergo cryopreservation. This is also the semen evaluation goal of principal interest to the veterinarian performing the breeding, or the client paying for the cryopreserved semen. The breeder would certainly like to have some prediction of the fertility after cryopreservation, although fertility may not be the most important factor when selecting or buying cryopreserved semen. The concept of fertility, how fertility is evaluated, and evaluation methods that attempt to predict the fertility of cryopreserved canine semen, are explored in this manuscript.

3. Fertility

Fertility is a crucial concept that must have a common definition when comparing data among papers. Fertility is assumed to mean the percentage of females that get pregnant after breeding. Amann [2] recently published a paper examining reports using stallion fertility as the outcome of each study. His interpretations are rather shocking; he concluded that 76% (51/76) of the papers evaluated were flawed for one or more reasons. While some parts of the paper are not relevant to the canine, several aspects are highly applicable.

Male fertility cannot be evaluated independently of female fertility; fertility of the male or an ejaculate from a male is highly dependent on the fertility of the females bred. Amann [2] reports that a stallion with 95% fertility can have an observed fertility as high as 90% if bred to mares with 95% fertility, and as low as 24% if bred to mares with 25% fertility. Subsequently lower observed fertility is seen if the stallion's fertility is lower. For example, if the stallion has 50% fertility, the observed fertility is only 48% if bred to mares with 95% fertility and a dismal 6% if bred to mares with 25% fertility. Because of this female factor in fertility [2], using a single male on a group of females to ascertain fertility is not sufficient to apply the results to a population. Even if the observed fertility of a dog were known because of a large group of test-breeding on fertile females, the fertility of the dog could

not be predicted on a different group of females that have different fertility. Using many different ejaculates from the same male makes this prediction even more difficult. It is very expensive to purchase and maintain breeding females in a research situation. It is even more difficult to know if their fertility is normal. The selection of females for breeding with cryopreserved semen in practice situations rarely has fertility as a primary selection criterion, so even if normal fertility were known, it probably would not be used in selecting the female. Furthermore, there is no accepted standard for normal fertility for a dog. However, with estrus occurring, on average, only twice yearly, it would seem that each estrus is too precious for them to be non-productive.

There are many variables to consider when assessing fertility of frozen-thawed canine semen. There is the fertility of the female, the variability of each female's estrous cycle, the insemination method (surgical, 'Norwegian' transcervical, 'New Zealand' transcervical, vaginal), the timing method (LH and/or progesterone), differences among laboratories in hormone concentrations, timing of insemination (inseminating on days 5–6 after LH or based on progesterone concentrations), the number of inseminations, and the number of cells in the insemination (or total normal motile cells inseminated). Unless all of these variables are controlled by the investigator, there is little chance that meaningful data will be generated. Heterospermic AI or sex-sorted AI of different males into individual females are suggested by Amann as a better control of variables, but are still not a true measure of fertility [2]. Even very well intended shared data bases, as proposed by Roskin [3], or large clinical trials using many animals from many sources [4] that are expected to have sufficient numbers to overcome problems in variability 'will have so much "noise" as to have little value' at determining fertility [2]. Despite that, it is one of the few ways the canine industry has to generate enough numbers to show statistical significance or even to detect trends when using cryopreserved sperm.

4. Statistics

Statistics are used to evaluate the outcome of experimental procedures in order to determine if the results are truly significant. The term most often examined is the α level. This expresses the chance of making a false positive conclusion, for example saying that 'ejaculate A' has better fertility than 'ejaculate B', when in fact it does not. The term β is not looked at as critically by many, but expresses the chance of making a false negative conclusion, for example saying that 'ejaculate A' does not have better fertility than 'ejaculate B' when in fact it does. The term power is defined as $1 - \beta$. Table 1 shows how many females are needed in each group to have statistical significance. The smaller the difference in the outcomes of the two groups, the more animals are needed to have statistical difference at a given α or β . Although there are very few small animal studies that have 37 animals in each group, that is how many are required to have statistical significance for a 30% difference (80% versus 50%) between two groups at $\alpha = 0.1$ and a $\beta = 0.2$ (power = 0.8). Trying to make meaningful conclusions using too few animals or poorly designed studies is worse than not doing the study at all.

As can be seen with the problems and limitations stated, it is difficult and perhaps impossible to generate statistically significant data on canine fertility, let alone a single

Table 1

Number of animals needed to show significance using Chi-square analysis^a

Expected difference (expected outcome of each group in %)	(α)	Power ($1 - \beta$)	Animals/group needed
10 (70–60)	0.05	0.8	376
30 (80–50)	0.05	0.8	45
40 (90–50)	0.05	0.8	24
10 (70–60)	0.1	0.8	300
30 (80–50)	0.1	0.8	37
40 (90–50)	0.1	0.8	20

^a Calculated at: <http://members.aol.com/johnp71/proppowr.html>.

ejaculate using normal breeding methods. However, Amann [2] states that ‘quantitative measurements of sperm function are better than fertility estimates’. These function estimates include motility (bright field, phase contrast, computer analysis), morphology, membrane integrity, capacitation and sperm function tests.

5. Evaluation of semen quality

Bright field microscopy is most often used to analyze the ‘quality’ and predict the fertility of the ejaculate. Visual assessment requires good equipment (heated stage and slides) and an experienced evaluator, but is subject to evaluator bias. In a study with 67 dogs breeding 42 bitches with fresh semen, 45 dogs with an average motility of $75.6 \pm 15.2\%$ had a pregnancy rate of 60.9% (14/23), whereas dogs having an average motility of $57.2 \pm 24.3\%$ had a pregnancy rate of 13.3% (2/15; $P < 0.05$) [5]. The numbers appear large enough to be significant, but the standard deviation for the infertile group was very large. However, fertility groups were really classified based on greater than or less than 60% morphologically normal sperm cells. Cryopreserved semen with an average of $58.5 \pm 6.1\%$ motile spermatozoa from two dogs bred to nine bitches had a 100% conception rate [6]. Nine is not enough to show any statistical differences, and in fairness to the author of the paper, no statistics or comparisons were actually done looking at motility and fertility in that study.

Some studies compared several extenders, picked the ‘best’ one based on motility, and then used that extender in a fertility trial. In one of these papers, the extender selected had 50% post thaw motility, but only seven dogs were bred, yielding a pregnancy rate of 57% (4/7), so no real comparisons were made to evaluate fertility [7]. Furthermore, motile sperm is not necessarily fertile, because acrosomal and membrane changes may occur after cryopreservation and thawing that affect fertility, but not motility.

To decrease subjectivity in sperm motility analysis, computer-aided semen analysis (CASA) systems have been used to investigate the appropriate settings to measure sperm motility of fresh canine semen [8–11], to compare fresh semen treatment with sugars [12], and to compare different treatments of Equex paste on cryopreserved canine sperm motility [13]. However, none of the studies considered fertility.

Sperm morphology is investigated to help predict fertility. The same study by Oettlé that was previously cited classifying fertility groups of females based upon the males having

more or less than 60% morphologically normal sperm cells looked at sperm morphology [5]. Using 67 dogs and breeding 42 bitches with fresh semen, 45 dogs with an average of $79.3 \pm 11.4\%$ normal sperm cells had a pregnancy rate of 60.9% (14/23) whereas dogs having an average of $33.4 \pm 21.0\%$ normal sperm cells had a pregnancy rate of 13.3% (2/15; $P < 0.05$) [5]. It was noteworthy that the standard deviation for the infertile group was very large.

Computerized determination of morphology has been described and has shown to give accurate morphometric sperm measurements when the technical settings were standardized; however, no breeding was done so no fertility data are available [14]. Other workers using computerized morphometry investigated fresh semen from fertile normospermic and subfertile teratospermic dogs, but it was stated that no relation to fertility was known in dogs based on morphometric analysis [15]. There is no information regarding morphometric analysis of cryopreserved sperm.

Membrane integrity assesses whether the sperm cell membrane has been disrupted and can be analyzed several different ways. The most common is the eosin–nigrosin stain that is also used to examine morphology. The dead cells stain red and the live cells are not stained; the dead cells allow stain to be taken up after membrane damage. Even though it is probably the most common stain used in practice, there are apparently no reports on its use in thawed, cryopreserved canine sperm to estimate fertility. Membrane integrity using several stains and visualizing the cells with fluorescence or flow cytometry have been used to analyze fresh [16] or cryopreserved canine sperm cells [17,18]. However, no fertility testing was done. The hypo-osmotic swelling test evaluates the ability of the cell membrane to swell when exposed to hypo-osmotic conditions, thereby indicating the cell membrane is intact and can transport water normally. Several studies have looked at hypo-osmotic swelling of canine semen, but none have evaluated cryopreserved canine sperm or investigated fertility.

The acrosome reaction is the fusion of the plasma membrane with the outer acrosomal membrane, resulting in vesiculation and release of acrosomal enzymes that aid in penetration of the zona pellucida. After the acrosome reaction, only the inner acrosomal membrane is intact. The physical change of the acrosome reaction after cryopreservation is indicative that the cell cannot fertilize, even though the cells may be motile. Acrosome integrity can be assessed with eosin nigrosin stain. In a live sperm cell having undergone the acrosome reaction, the acrosome will stain; however, the cell will remain unstained against the black background. Acrosomal status after cryopreservation of dog sperm has been investigated, but no fertility trials were done on the semen [17,18]. Some studies compared several extenders and picked the ‘best’ based on post thaw acrosome integrity and then used that extender to inseminate. In one of these, the extender selected had $66 \pm 2.0\%$ post thaw acrosome integrity, but only seven dogs were bred, yielding a pregnancy rate of 57% (4/7) [7].

Sperm function tests such as zona pellucida binding have also been used to examine fresh semen in fertile and infertile dogs [19] or cryopreserved canine sperm cells [20,21]. The study using fresh semen showed lower binding in the infertile dogs, but the definition of fertility in the dogs was not ideal. It was shown that binding was poorer in cryopreserved samples than fresh samples of some dogs [21]. Again, fertility was not assessed in either of the studies using cryopreserved sperm.

6. Conclusions

Based on the preceding information, it appears that little real progress has been made in assessing cryopreserved dog spermatozoa; furthermore, progress in assessing fertility will be difficult. This seems much more true in the dog than in cattle and horses. In cattle, a single ejaculate may yield 250–400 insemination units and bulls may have ejaculates collected twice weekly for processing. In dogs, it is common to have only one insemination dose from a cryopreserved ejaculate. This makes fertility comparisons within a single ejaculate impossible. In addition, few if any, dogs will have 50–100 ejaculates cryopreserved and bred to 100 bitches using similar breeding techniques. Another problem that exists in cryopreservation of canine sperm is standardization. Currently there is no consensus on minimum sperm numbers needed for optimal fertility, cryopreservation extenders, cryopreservation techniques, cryoprotectants, freeze rates, thaw rates, packaging methods, timing of insemination, or insemination method. Furthermore, there is no standardized cryopreservation form or a standardized reporting system for breeding bitches. Without some type of standardization, canine breeding will continue to be a cottage-industry permeated by proprietary information.

Perhaps the researchers in canine cryopreservation have done a better job than those studying the horse, in that we have not overstated fertility when looking at these techniques. Workers continue to use terms such as ‘quality’ or ‘viability’ of a sample or correlate one parameter with another (usually motility) when using these all these different techniques to analyze cryopreserved canine semen. An index of several parameters may result in being more useful than any single parameter. Eventually, true fertility, as it relates to these estimates, will have to be defined. True fertility has not really been measured, however. If true fertility cannot be measured in the dog, researchers and veterinarians currently must interpret the data obtained in a realistic manner to make decisions and predictions using cryopreserved canine sperm cells.

The goal still remains when a semen-processing center freezes semen or a veterinarian wants to use a cryopreserved sample. That goal is to answer the question “How fertile is this sample?” The question is not “How fertile is the male?” or “How fertile have other samples been?” The ultimate goal is to answer the question “How fertile is this sample?” Users want assurance that a sample is ‘fertile’. Currently, it can barely be stated that the sample has parameters that are associated with ejaculates that have yielded pregnancies. More than 10 years ago, Amann published the opinion that we may be able to predict >60% fertility in a male using *in vitro* evaluation of sperm quality, but we would never be able to predict if a male’s fertility will be greater than the mean fertility of the top males [22]. Much more intensive work is needed to achieve this quixotic goal. Some conclusions by Amann are: (a) most published fertility studies reporting that no significant difference was found are flawed because an insufficient number of mares was used; (b) researchers should carefully think out the design of their fertility studies and the stringency of the test for statistical significance; (c) researchers should be more candid in presentation of results based on 10–150 mares per treatment group and, when appropriate, explicitly alert the reader that use of an insufficient number of mares, rather than the nature of the new procedure might have caused failure to detect a statistically significant difference. Also, recognize that not all statistically significant differences are clinically important [2]. These

conclusions probably can be applied even more in the canine research community than in the equine research community.

In closing, this paper is not an attempt to criticize any of the papers or workers who put so much time and effort into their research. Quite to the contrary, it is a plea to have them continue their efforts to achieve that final goal in the fertility question.

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