Semen Collection, Assessment and Processing for *in vitro* Fertilization in Dog – a Review

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Abstract
The attainment of a feasible *in vitro* capacitation is clearly dependent on the maintenance of suitable energy levels of mammalian spermatozoa. This is because of the fact that all the sperm changes related to capacitation, such as the increase in tyrosine phosphorylation or changes in motility patterns, are energy-consuming. Canine semen can be capacitated and undergoes acrosome reaction *in vitro* and spermatozoa are able to fertilize homologous oocytes in *in vitro* culture conditions. The aim of this paper is to describe the recent research of the authors in the field. Recent and classical reviews and new trends regarding semen prelevation, evaluation and preparation for *in vitro* fertilization in dog.

Keywords: Capacitation; Cryopreservation; Dog sperm; Evaluation; Prelevation

1. Introduction

In recent years pure dog breeding is experiencing growing interest and the request for assisted reproductive technologies is increasing. The existence of sperm subpopulations defined based both on kinematics and morphometric characteristics of the spermatozoa are now widely accepted by the scientific community. New techniques to evaluate early membrane changes have also been recently developed [1].
The application of ART to the management of endangered wildlife populations requires sufficient species-specific information on gamete and embryo physiology for an intricate series of laboratory procedures to be successfully and efficiently completed. For example, the development of a successful IVF protocol requires safe and effective methods for collecting viable spermatozoa and mature developmentally competent oocytes. If spermatozoa are to be cryopreserved before use, methods also must be available to maximize the recovery of motile acrosome-intact spermatozoa capable of fertilizing oocytes after sperm thawing. In addition, culture conditions used for IVF must support a variety of complex cellular functions necessary for successful fertilization, including sperm motility, sperm capacitation, the acrosome reaction, oocyte viability, sperm-oocyte recognition, oocyte activation, and the initiation of embryonic development [2].

2. Semen collection

The most commonly used method for semen collection in dogs is the manual one, and in cats is electroejaculation. Sperm can also be collected from the epididymis after surgical sterilization, post-mortem, or vaginal lavage after natural mating [3]
Semen can be used immediately after harvest, fresh and undiluted, can be refrigerated for a short term storage or can be frozen and thawed if to be stored for a longer period of time.

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3. Semen assessment

The spermatozoa, is a complex and very specialized cell adapted to transport the male genome to the female genital tract to fertilize the oocyte. Because of its complexity, a simple test cannot give complete information of the fertilizing potential of a sample and different tests are necessary. To date, canine ejaculates have been evaluated through different tests, using fluorescent probes for evaluation of sperm membranes, acrosomal and capacitation status [4].

General evaluation includes organoleptic examination (color, smell, viscosity, and semen liquefaction), biochemical (density, ph, alkaline phosphatase concentration specific for epididimar fluid) and microscopic analysis.

Despite the widespread use of conventional semen analysis under light microscopy in which semen samples are analyzed for characteristics of motility, morphology and abnormalities, concentration and the total sperm count, the method is subjective and may be unreliable [5]. The ultimate functional test is the ability of the spermatozoon to fertilize the oocyte [6].

Functional tests for dog sperm include techniques for sperm binding assay and sperm penetration assays using entire, hemi-zonae or intact either fresh or cooled oocytes [7]. Semen may be collected from fresh or cooled epididymis up to 8 days [8] and be able to bind to homologous zone in a time dependent manner [9].

The oocyte penetration assay is a more rapid test of spermatozoa function than in vitro fertilization as simply assesses the presence of decondensing spermatozoa heads within the oocyte [10].

Tests to determine the capacity of spermatozoa to bind and penetrate intact zona pellucida or hemizonae have been applied in the canine species [11-13].

In vitro methods for the prediction of the fertilizing potential of a semen sample are important both when developing new methods for cool storage or deep freezing of semen and when evaluating the fertility of an individual male.

Protocols for canine semen freezing had been evaluated by sperm–oocyte interactions test [14, 15].

In dogs, ejaculates with high incidence of proximal droplets have been reported to have low fertility [16, 17].

Peña et. al. (2007) performed a zona pellucida-binding assay, using canine oocytes derived from frozen-thawed ovaries in order to investigate the zona-binding ability of dog spermatozoa with proximal cytoplasmic droplets. Cytoplasmic droplets are small spherical masses of cytoplasm commonly found in the tail of a small percentage of ejaculated spermatozoa. Droplets can be found in a proximal or a distal position to the sperm head and neck [19]. On electron microscopy, the ultrastructure of the cytoplasmic droplet consists of a system of vesicles, tubules, vacuoles and gastrula-like bodies [20] thought to be derivatives of degenerating Golgi apparatus and endoplasmic reticulum, and remnants of the nuclear membranes from the precursor round spermatid [19]. The cause of which spermatozoa with a proximal cytoplasmic droplet have low fertilizing potential remains to be elucidated. Histochemical studies showed that droplets contain several hydrolytic enzymes similar to those found in lysosomes, and the presence of such hydrolases was suggested to be the cause for the failure of the fertilization process [21]. It has also been suggested that a high proportion of sperm with retained cytoplasmic droplets would enhance the production of reactive oxygen species (ROS), which are known to be detrimental for fertilization [22, 23].

Thundathil et al. (2001) proposed that sperm with proximal droplets may be deficient in zona-binding receptors or they may be structurally abnormal and cannot normally participate in egg binding. It was concluded that dog sperm with proximal cytoplasmic droplets seem to lack normal capacitating ability in vitro, and consequently, they may have reduced capacity to bind to the zona pellucida of canine oocytes [18].

In recent years, a number of different tests have been developed to achieve more information regarding the fertilizing ability of a semen sample, or the outcome of a freezing–thawing procedure [25].

Also **computer-assisted sperm analysers** have been included in the investigation of the spermiogram and its use is becoming more popular in canine andrological laboratories [26]. It is widely accepted that sperm morphology is a strong indicator of semen quality. As the sperm head mainly comprises the sperm DNA, it is have been proposed that subtle changes in sperm morphology may be related to abnormal DNA content.

Núñez-Martinez et. al. described for the first time in 2005 the SCSA assay in dog semen. Both SCSA and sperm head morphometry may be powerful tools to improve the canine spermiogram. Two main factors explain this statement: first the great variability in both parameters observed in canine ejaculates, second the strong statistical correlation observed. These facts together with the lack of objective criteria on the morphological classification of canine spermatozoa and the great variability observed among dogs and ejaculates points out the utmost importance of further studies to standardize sperm head morphometry in the canine species.

Significant differences were found in all CASMA-derived parameters among dogs. Linear regression models including sperm head shape factors 1, 3 and 4 predicted the extent of DNA denaturation [27].

It is plausible that variation in sperm morphology arises during spermatogenesis, when genotypic effects influence sperm structure. Sperm morphology phenotype appears to be controlled by genes transcribed in the pre-meiotic phase of development [28].

Inbreeding coefficients have been related to poor ejaculate quality further demonstrating the genetic control of sperm morphology [29]. This fact, together with the easy identification (albeit few dogs were used) of differences on sperm ejaculates between dogs and ejaculates within the population of normal spermatozoa, points out the possibility of identifying those dogs or ejaculates more suitable for biotechnological procedures such as sperm cryopreservation [27]. In fact in boars [30] and bulls [31] significant relationships have been found among morphometry, freezability and field fertility. Also in humans sperm head morphometry is considered a reliable predictive tool in the assessment of the outcome of most of the assisted reproductive technologies [32].

The **nucleus** of the spermatozoon is the key structure of the cell, and is considered as the strongest indicator of semen quality. In spite of this, the investigation of the canine sperm DNA have, to date, being neglected. The nucleus of a normal spermatozoa has a highly condensed chromatin built by association of double-stranded DNA with proteins, protamines and histones. The condensed and insoluble nature of sperm chromatin protects the genetic integrity during transport of the paternal genome through the male and female reproductive tracts [27].

The sperm chromatin structure assay (SCSA) was first described in mammalian by Evenson et al. (1980). In this procedure sperm are first treated for 30 s at pH 1.2, to potentially denature DNA in situ. With normal chromatin structure sperm DNA does not denature under such conditions [27].

The sperm are then stained with the metachromatic DNA stain acridine orange (AO). When intercalated into native, double-stranded DNA, AO fluoresced green whereas AO associated with single-stranded DNA fluoresced red. The amount of red and green fluorescence emitted by each of 5000 spermatozoa is measured per sample with a flow cytometer and provides an index of the percentage of cells with denatured DNA (% cells outside the main population, %COMP). The extent of DNA denaturation in each individual spermatozoa is expressed as at and is red/(red + green) fluorescence [27].

SCSA data are expressed as the mean of these parameters as well as the variation. The data showed a very significant difference between proven fertile and sub/infertile men and bulls in their susceptibility of sperm nuclear DNA to denaturation [33].

Since this first publication the SCSA have demonstrated to be a strong indicator of semen quality in man, bulls, pigs, stallions [34] and rams [35].

Unfortunately, the need for expensive flow cytometers restricts the use of this technique in clinical situations in companion animals. Because sperm heads consist almost entirely of DNA, their shape should be related to sperm chromatin and organization, and potential problems in DNA, may result in subtle changes in sperm head shape, probably not detectable with a traditional sperm head morphology evaluation, but could be detectable with a computer-assisted morphology assessment (CASMA) [27].

4. Semen processing

Fresh semen is commonly used for in vitro insemination, with the use of special media for capacitation. It has been demonstrated that ejaculated sperm capacitation occurs in vitro after 7 hours and that Ca++ is essential for this process [36]. In vitro capacitation may be achieved in

Canine Capacitation Medium (CCM) [36] or in a modified Tyrode´s [37]. When CCM was used, removal of proteins was detrimental to sperm motility and glucose withdrawal reduced the percentage of acrosome reacted sperm [36]. Calcium ionophore A23187 can promote capacitation and acrosome reaction in a similar manner as Ca++ acts in vitro [38].

Bitch follicular fluid may also induce capacitation of dog sperm [39, 9].

Semen samples are diluted (1:1 with Hepes-buffered [40]. At the conclusion of semen collection, all diluted samples are pooled, and aliquots (3–5 μl) are stained with Rose Bengal-Fast Green stain [41] to determine initial acrosomal integrity (200 sperm per ejaculate at 400 x magnification) and sperm concentration using a hemocytometer. The remaining sample was centrifuged at 300 x g for 10 min.

To ensure maximal sperm recovery, the supernatant was recentrifuged at 1100 x g for 10 min, and 20 μl from the bottom of the tube, assumed to contain the majority of remaining spermatozoa, was combined with the sperm pellet from the initial centrifugation [40].

Energy management of dog sperm has some very remarkable aspects. Thus, these cells have a fully functional glycogen metabolism, which implies that they are able to accumulate important energy stores after ejaculation for incubation times of at least 1 h [42]. Moreover, several other anabolic pathways, like the pentose phosphate cycle, seem to be active in mature sperm from fresh ejaculates, indicating that these cells also have the ability to maintain reduction potential for a long time [43]. All these indicate that dog sperm will be able to regulate its energy levels not only by modulating energy consumption, but also energy storage. This would imply that, at least under some conditions, dog sperm could maintain its energy status using its own internal reserves. This metabolic characteristic would be on the basis of the described capacity of dog sperm to maintain its functionality for a long time in sugar-free media, like its own seminal plasma [44], and could also probably be one of the reasons to explain the long life-span that these cells have inside the female genital tract after ejaculation [45]. Thus, it is a little surprising that this ability to maintain energy levels during relatively long periods of time does not allow for the maintenance of dog sperm in vitro capacitation in a medium without glucose. The reason could be that glucose and fructose are utilized by dog sperm not only as energy substrates, but also as function regulators, as the specific changes in motility patterns and tyrosine phosphorylation described after the incubation with these sugars indicate [43]. However, all these data clearly indicate that questions arising from the ability of achieving in vitro capacitation in dog sperm are not completely solved [46]

The process of capacitation is a time-dependent phenomenon and in vitro is different among species [47]. Preliminary studies using fresh semen, have found that canine spermatozoa capacitated in vitro are able to penetrate ZP within 7 h after initiation of incubation [36]. However, later studies demonstrated that canine sperm penetrate ZP around 2–4 h after incubation [48, 49, 50, 51].

It is generally accepted that capacitation induces great changes in the tyrosine phosphorylation pattern of mammalian sperm because these changes are strongly related to the functional changes of proteins related to this process [52]. Recently, this change of phosphorylation pattern in in vitro capacitation has been described in dog sperm [53].

The endpoint for capacitation is the attainment of spermatozoa, which are able to perform acrosome reaction in the presence of the oocyte. Thus ,the success for a concrete in vitro capacitation procedure will be marked by its capacity to produce sperm that would attain acrosome reaction after appropriate stimulation. Our results indicate that incubation in the l-CCM is able to produce a significant percentage of spermatozoa able to gain acrosome reaction in the presence of oocytes, thus indicating the success in the attainment of in vitro capacitation. This is clearly indicated, first, by the high number of sperm attached to oocytes and, secondly by the very high values of altered acrosomes observed which were accompanied by relatively high percentages of viable and motile sperm. This indicates that the loss of acrosomes was not a direct consequence of the death of the sperm. Moreover, the other indicators, such as motion parameters and lectin distribution in sperm detached from oocytes, are fully compatible with the attainment of acrosome reaction. All these data, together, indicate the establishment of a functional acrosome reaction in a very significant number of sperm.

The acceptance of the ability of dog sperm to attain in vitro capacitation in a glucose-free medium would seem to create, at first glance, a paradox in the commonly established theory about the absolute necessity of glucose to attain feasible levels of in vitro capacitation. However, the results can be explained if we consider the peculiar characteristics of dog-sperm energy metabolism. These cells are able to store relatively elevated intracellular glucose levels in the form of metabolites like glycogen or even glucose 6-phosphate, and the intracellular levels of these metabolites remain high for a relatively long time without the addition of exogenous sugars [43]. This indicates that dog spermatozoa are able to maintain elevated intracellular levels of glucose-derived metabolites despite the lack of extracellular sugars. Thus, we infer that the attainment of a functional in vitro capacitation by dog sperm depends more on their intracellular levels of glucose-derived metabolites than on the presence of extracellular glucose itself. This would also explain the existence of contradictory results in the bibliography about lack of in vitro capacitation in the absence of extracellular glucose [36], and, in fact, differences in the dog sperm intracellular levels of metabolites like glycogen and glucose 6-phosphate would explain these discrepancies. Furtherm more, these differences could be the result of either different metabolism rates of freshly obtained spermatozoa or different capacitation medium composition. In any case, we can indicate that the l-CCM medium is well designed to allow the attainment of in vitro capacitation of dog sperm, although the mechanism(s) by which this medium is effective are not known.\

5. Sperm Cryopreservation and Thawing

Semen cryopreservation is a useful tool for extending the availability of spermatozoa for long or short periods (frozen or chilling, respectively); nevertheless, cryopreservation has been associated with reduced fertility. It has been hypothesized that during cryopreservation, the sperm acquire capacitation-like changes [54-58] and these changes are frequently cited as a factor associated with the reduced longevity in vivo [57] and in vitro [59]. While alterations to the acrosome and plasma membrane have been demonstrated after chilling and freezing steps [60, 61], damage appears to occur first during the dilution and cooling [62, 63]. Induction of cryocapacitation has been attributed to plasma membrane reorganization and to increased intracellular calcium levels [58, 50]. Cryopreservation has also been shown to change the ability of spermatozoa to regulate internal Ca^{2+} [64, 55].

These changes in cryopreserved sperm may not only affect the final percentage of fertilized oocytes, but also the time course of sperm penetration through the oocyte envelop as reported previously in frozen/thawed sperm from other species [65, 56].

On the other hand, early studies of canine gamete interaction in vitro, suggested that the maturation stage of dog oocytes does not affect sperm penetration [36, 66], which has been corroborated in later reports [10]. However, even though fresh [36, 10] or cryopreserved dog sperm [67, 10] are able to bind and penetrate canine oocytes in vitro regardless their maturation state, it is unclear if the dynamic of this process is modified by the maturation state of the oocyte.

Most current protocols for sperm cryopreservation and thawing in felids, involve slow initial cooling to retain acrosomal integrity and gradual addition of hypertonic cryopreservation medium to maintain sperm motility before freezing and then (following thawing) slow dilution of cryoprotectant with culture medium to preserve plasma membrane structure and sperm motility [69, 70, 71].

In 2009, De los Reyes et. col. have published a study with the aim to compare the time course of frozen/thawed, chilled/rewarmed or fresh dog sperm in the penetration of the zona pellucida of immature and in vitro mature canine oocytes throughout different co-culture periods. Studies utilizing the chlortetracycline (CTC) assay with cryopreserved dog semen, have demonstrated a significant increase in the number of capacitated sperm between 0 and 2 h of incubation in capacitating medium [72].

Cryopreservation of canine semen using powder coconut water (ACP-106®) extender had only been evaluated by gross evaluation and the post-thaw values for these characteristics were acceptable for artificial insemination [73]. ACP-106® can be another alternative for canine semen freezing as it presents good extender characteristics such as atoxicity, isotonicity,

buffering system and elements necessary for sperm metabolism. However, other more accurate methods are necessary to check the efficiency of ACP-106®, as a combination of tests measuring different aspects of sperm function provides information about several different sperm characteristics required for fertilization. The commercialization of ACP-106® will take place earlier if its efficiency is confirmed. The aim of the study was to evaluate the functional status of cryopreserved dog spermatozoa that had been frozen in ACP-106® extender by means of sperm–oocyte interaction assay (SOIA) [74].

Cryopreservation procedures must be adaptable to suboptimal conditions frequently encountered with mobile laboratory applications, especially for the collection and freezing of spermatozoa from wild (free-ranging) males in the field. For example, some cryopreservation procedures involve cooling the spermatozoa to -58°C before adding glycerol and loading straws, but it is difficult to maintain stable sample temperature (-58°C) while performing these steps unless a walk-in cold room is available [8, 15]. Similarly, liquid nitrogen stored in an insulated dewar is less labile than dry ice, extending its availability for sperm cryopreservation procedures in remote areas. From a functional perspective, spermatozoa frozen in straws in liquid nitrogen vapor have been assessed using homologous and/or heterologous IVF in several cat species, resulting in fertilization percentages that are similar or superior to those following IVF with spermatozoa frozen in pellets [2, 7, 8, 10].

6. Conclusions

Most of these studies have been focused on farm animals, and the development of new tests, for evaluation of canine ejaculates, have received little attention compared with other species, including humans.

Although canine biotechnologies are being developed at a much lower rate than in other species, research in the last years has substantially increased and most of the reproductive mysteries of this species will probably be unveiled in the near future.

Dog semen presents a great heterogenity, especially compared with other domestic species as bulls and boars. This fact, makes difficult the morphological classification of dog spermatozoa, but makes dog sperm as a valuable model for the study of human semen.

In vitro capacitation of dog spermatozoa can be efficiently achieved in a medium without sugars. The capacity to achieve in vitro capacitation in these conditions seems to be linked to the intracellular levels of glucose-derived metabolites that dog sperm can accumulate, and, thus, the medium is able to maintain these levels enough time to induce capacitation.

Sperm morphometry has been claimed to be one of the strongest indicators of semen quality, as the sperm cell is considered mainly a highly specialized transporter of the male genome in the sperm head. Modifications of the head may reflect anomalies in the DNA content of the spermatozoa. The development of standardized protocols of computerized analysis of sperm morphology is being considered a high priority for the investigation of human semen.

It was observed a great variability in all SCSA-derived parameters among dogs and ejaculates. Linear regression analyses revealed significant relationships among some sperm shape- derived factors and the percentage of denatured DNA in each individual spermatozoon (at).

The SCSA has been demonstrated to be a powerful tool to assess the fertilizing potential of a semen sample in humans and animal models.

Dog semen presents a great heterogenity, especially compared with other domestic species as bulls and boars. This fact makes difficult the morphological classification of dog spermatozoa. The inclusion of CASMA (Computer-Aided Sperm Motility Analysis) systems in the andrological evaluation of the canine spermatozoa may be a valuable tool to standardize the canine spermiogram.

Cryopreservation in straws appeared preferable to cryopreservation in pellets on dry ice, resulted in superior motility at the initiation of culture and tended to improve acrosome status compared with sperm pelleting.

Direct contact with dry ice produces more rapid freezing than exposure to liquid nitrogen vapor, and sperm pellets are thawed directly in warm culture medium, resulting in an immediate rapid dilution of the cryoprotectant solution. In contrast, semen cryopreserved in sealed straws are frozen more slowly and then thawed intact, allowing a gradual controlled rate of cryoprotectant dilution,

which is known to minimize loss of sperm motility and membrane integrity. Straw freezing also is the more ‘‘field friendly’’ of the two methods and provides superior biosecurity against potential pathogens.
In felids, cryopreserved spermatozoa typically undergo capacitation more rapidly than nonfrozen samples.

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