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New techniques for the assessment of canine semen quality: A review

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Abstract

Until recently, canine semen assessment was routinely performed by conventional light microscopic techniques. The limitations of these methods include subjectivity, variability, the small number of spermatozoa analyzed, and poor correlation with fertilizing potential. The last decade, several new in vitro techniques have been introduced for canine semen assessment that enable a more detailed evaluation of several sperm characteristics. Numerous fluorescent staining techniques have been developed for the evaluation of specific sperm characteristics and functions, including plasma membrane integrity, capacitation status and the acrosome reaction. By combining fluorescent stains, several functional sperm characteristics can be assessed simultaneously. Moreover, by means of flow cytometry, large numbers of fluorescently labelled spermatozoa can be analysed in a short interval. Following thorough standardization and validation, computer-assisted sperm analysis systems provide objective and detailed information on various motility characteristics and morphometric dimensions that cannot be identified by conventional light microscopic semen analysis. In vitro assays, evaluating the capacity of canine spermatozoa to bind to the zona pellucida or oviductal explants, or to penetrate the oocyte, provide additional information on canine gamete interaction that may be useful in predicting the fertilizing potential of spermatozoa. Although substantial improvements have been made in canine semen assessment, surprisingly few parameters were correlated with in vivo fertility. Therefore, further research is required to determine which sperm characteristics are of clinical value for predicting the in vivo fertility in dogs.

Keywords: Dog semen; Sperm quality; Fluorescent staining; CASA; Zona pellucida binding

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1. Introduction

Although evaluation of conception rates remains the ultimate test to assess the fertilizing capacity of canine semen, this method is time-consuming and results in the birth of unwanted puppies [1,2]. Therefore, alternative methods to assess the functional capacity of spermatozoa in vitro are required. Until recently, light microscopy was routinely used to evaluate the principal parameters of dog semen, i.e. concentration, motility and morphology [3]. Concentration is usually determined using a counting chamber [3]. Motility is assessed subjectively on a pre-warmed glass slide [3–5], whereas morphology is assessed with various staining techniques [1,3].

The primary limitations of light microscopical methods are subjectivity and variability [1,5,6]. Visual sperm motility assessment is difficult and is influenced by the temperature and the evaluator's skills, leading to high variability among laboratories and observers [7]. Assessment of morphology depends on the fixation and staining technique [8,9], the quality of the microscope, and the observer's experience. An additional source of laboratory variation is the relatively low number of spermatozoa analysed with these conventional techniques [10]. Furthermore, these parameters often have poor correlation with fertility potential, making the interpretation of the data very difficult [6,11–13]. Although several reports related specific sperm defects to canine infertility [14–16], actual threshold values below which in vivo fertility is impaired, are rarely described for dogs [1].

Recently, several techniques have been described which may enable more accurate prediction of the fertilizing capacity of a canine semen sample [2,17]. These new techniques allow for the assessment of several functional characteristics of spermatozoa which are related to the capacity to reach, bind, penetrate, or fertilize an oocyte [2,17]. Additionally, conventional light microscopic semen assessment is increasingly being replaced by fluorescent staining techniques, computer-assisted sperm analysis (CASA) systems, and flow cytometry [18,19]. Therefore, the aim of the present review is to discuss several recent techniques for the assessment of canine semen quality.

2. Recent techniques for assessment of canine sperm quality

To fertilize an oocyte, a sperm cell should have the capacity to perform several functions since successful fertilization requires a succession of reactions. Recently, considerable information has been accumulated on the assessment of several sperm functions allowing for a more detailed evaluation of dog sperm quality [17]. These new techniques can roughly be divided into five different categories: (1) fluorescent stains; (2) computer-assisted sperm analysis; (3) zona pellucida-binding assay; (4) oocyte penetration assay/in vitro fertilization; and (5) sperm–oviduct interaction.

2.1. Fluorescent stains

During the last decade, numerous fluorescent staining techniques have become available for the assessment of specific sperm functions in dogs, such as the integrity of the plasma membrane, the capacitation status and the acrosome reaction.

2.1.1. Membrane integrity

The integrity of the plasma membrane is essential for the fertilizing capacity of spermatozoa. Until recently, the membrane intactness of dog spermatozoa was routinely assessed by means of light microscopic stains, such as eosin/nigrosin [20,21] or trypan blue [22]. The major problem with these techniques is that spermatozoa may show partial staining, making interpretation difficult [23]. Furthermore, several ingredients, such as glycerol or fat globules (present in most cryopreservation media), may interfere with these stains [24]. An indirect method to evaluate the membrane integrity is by exposing spermatozoa to hypo-osmotic conditions (i.e. the hypo-osmotic swelling test) [25], since the number of swollen spermatozoa was shown to be inversely proportional to the number of spermatozoa with damaged membranes [26].

During the last decade, several fluorescent dyes were used and validated for the assessment of the sperm membrane integrity in dogs: carboxyfluorescein diacetate (CFDA) in combination with propidium iodide (PI) [10,27], SYBR-14 in combination with PI [28,29], carboxy-seminaphthorhodfluor (Carboxy-SNARF) in combination with PI [8], calcein-AM in combination with ethidium homodimer (Calcein-AM/EthD-1) [30] and Hoechst 33258 [31]. Both CFDA and SYBR-14 are membrane-permeant, non-fluorescent compounds which are rapidly converted by intracellular esterases into highly fluorescent, membrane impermeant green fluorophores which are maintained intracellular by intact membranes [10]. Upon cell death, spermatozoa lose their ability to resist the influx of red fluorescent PI, which replaces or quenches CFDA or SYBR-14 [32]. Viable and intact spermatozoa membranes show a green fluorescence (CFDA; SYBR14; Calcein) whereas the membrane of damaged (dead) spermatozoa stain red (PI; EthD-1) [10,28]. Carboxy-SNARF-1 is an intracellular pH indicator staining live spermatozoa orange [8]. The bisbenzimide stain Hoechst 33258 labels non-viable (dead) spermatozoa bright blue, whereas viable cells are not stained [31].

When comparing SYBR14-PI with eosin/nigrosin, Rijsselaere et al. [28] were inclined to consider SYBR14-PI as the most sensitive method, since minor membrane damages caused by centrifugation could be detected by this fluorescent technique but not with conventional eosin/nigrosin staining. Previous studies on fowl [33] and bovine [34], using known proportions of killed and viable spermatozoa, also confirmed the efficacy of SYBR14-PI to detect dead spermatozoa. The major advantage of fluorescent staining techniques is the possibility to analyse fluorescently labelled spermatozoa by means of flow cytometry, enabling the evaluation of larger numbers of spermatozoa in a short interval. Peña et al. [10,18] found high correlations between flow cytometry and epifluorescence microscopy for the percentage of live and dead spermatozoa as determined by a CFDA-PI staining. Additionally, these fluorescent methods were highly correlated with the results obtained from eosin/nigrosin staining. The proportion of non-viable cells labelled with Hoechst 33258 was highly correlated with the dead spermatozoa stained with eosin/nigrosin, if Hoechst 33258 was added at a concentration of 70 μg/mL [10]. The results obtained by the combination SNARF/PI were highly correlated with the CFDA/PI staining [18]. Other advantages of fluorescent stains are the possibility to assess the function of the semen sample without the interference of fat globules or other non-stained material [10] and that differences between the sperm populations are more consistent and clear [28]. Furthermore, several functional characteristics (e.g. membrane integrity and

acrosomal status) may be evaluated simultaneously, e.g. by combining these stains with the fluorescently labelled lectines FITC-PSA or FITC-PNA [8]. Finally, using SYBR14-PI, a more subtle distinction between spermatozoa can be made since three cell populations can be identified (live, dead, and moribund spermatozoa), whereas the conventional eosin/nigrosin stain only discriminates between two groups (live and dead spermatozoa) [32].

2.1.2. Capacitation status

Although spermatozoa are motile upon release from the male genital tract, they require a complex sequence of membrane alterations, known as capacitation, before they can fertilize an oocyte [31,35]. Once they are in contact with the oocyte, capacitated spermatozoa are able to undergo the acrosome reaction [31].

In dogs, several methods have been described for the evaluation of the capacitation status of spermatozoa: the chlortetracycline assay (CTC) [28,36,37], a fluoresceinated lectin stain [38], and measurements of sperm motility characteristics by a CASA system which are indicative of sperm capacitation or hyperactivation [37]. Recently, Petrunkina et al. [35,39] described the use of the fluorescent staining 'Cy3-conjugated anti-mouse IgG' to study the kinetics of tyrosine phosphorylation, a crucial step in capacitation.

The fluorescent antibiotic CTC-staining has been utilized to assess the degree of destabilization of the sperm membrane. Upon entering the sperm cell, CTC binds free calcium ions (Ca^{2+}). These CTC-Ca^{2+} complexes become fluorescent and bind to hydrophobic regions of the cell membrane [36]. In addition to the determination of acrosomal status, CTC staining enables the spermatozoa to be designated as capacitated or uncapacitated [31,37]. As originally described in mouse [40] and confirmed in several other mammalian species, canine spermatozoa stained with CTC display three fluorescent patterns: uncapacitated and acrosome intact (F-pattern), capacitated and acrosome intact (B-pattern), and capacitated and acrosome reacted (AR-pattern) [31]. By combining this CTC staining with Hoechst 33258, the sperm viability can be assessed simultaneously making sure that only viable spermatozoa are evaluated for capacitation status [31,37,41].

2.1.3. Acrosomal status

The acrosome reaction is a prerequisite for the successful penetration of the oocyte. By fusion of the sperm plasma membrane with the outer acrosomal membrane, spermatozoa release their acrosomal enzymatic content by a modified form of exocytosis, enabling them to penetrate the zona pellucida [42].

Acrosomal status can be determined with several non-fluorescent stainings such as eosin/nigrosin [21], Giemsa [43], Trypan blue/Bismark brown/Rose Bengal [44] or Spermac® [1,45]. Recently, several fluorescent dyes have been validated which are considered, due to greater contrast, to be superior for the discrimination between acrosome intact and acrosome damaged spermatozoa [46]. Acrosomal status is mostly shown by lectins, which are conjugated with a fluorescein isothiocyanate, such as Peanut Agglutinin (FITC-PNA) [30,47,48] or Pisum Sativum Agglutinin (FITC-PSA) [8,9,42,48]. These lectins bind specifically to the acrosomal contents by interacting with the glycoconjugates of the outer acrosomal membranes (Peanut agglutinin, PNA) [30,47] or with the saccharide groups of the glycoprotein pro-acrosin (Pisum sativum agglutinin, PSA) [9]. FITC-PSA has been reported to stain the acrosomal region of canine testicular [48], epididymal [49] and ejaculated [38,50]

spermatozoa, demonstrating that the lectin-binding capacity does not change during epididymal transport. Labeling permeabilized spermatozoa with FITC-PSA or FITC-PNA renders acrosome-intact cells brightly fluorescent over the entire acrosomal region of the sperm head, while acrosome-reacted spermatozoa have either no acrosomal labeling, or only have FITC fluorescence of the equatorial segment [30,42]. Indirect immunofluorescence using monoclonal or polyclonal antibodies [42,51] and CTC-staining [36] have also been used to evaluate the acrosomal status of dog spermatozoa. FITC-PNA and FITC-PSA have been successfully combined with fluorescent stains, such as Hoechst 33258 [42], carboxy-SNARF/PI [8] and ethidium homodimer [47], allowing for the simultaneous assessment of the membrane integrity by epifluorescence or flow cytometry.

Assessment of acrosomal status, as determined by a FITC-PSA stain, correlated significantly with that of samples stained with Spermac® [18], the triple staining Trypan blue, Bismark brown and Rose Bengal [44], and with indirect immunofluorescence with an antisperm antiserum [42]. Guérin et al. [36] suggested that the CTC staining was a more reliable method for the detection of acrosome reaction than Spermac®, since the latter stain systematically overestimated the percentage of acrosome reacted spermatozoa when compared with CTC staining. Flow cytometric assessment of the acrosomal status by a FITC-PSA/PI staining was highly correlated with the results obtained with Spermac® [9]. However, some of the initially reacting spermatozoa were undetected by the Spermac® stain, whereas FITC-PSA bound sufficiently to be detected by epifluorescence microscopy. Additionally, Peña et al. [9] found that epifluorescence microscopy was less precise than flow cytometry for detecting the percentage of spermatozoa with damaged acrosomes, probably due to the difference in sample size.

The percentage of sperm cells having an intact acrosome and being able to undergo the acrosome reaction upon triggering by treatment with calcium ionophores [47] or lysophosphatidyl choline [18] is regarded as an important semen characteristic in dogs. Interestingly, in dogs, the artificial induction of the acrosome reaction of a fresh semen sample with calcium ionophore may be useful to predict whether the sample can be successfully cryopreserved [47].

3. Computer-assisted sperm analysis (CASA)

The high variations reported in the estimation of sperm motility [52–54] and morphology parameters [55,56] of the same ejaculate assessed by different observers implicate an absolute need for objective and standardized methods, both for clinical and research purposes [7,57]. This has led to the development of several semi-computerized [5,58,59] and computerized measuring devices [5,7,57,60–62] for the evaluation of canine motility, morphology, and concentration. The use of CASA systems for veterinary purposes has recently been discussed in a review by Verstegen et al. [19].

3.1. Motility assessment

During the last decade, several commercial CASA systems (i.e. CellSoft computer videomicrography, Strömberg-Mika Cell motion analyzer, Hobson Sperm Tracker, and

Hamilton–Thorne), based predominantly on individual spermatozoon assessment, were validated for use in dogs [5,7,57,60,61]. These systems offer an accurate and rapid calculation of different semen parameters, such as total and progressive motility, slow, medium and rapid moving spermatozoa, linearity of sperm movement, the beat cross frequency, the amplitude of the lateral head displacement, and several velocity parameters [5,7,57,60,61]. Several studies found high correlations between the computer-calculated motility, progressive motility and concentration, and the conventional light microscopic evaluation [7,60,61]. Additionally, these computerized measuring devices proved to be useful to assess various semen characteristics simultaneously and objectively, and are valuable for the detection of subtle changes in sperm motion that could not be identified by conventional semen analysis [19,37,60,61]. Finally, large numbers of spermatozoa can be analyzed individually in a short interval [5,61].

The main problems using these computerized measuring devices are the high investment costs and the extreme need for standardization and validation of the system before any practical use is possible [7,19,57,61]. Indeed, the choice of internal image settings (e.g. minimum contrast, frame rate, analysis time), which are important to identify and reconstruct the trajectory of the different spermatozoa accurately, clearly influences the results obtained [57,60,61]. In dogs, significant alterations of the motility characteristics measured by CASA systems have been described due to the dilution of the semen sample [57,61], the diluent used [57,61], the analysis temperature [7,57] and the counting chamber [7]. Once these systems have been standardized, a high degree of repeatability can be achieved with inter- and intra-assay coefficients of variation of $<12\%$ for most parameters [7,57].

As a consequence, the computer parameters selected, the software used and the microscopy conditions might lead to a new source of subjectivity among laboratories. These technical settings should, therefore, be standardized [7,57,61]. However, due to the lack of uniformity among users and due to the use of different instruments, a definition of standard accepted values for motility and sperm velocity is difficult to determine in dogs [19]. Moreover, standardization of the technical settings is required in order to compare results among laboratories and andrologic centers, which is becoming increasingly important as international exchange of diluted and frozen dog semen expands [60]. Although Iguer-ouada and Verstegen [7] and Rijsselaere et al. [61] provided datasets of CASA measurements obtained from proven fertile dogs that could serve as preliminary references, the specific sperm movement characteristics that are of clinical value for the prediction of in vivo fertility in dogs are yet to be determined.

Recently, an easy to use and inexpensive device, the Sperm Quality Analyzer (SQA), was introduced and validated for the assessment of fresh [5,59] and cryopreserved dog semen [59]. This semi-automated instrument detects variations in optical density, resulting from a light beam passing through a capillary tube with moving spermatozoa. A photometric cell registers these fluctuations in optical density and converts this information, through mathematical algorithms, digitally to a numerical output called the sperm motility index (SMI). The manufacturer claims the SMI expresses the overall sperm sample quality, taking into account the concentration, the progressive motility and the percentage spermatozoa with normal morphology. The results obtained by the SQA were compared with Hamilton–Thorne measurements [5] and with conventional light microscopical evaluation [59]. The device displayed a good repeatability of measurements

for semen of medium and high quality, whereas higher variations were obtained for poor quality dog semen [5,59]. For sperm concentrations below 150–200 $\times 10^6$ mL^{-1}, highly significant correlations were established between SMI values and the sperm concentration, and several semen parameters assessing overall semen quality, respectively [5,59]. At higher sperm concentrations, the system appeared to saturate, probably because spermatozoa are so condensed, making free movement within the capillary more difficult [59]. However, whether the correlation between SMI and in vivo fertility in dogs has not been determined.

3.2. Morphometry

Automated sperm morphometric evaluation has the potential to eliminate several drawbacks inherent to the current methods of sperm morphology evaluation, and allows for the identification of subtle sperm characteristics that cannot be detected by visual light microscopic evaluation. Previously, canine sperm morphology assessment has been attempted by means of scanning (SEM) and transmission electron microscopic (TEM) images [1,63–65]. These studies mainly evaluated membrane and acrosomal damage that occurred following cryopreservation, elucidating major changes in the acrosomal region after freezing and thawing. Afterwards, several canine sperm head dimensions (length, width, area, roundness) were measured automatically using a Leica computer system and were compared with the results obtained by TEM [43]. Recently, the Metrix Oval Head Morphology software (Metrix) was implemented in the Hamilton–Thorne (HTR) and validated for the evaluation of dog sperm morphometric dimensions and morphology [62]. This system provides very detailed information on sperm head dimensions (length, width, area, roundness, perimeter), tail length and tail abnormalities (bent, coiled, absent). Based on these studies, dog sperm heads appeared to be smaller than bull, ram, goat and rabbit sperm heads but, interestingly, were larger than horse sperm heads [43,62]. Although Dahlbom et al. [43] provided data for proven fertile dogs, large variations in sperm morphometry were reported among individual dogs, suggesting that the evaluation of larger numbers of dogs might be necessary to obtain a reliable and accurate estimation of the normal limits of canine sperm dimensions [43,62]. Furthermore, validation and standardization of these systems before use are required, as the magnification level of the objective, sperm concentration and the staining method influence most of the morphometric dimensions obtained [43,62]. High correlations were established for the percentage of normal spermatozoa assessed by light microscopic evaluation (eosin/nigrosin and Diff-Quick-staining) and by the HTR-Metrix [62]. However, most of these automated systems are very time-consuming [43,62]. Additionally, further research is needed to determine which sperm morphometric parameters are correlated with the in vivo fertility in dogs.

4. Zona pellucida binding assay

Sperm binding to the zona pellucida (ZP) is a crucial step in fertilization [66]. Therefore, in vitro tests investigating the ability of spermatozoa to bind to the homologous ZP may be useful in predicting the fertilizing potential of spermatozoa. Moreover, since ZP

binding is receptor-ligand mediated, the zona binding assay is believed to detect sperm damage at a molecular level which is not visible by conventional semen analysis techniques [67,68].

The zona binding capacity of canine spermatozoa has been evaluated by two types of assays: one using intact homologous oocytes (ZP-binding assay, ZBA) [66,68,69], the other using bisected hemizonae (hemizona binding assay, HZA) [13,67]. Briefly, in the ZBA, spermatozoa are co-incubated with oocytes that are usually obtained from sliced ovaries removed at ovariohysterectomy [66,70]. Subsequently, the number of spermatozoa bound to the ZP is counted using phase-contrast [70] or fluorescence microscopy [66,71]. Using the ZBA, Ström Holst et al. [69] showed that binding of dog spermatozoa to the ZP is a specific feature of living spermatozoa. Since canine oocytes are not routinely available, several types of oocyte storage have been evaluated [66,69]. Salt-stored oocytes and oocytes from frozen–thawed ovaries appeared to have a reduced sperm binding capacity compared to that of fresh oocytes [66,71,72]. Attachment of spermatozoa to the zona did not differ significantly between fresh and cooled (5 °C) oocytes [71]. Another drawback of the ZBA is the variation of sperm binding capacity among oocytes, requiring large numbers of oocytes and replicates [13,68,69]. This variation can partly be overcome by the HZA. Using this assay, the canine oocyte is bisected by means of micromanipulation and the dense ooplasma is dislodged [13,67]. The two parts of the zona are subsequently incubated with a semen sample and the number of bound spermatozoa is determined using phase-contrast microscopy [13,67]. The advantages of the HZA are the possibility to compare the binding capacities between a control male and a test male, and that the zonae may be stored either chilled or frozen [13]. Significant differences in sperm binding capacity between infertile and fertile dogs [13], and between fresh and frozen-thawed spermatozoa have been described using the HZA [67]. However, the HZA is time-consuming and technically more demanding [68], and various factors may affect the results, such as the quality of the zona and the concentration of motile spermatozoa used in the assay [13]. Moreover, in humans the HZA is recently questioned since the sperm-binding capacity of oocytes appears to be unevenly distributed, which is in contradiction with the basic assumption of the HZA that the sperm binding affinity is evenly distributed along the zona [68,73].

5. Oocyte penetration assay and in vitro fertilization

The ultimate laboratory test is to assess whether spermatozoa are capable of fertilizing oocytes in vitro since pronuclear formation requires both zona binding and penetration, and sperm head decondensation [6]. In several mammalian species, in vitro fertilization (IVF) of matured oocytes is well established and used routinely [74–78]. In dogs however, in vitro maturation (IVM) and fertilization (IVF) of oocytes is difficult to achieve (for review see [79,80]) and, despite the numerous attempts to improve the results (for review see [17,80]), the percentages of canine oocytes which are fertilised in vitro are generally very low [6,41,71,81–84]. Additionally, further embryonic development did not occur except in studies performed by Yamada et al. [83] and Rodrigues et al. [41] in which, respectively 2 and 0.75% of the fertilised oocytes reached the eight-cell stage. Recently, one study produced a single blastocyst [85] and England et al. [86] reported a pregnancy following

IVF in the bitch with subsequent embryo resorption. The use of intracytoplasmatic sperm injection (ICSI), involving the injection of one sperm cell directly into the ooplasm of the oocyte, has been described in dogs by Fulton et al. [87], yielding 8% of oocytes with both male and female pronuclei.

Unlike most other mammalian species, capacitated dog spermatozoa are able to penetrate immature oocytes [6,81] subsequently inducing chromatin decondensation [88] and resumption of meiosis [89]. Consequently, in dogs, both immature and in vitro/in vivo matured oocytes could be used for an in vitro penetration test [6,71,90]. However, due to the difficulties associated with the in vitro maturation of canine oocytes, the use of immature oocytes has been better documented [72]. Moreover, no significant differences were found in oocyte penetration following sperm interaction with mature and immature oocytes, demonstrating that culturing oocytes has no beneficial effect on this penetration test [17,81]. The zona pellucida/oocyte penetration test (OPT) evaluates by means of fluorescence (e.g. Hoechst 33258 or PI in combination with WGA-FITC) [6,71,72,90] or light microscopy (e.g. aceto-orcein) [6] the presence of canine sperm heads in the perivitelline space and ooplasm of the oocyte after several hours of sperm–oocyte co-incubation [6]. To avoid ambiguity about the location of spermatozoa (i.e. bound or penetrated) the observations obtained by fluorescence microscopy were compared with those obtained by confocal microscopy, confirming that fluorescence microscopy correctly identified 98% of the spermatozoa that had penetrated the oocyte [91]. Higher levels of ZP penetration were observed in oocytes with an intact cumulus oophorus, suggesting an important role of cumulus cells in canine gamete interaction [72].

The OPT is a less time-consuming technique than IVF, since maturation of the oocytes is not required and only the penetration of the oocyte is evaluated and not the further development [6]. Sperm penetration of the zona and/or the ooplasm was shown to be reproducible [71] and to be closely associated with the sperm motility [90]. No correlation was found between the acrosomal status of the spermatozoa and sperm penetration [6,41]. Additionally, the OPT proved to be a useful alternative method to evaluate several freeze-thawing and chilling procedures for canine semen preservation more effectively [90] and might even provide an alternative for the assessment of the sperm quality of wolves [71]. Indeed, preliminary investigations suggested that oocytes from domestic dogs can be used to evaluate the sperm quality from wild canidae such as the timber wolf (*Canis lupus*) and the red wolf (*Canis rufus*) [71].

6. Sperm–oviduct interaction

The fertile lifespan of spermatozoa in the reproductive tract of the bitch is considerably longer than in most other domestic species [92,93]. To remain functionally competent until the time of fertilization, storage of spermatozoa in a sperm reservoir is required [93]. Several in vivo studies reported that the major sperm reservoirs in the dog are located in the uterine crypts [94,95] and the utero-tubal junction [95,96]. However, several authors suggested that the sperm reservoir might be located in the oviduct since a number of in vitro studies showed that sperm survival was prolonged by co-incubating canine spermatozoa with homologous [39,97–99] or heterologous [35] oviductal epithelium.

One can imagine that if a dog is unable to populate the sperm reservoir with a sufficient number of spermatozoa, this might interfere with its fertility. Additionally, dogs may differ in their capacity to establish a sperm reservoir. In vitro tests investigating the interaction of spermatozoa with oviductal explants might, therefore, not only provide information about the physiology and mechanisms of sperm–oviduct binding in dogs [35,39,97–99] but may also be useful to evaluate and compare the binding capacity of individual ejaculates [39]. Our laboratory recently optimised a reliable in vitro approach to study sperm binding to oviductal epithelium in bovine [100]. By means of this in vitro model, it was shown that the capacity of spermatozoa to bind to oviduct explants varied among bulls and that the number of spermatozoa bound to oviduct epithelial explants was positively correlated with the non-return rates (i.e. preliminary pregnancy rates) [100]. Despite the fact that various aspects of sperm–oviduct binding (i.e. effect of stage of the oestrous cycle, effect of the region of the oviduct, homologous versus heterologous oviductal epithelium) [35,39,98,99] have been investigated in dogs, the capacity of canine spermatozoa to bind to oviduct explants has not yet been correlated with in vivo fertility. Moreover, regarding the conflicting findings on the location of the sperm reservoir in the dog, it might also be interesting to evaluate whether canine sperm interaction with epithelial explants from the uterine body or uterine horn also leads to a prolonged flagellar activity and viability of spermatozoa and whether this in vitro model is correlated with the in vivo fertility in dogs [95].

7. Conclusion

The last decade, canine semen quality assessment has progressed considerably. Several new techniques have been introduced which allow for a very detailed and rapid evaluation of various specific sperm functions that cannot be visualized by subjective light microscopic techniques. Moreover, by means of CASA systems or flow cytometry, large numbers of spermatozoa can be analysed simultaneously. However, few studies in dogs have correlated sperm characteristics with in vivo fertility or defined threshold values. Since successful fertilization is the result of a succession of various reactions, laboratory assays which assess a single sperm function are unlikely to accurately predict potential fertility. A combination of several sperm quality measurements probably has a better correlation with in vivo fertility [27] than any single measurement. Therefore, futurer research in canine andrology should determine which sperm characteristics are of the most useful for the prediction of in vivo fertility. The difficulty in establishing correlations with in vivo fertility in the dog, however is the rather limited number of breedings per dog in comparison with other species (e.g. cattle), and the high incidence of conception failure due to poor breeding management in dogs, which frequently results in mistimed breedings [101].

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