Chromatin Assays for DNA Fragmentation Evaluation in Canine Sperm

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Abstract: The aims of this study was to evaluate efficacy of detecting male infertility in dogs using conventional Computer Assisted Sperm Analysis (CASA) for morphological analysis of spermatozoa and the Sperm Chromatin Structure Assay (SCSA) for level of DNA fragmentation in the spermatozoa. This study found percentage of morphological abnormalities and motility in infertile dogs were statistically significantly poorer than those in healthy control (p<0.05). Also percent DNA Fragmentation Index (DFI%) were significantly higher in infertile dogs. There is correlation between morphological mortality indices and DFI%. This result strongly suggested that assays such as SCSA could be a good alternative test for canine male infertility and for evaluating quality of sperm before artificial insemination as reported previously.

Key words: DNA fragmentation, dog, infertility, SCSA, sperm viability, canine, morphology

INTRODUCTION

Poor semen quality can reduce litter size or result in conception failure. Therefore, semen evaluation is the single most important test for male infertility in dogs. The semen evaluation includes spermatozoan concentration, motility characteristics and morphological classification of spermatozoa. The proportion of abnormal size and shape of spermatozoa has been associated with clinical infertility and conception failure.

Although, the methods for evaluation of male infertility have typically been limited to conventional semen analysis (i.e., spermatozoan concentration, motility and morphology of the sperm, recent studies suggest that sperm with certain levels of DNA fragmentation serve as a strong predictor of reduced integrity of spermatozoa male fertility (Evenson and Jost, 1994; Evenson et al., 1999). According to those researches, sperm with high levels of DNA fragmentation have a lower probability of producing a successful conception. Also human studies found that patients with a DNA fragmentation level of >30% are likely to have significantly reduced fertility potential including a significant reduction in term pregnancies and a doubling of miscarriages (Spano et al., 2000; Boe-Hansen et al., 2006).

There are several methods for evaluating sperm DNA fragmentation. The Sperm Chromatin Structure Assay (SCSA) measures the intensity of Acridine Orange (AO) fluorescence using flow cytometry (Evenson and

Jost, 1994). AO fluoresces green when binding to native DNA and red when it binds to the fragmented DNA. The ratio of red/red+green yields the percentage of DNA fragmentation, referred to as DNA Fragmentation Index (DFI).

In this study, the accuracy of SCSA assays over conventional Computer Assisted Sperm Analysis (CASA) is evaluted in male dogs with healthy or abnormal fertility.

MATERIALS AND METHODS

Semen collection and evaluation of fresh semen: The sperm-rich fractions of ejaculates from 5 healthy control dogs (toy dog breeds, age 3.4±2.5, body weight 3.5±3.1 kg) and 5 dogs with history of male infertility dogs (toy dog breeds, age 4.7±1.5, body weight 4.1±2.2 kg) were collected by manual stimulation into prewarmed glass tubes (+37°C) as described by Seager and Fletcher (1972). The motility and viability were assessed in a Computer Assisted Sperm Analyzer (CASA; SpermVision; Minitub, Germany) according to Schafer-Somi and Aurich, (2007). The following motility parameters were assessed: Curvilinear velocity (VCL, μm sec^{-1}), Linear velocity (VSL, μm sec^{-1}), Mean velocity (VAP, μm sec^{-1}), Distance Curved Line (DCL, μm), Distance Straight Line (DSL, μm), Distance Average Path (DAP, μm). For the assessment of spermatozoa viability, 100 μL of semen were placed in a vial with 2 μL of SYBR-14/PI and incubated for 10 min at

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room temperature in darkness. One droplet was placed on a glass slide, covered with a glass coverslip and evaluated via fluorescence microscopy at magnification 400×(Olympus AX70, Japan; U-MWB filter block, BP420-480 excitation filter, BA515 suppressor filter, dichromatic mirror: DM500). The term morphologic aberrations describes the percentages of head, acrosome, neck, midpiece and tail abnormalities inclusive of the percentage of juvenile and round cells as well as double malformations. At least 15 fields were evaluated and the average value was calculated by the CASA. Results were given as percent of membrane intact cells.

Sperm Chromatin Structure Assay (SCSA): The SCSA was done with a commercial DNA fragmentation test (Halosperm®, Halotech DNA SL, Spain) as described in manufacturer's manual. About 500 sperms were manually evaluated with phase array microscope (BX-51, Olympus, Japan) under ×400 scale using DNA fragmentation image analyzing computer software (SAIS plus, Medical Supply Co. Ltd., Korea). Each semen sample and the results were expressed as percent DNA Fragmentation Index (DFI%).

Statistical evaluation: Data were analyzed with Student's t test, Analysis of Variance (ANOVA) and linear regression where appropriate using Sigma Stat program (SPSS, Chicago, Ill). The p value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Spermatozoa concentration averaged 250.3 ± 32.1 mL^{-1} in control dogs (n = 5) and 125.9 ± 38.7 in infertile dogs (n = 5). Abnormalites in head and acrosome were 1.7 ± 2.4 and $4.8 \pm 1.2\%$, respectively in control dogs whereas those were 5.1 ± 4.0 and $15.5 \pm 7.6\%$, respectively in infertile dogs. Percentage of morphologic aberrations averaged $16.6 \pm 7.5\%$ in control dogs and $36.5 \pm 5.3\%$ in infertile dogs (Table 1). Motility (M) and Progressive motility (P) as determined by CASA were $87.2 \pm 12.8\%$ in control dogs and $65.5 \pm 21.7\%$ in infertile dogs. Other motility parameters such as VCL (μm sec^{-1}), VSL (μm sec^{-1}), VAP (μm sec^{-1}), DCL (μm), DSL (μm) and

DAP (μm) were shown in Table 2. For the SCSA, the average DFI% from 5 control dogs was $15 \pm 3.7\%$ whereas that from 5 infertile dogs was $45 \pm 11.7\%$.

Evaluation of DNA fragmentation is alternative method for predicting integrity of spermatozoa (Evenson and Jost, 1994; Evenson *et al.*, 1999) and is widely used for detecting male infertility in humans and domestic animals including dogs (Henkel *et al.*, 2003; Koderle *et al.*, 2009; Eulenberger *et al.*, 2009). There are several methods for evaluating sperm DNA fragmentation. The Sperm Chromatin Structure Assay (SCSA) measures the intensity of Acridine Orange (AO) fluorescence using flow cytometry (Evenson and Jost, 1994). AO fluoresces green when binding to native DNA and red when it binds to the fragmented DNA. The ratio of red/red+green yields the percentage of DNA fragmentation referred to as DNA Fragmentation Index (DFI). TdT-mediated-dUTP Nick End Labeling (TUNEL) assay is another way to evaluate sperm DNA integrity. The TUNEL assay detects both single and double-stranded DNA breaks by labeling the free 3'-OH terminus with modified nucleotides in an enzymatic reaction with Terminal deoxynucleotidyl Transferase (TdT) and can be analyzed microscopically or using flow cytometry. The Sperm Chromatin Dispersion test (SCD) has been recently developed for evaluating sperm DNA fragmentation (Fernandez *et al.*, 2003). The SCD test is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops that is observed in sperm with nonfragmented DNA following acid denaturation and removal of nuclear proteins. The aims of this study was to evaluate efficacy of detecting male infertility in dogs using conventional CASA method for morphological analysis of spermatozoa and SCSA method for level of DNA fragmentation in the spermatozoa.

This study found percentage of morphological abnormalities and motility in infertile dogs were statistically significantly poorer than those in healthy control (p<0.05). Also percent DNA Fragmentation Index (DFI%) were significantly higher in infertile dogs. There is correlation between morphological mortality indices and DFI%. This result strongly suggested that assays such as SCSA could be a good alternative test for canine male infertility and for evaluating quality of sperm before artificial insemination as reported previously (Koderle *et al.*, 2009; Eulenberger *et al.*, 2009).

Table 1: Concentration and morphological aberrations of sperm from control and infertile dogs

Specimen	Conc. (×106 mL^{-1})	Head (%)	Acrosome (%)	Total (%)
Control	250.3±32.1	1.7±2.4	4.8±1.2	16.6±7.5
Infertile	125.9±38.7	5.1±4.0	15.5±7.6	36.5±5.3

Table 2: Motility parameters of semens from control and infertile dogs measured by Computer Assisted Sperm Analysis (CASA)

Parameters	DAP (μm)	DSL (μm)	DCL (μm)	VAP (μm sec^{-1})	VSL (μm sec^{-1})	VCL (μm sec^{-1})
Control	45.1±8.7	42.0±8.3	76.1±3.2	100.1±7.4	89.1±10.7	139.8±20.3
Infertile	38.1±4.1	29.1±2.1	42.8±5.1	75.2±7.8	65.7±3.2	107.0±30.1

CONCLUSION

In the study, the data show that SCSA is sensitive diagnostic tools for detecting DNA fragmentation in sperm and correlates with data from CASA.

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