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Prognostic value of a pre-freeze hypo-osmotic swelling test on the post-thaw quality of dog semen

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ABSTRACT

Throughout cryopreservation, sperm are exposed to major osmotic challenges. Only intact membranes of sperm cells are able to regulate these volumetric changes, which can be determined by the hypo-osmotic swelling test (HOS test). Correlations between the HOS test and conventional semen variables are inconsistent. Therefore, the objectives of this study were (1) to examine relationships between HOS test results and standard semen variables before freezing and after thawing and (2) to evaluate the prognostic value of the HOS assessments on post-thaw quality of dog semen. Semen of 35 dogs was collected and analyzed before freezing and after thawing following a 7-day freeze–thaw interval. Conventional semen variables such as sperm cell motility, membrane integrity morphology were evaluated and the HOS test was conducted with results from this test being recorded. In fresh semen the HOS test was positively correlated with progressive motility of sperm cells: $r = 0.52$, sperm cell membrane integrity: $r = 0.50$ and normal sperm cell morphology: $r = 0.46$ ($P < 0.05$). In frozen–thawed semen, the data obtained with the HOS test were positively correlated with progressive sperm cell motility: $r = 0.67$ and membrane integrity: $r = 0.86$ ($P < 0.05$). The data obtained with the HOS test in fresh semen were positively correlated with sperm cell membrane integrity: $r = 0.50$ normal sperm cell morphology: $r = 0.55$ and data from the HOS test ($r = 0.43$; $P < 0.05$) with frozen–thawed semen. For the prediction of individual cryopreservation capacity, results from assessment of the fresh semen variables of good and poor semen quality were statistically compared. Based on these results, it is not possible to predict the quality of frozen–thawed dog semen using the HOS test.

1. Introduction

Freezing dog semen is an extensive, time-consuming procedure consisting of multiple steps, which have to be followed accurately. In general, cryopreservation of dog semen results in few insemination doses in comparison to freezing semen of farm animals. Successful classification of the ejaculates before freezing to assess expected cryopreservation capacity would be thus be a great asset for use of frozen semen for dog breeding (Pena et al., 2006).

Cryopreservation implies osmotic stress on sperm as does the formation or reshaping of intracellular ice (Dorado et al., 2011). When the temperature decreases below the freezing point, ice crystals form. Extracellular water crystallizes, thus leaving the remaining solutes at a greater ion concentration (Pena et al., 2006). A major osmotic gradient across the sperm cell membrane is generated (Petrunkina et al., 2004) resulting in an efflux of water from the sperm. Sperm cells shrink because of dehydration. To withstand these osmotic challenges functional membranes of sperm

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cells are able to regulate volume changes through modulation of intracellular ion concentrations (Jeyendran et al., 1984).

Throughout the period of conducting the HOS assessment sperm cells are exposed to hypo-osmotic solutions. When the intracellular solutes are at a greater concentration, an osmotic gradient is established. To compensate, for this ion gradient, sperm cells with intact membranes swell due to a water influx, resulting in the curling of the tails of sperm cells (Rodriguez-Gil et al., 1994). Under the optical microscope, this phenomenon is easier to observe in the tail than in the head of the sperm cell as the plasma membrane of the tail seems to be less well attached than at the head (Bencharif et al., 2010). Essential changes for maintaining sperm cell viability take place with major osmotic gradients across membranes occurring during cryopreservation. Therefore, the responsiveness of sperm cells to osmotic challenges as well as the capacity of the cells to regulate cell volume is a characteristic closely related to cryopreservation capacity (Petrunkina et al., 2004). The HOS test has been used to assess the quality of both fresh (England and Plummer, 1993) and frozen-thawed dog semen (Kumi-Diaka, 1993). However, there have been no publications on a possible prognostic value of the HOS test to evaluate the freezing capacity of semen in dogs. Furthermore, correlations between data from the HOS assessment and conventional semen variables are inconsistent (Goericke-Pesch and Failing, 2013).

Therefore, the objectives of this study were (1) to examine relationships between HOS test results and conventional semen variables before freezing and after thawing and (2) to evaluate the prognostic value of the HOS test on the post-thaw quality of dog semen.

2. Materials and methods

2.1. Animals

From September 2012 to October 2013, semen of 46 client owned male dogs was collected. The dogs were selected irrespective of breed (22 different breeds, one mixed breed), age or fertility status (convenience sample). Clients had registered the dogs for the study in advance and a written consent to conduct this research was obtained. The animals were kept in kennels or in houses by the private owners. They were fed individually. All animals were clinically and andrologically healthy as confirmed by a thorough examination of each animal immediately before semen collection including ultrasonic assessments of the prostate and testes. The dogs were between 1 and 11 years ($\bar{x} = 3$) and weighed between 2.6 and 65 kg ($\bar{x} = 19.8$). One ejaculate was collected from each dog after a sexual rest of at least 7 days.

2.2. Semen collection and evaluation

A “teaser” bitch in estrus or stocked vaginal swaps from bitches in estrus were used for sexual stimulation before the time of semen collection. One ejaculate from each dog was obtained by digital manipulation. Throughout sampling, three semen fractions were collected into three different warmed (37 °C) sterile glass funnels (Ludwig Bertram GmbH, Germany) using the previously reported procedures of Riesenbeck et al. (2001) and Goericke-Pesch and Failing, 2013. These consisted of the transparent pre-sperm, milky-white sperm-rich, and slightly opaque to transparent prostatic fractions and these fractions were stored in a water bath (Julabo 5A, Julabo GmbH, Germany) at 37 °C. Only the sperm-rich fraction was used for further analysis and it was separated into two equivalent aliquots. The first aliquot was evaluated immediately after semen sampling. The second aliquot was prepared for the freezing process. Semen variables such as sperm motility, morphology, and membrane integrity as well as results from the HOS-test were evaluated in fresh and frozen-thawed semen samples. Each ejaculate was tested individually and the results were documented on evaluation forms. Semen volume, color and viscosity were determined using standard techniques. The pH was measured immediately after semen collection from the second fraction using pH-indicator strips (Merck KGaA, Germany; Goericke-Pesch and Failing, 2013).

Immediately after semen collection, sperm motility was assessed at 37 °C in a Makler chamber (Sefi-Medical Instruments, Haifa, Israel) under a phase contrast microscope (Olympus BH-2, Olympus Corporation, Tokyo, Japan) at X 200 (Rota et al., 1995). Sperm cells ($n = 200$) were examined and the percentage of progressive motile (forward movement), local motile (circular movement) and immotile (no movement) were ascertained.

Concentration of sperm cells was determined cytometrically using an improved Neubauer haemocytometer counting chamber (LO—Laboroptik GmbH, Germany; England and Plummer, 1993). The numbers of sperm cells within 10 squares were counted under a light microscope at X 200 and multiplied by a factor of 5000 to obtain the number of sperm/μL.

For assessment of sperm cell membrane integrity Eosin Y solution 1% in water (Carl Roth GmbH & Co. KG, Germany) was used. Immediately after mixing two drops of Eosin with one drop of the ejaculate, a smear was prepared and air dried. Sperm with damaged plasma membranes are stained by Eosin whereas sperm with intact plasma membranes remain unstained (Goericke-Pesch and Failing, 2013). Sperm cells ($n = 200$) were evaluated for cell membrane integrity under a light microscope at X 400.

For sperm cell morphology assessment, a smear of ejaculate was prepared and assessed using Spermac™ stain (Fertipro NV, Beernem, Belgium) using the manufacturer’s instructions (Goericke-Pesch and Failing, 2013). After fixation in formalin (4%) the specimen was dyed with three different stains and allowed to air dry before examination. The various parts of the sperm cell take up the stain differently, thus these fixation and staining techniques were used to facilitate detection of any anomalies of each cell part. As a result of morphological assessments, sperm cells ($n = 200$) were classified either as normal or abnormal under a light microscope (Olympus BH-2, Olympus Corporation, Tokyo, Japan) at X 1000 using oil immersion. Abnormalities were specified as defects in the head, midpiece or tail region of sperm cells.