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Effects of staining method and clinician experience on the evaluation of stallion sperm morphology

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ABSTRACT

Evaluation of sperm morphology is part of the assessment of fertility in human and animal reproduction. Analyses can be performed using different techniques, including the use of staining methods In our prospective study, the morphology of equine sperm was evaluated using 3 staining methods Diff-Quik, eosin-nigrosin, and SpermBlue, the latter being a relatively new stain commonly used for human sperm. Our hypotheses were that (1) SpermBlue allows easier assessment of horse sperm morphology and facilitates better identification of sperm abnormalities, and (2) sperm morphology classification differs depending on the evaluator's experience. Semen was obtained from various horse breeds; 40 samples from stallions between 2 and 15 years of age were collected during the 2016 breeding season and stored in a 2% buffered formaldehyde solution until processing. For each sample, 3 semen smears were made and stained with Diff-Quik, eosin-nigrosin, and SpermBlue. All morphological parameters were then evaluated blindly using a light microscope by a novice evaluator and a more experienced evaluator. For each slide, 200 spermatozoa were examined randomly and classified according to their characteristics. For the identification of morphologically normal spermatozoa, no significant difference between evaluators was found with any of the staining methods used. In contrast, significant differences between evaluators were observed in the classification of some anomalies affecting mainly the midpiece and the tail. Poor dye fixation was observed with SpermBlue.

1. Introduction

Sperm morphology evaluation is an important parameter for determining the quality of an ejaculate and predicting fertility in humans and animals. In equine reproduction, the Society for Theriogenology (SFT) recommends the use of phase contrast or differential interference contrast microscopy to assess the morphology of stallion spermatozoa (Kenney et al., 1983). Despite the fact that these technologies allow a very good assessment of the acrosome region, midpiece, and cytoplasmic droplets of spermatozoa, eosin-nigrosin—a "live-dead" stain recommended by SFT for bull sperm morphology assessment (Freneau et al., 2010)—is also widely used with stallion sperm (Brito et al., 2011). This stain, with its ease of use, has the advantage of allowing simultaneous assessment of membrane integrity and morphology (Varner and Johnson, 2007a, 2007b). In human reproduction, the World Health Organization (WHO) recommends the use of the Papanicolaou stain for the evaluation of sperm morphology (Menkveld, 2010), even

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though the method is time-consuming (the process involves more than 20 steps) and may cause cell shrinkage (Van der Horst and Maree, 2010a, 2010b). Since 1999, Diff-Quik staining, a simple, rapid, and inexpensive method, has also been recommended for use in the assessment of human sperm morphology (Henkel et al., 2008). This stain allows good visualization of the acrosome region in human spermatozoa (Kruger et al., 1987) but can overestimate the size of the sperm head (Graves et al., 2005). In the horse, it has been used to differentiate round cells from immature germ cells and to identify inflammatory cells in the ejaculate (Chenoweth and Lorton, 2014). In 2010, SpermBlue, a stain that fulfils the ideal criteria for a sperm staining procedure (easy to use, quick to perform, allows visualization of all sperm regions without affecting the cell, and can be used for most species) was developed and used in assessing human spermatozoa (Van der Horst and Maree, 2010a, 2010b).

Currently, although staining techniques have been standardized in laboratories specializing in fertility, a persistent concern is variability, which can affect the results. The method used to assess sperm morphology can be responsible for variations in the results due to interindividual differences in smearing technique or in reading the slides. The outcome of the evaluation can also be affected by a lack of quality control and improper training (Ombelet et al., 1998; Eustache and Auger, 2003; Graves et al., 2005; Brito et al., 2011).

The objectives of our study were to compare the results of semen staining using 3 different techniques and to evaluate the effect of clinicians' experience on the assessment of stallion sperm morphology.

2. Materials and methods

2.1. Preparation of semen samples

Forty semen samples from 15 stallions (1–3 samples per stallion) of various breeds, aged 3–15 years, were obtained upon presentation at the veterinary hospital (Centre Hospitalier Universitaire Vétérinaire) of the University of Montreal in Saint Hyacinthe, Quebec, during the 2016 breeding season for breeding soundness evaluation, breeding management, semen processing for shipment, or semen freezing. Semen was collected using a Missouri artificial vagina at a temperature of approximately 46 °C. For each ejaculate obtained, 1 mL of gel-free semen was preserved in a 10% buffered formalin solution (BFS) at a 1:4 ratio (D. Varner, personal communication), placed in tightly sealed tubes (Kenney et al., 1983), and carefully identified and stored until further examination. For each of the 40 samples collected and preserved in BFS, 3 smears were prepared and stained by the same clinician with eosin-nigrosin, Diff-Quik, or SpermBlue. All smears were prepared sufficiently thinly to avoid overlapping of spermatozoa and then air-dried (Kenney et al., 1983).

For the eosin-nigrosin staining method (RAL Diagnostics, Martillac, France), semen smears were prepared by gently mixing a drop of approximately 15 µL of eosin-nigrosin with a drop of about the same volume of semen on a microscopic slide maintained at 37 °C. The mixture was spread gently using a clean glass slide, and the smears were air-dried. For the Diff-Quik staining method (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA), semen smears were prepared using 15 µL of semen on a microscopic slide maintained at 37 °C, and then allowed to air dry. The slide was dipped 10 times for 1 s in fixative solution, 10 times for 1 s in stain solution I, and finally, 10 times for 1 s in stain solution II. The slide was then rinsed with distilled water to remove excess stain and allowed to air dry. Between each dip, the used solution was allowed to drain. For SpermBlue (Microptic Automatic Diagnostic Systems, Barcelona, Spain) staining, semen smears were prepared using the newly "improved formula" in accordance with the manufacturer's recommended protocol. After making smears using 15 µL of semen and allowing them to air dry, the smears were placed vertically into a staining tray containing the fixative for 1–2 min. Slides were then carefully removed from the tray and held at an angle of 80° to drain off the excess stain. The slides were finally dipped slowly, without agitation, into a container of distilled water for 3 s. Next, the slides were removed, and excess fluid was allowed to drain off before air-drying. If the staining was insufficient, the slide was placed in the staining tray for a further 20 s. Slides too intensely stained were washed for no more than 6 s in distilled water and then air-dried.

2.2. Evaluation of sperm morphology

All smears were blindly evaluated for morphology by 2 clinicians: 1 with less than a year of experience of sperm morphology evaluation (OBS-1) and 1 with 3 years of experience (OBS-2). For each slide, using the same light microscope (Will-Wetzlar, Wetzlar, Germany), sperm cells were identified at low power. Then, 200 spermatozoa were examined randomly at 1000 × magnification under oil immersion. According to their morphological characteristics, spermatozoa were classified into 13 categories: normal, abnormal acrosome, abnormal head, detached head, abnormal midpiece, bent midpiece, proximal droplet, distal droplet, abnormal tail, bent tail, coiled tail, round cells, and others.

2.3. Statistical analysis

Statistical analysis was performed by transforming the obtained percentages with the arc-sinus of the square root to normalize the distributions. A repetitive linear model was used with the type of staining method (3 levels), and clinicians were considered intra-subject factors (each horse was evaluated using all 3 techniques and by both clinicians). Contrasts were then performed to determine the differences between staining method for each clinician and to determine the differences between clinicians for each technique. The alpha threshold was adjusted for each comparison using the sequential method of Benjamini–Hochberg. Significance was set at $P < 0.05$.