Artifactual disruption of sperm midpiece in extended stallion semen

Anne Beckham,[a] Camilo Hernandez-Avilés,[a] Charles Love,[a] Larry Johnson,[b]
Sheila Teague,[a] Mariah Pearson,[a] Kat LaCaze,[a] Dickson Varner[a]
[a]Department of Large Animal Clinical Sciences, [b]Department of Integrative Biosciences
College of Veterinary Medicine and Biomedical Sciences
Texas A&M University, College Station, TX

Abstract

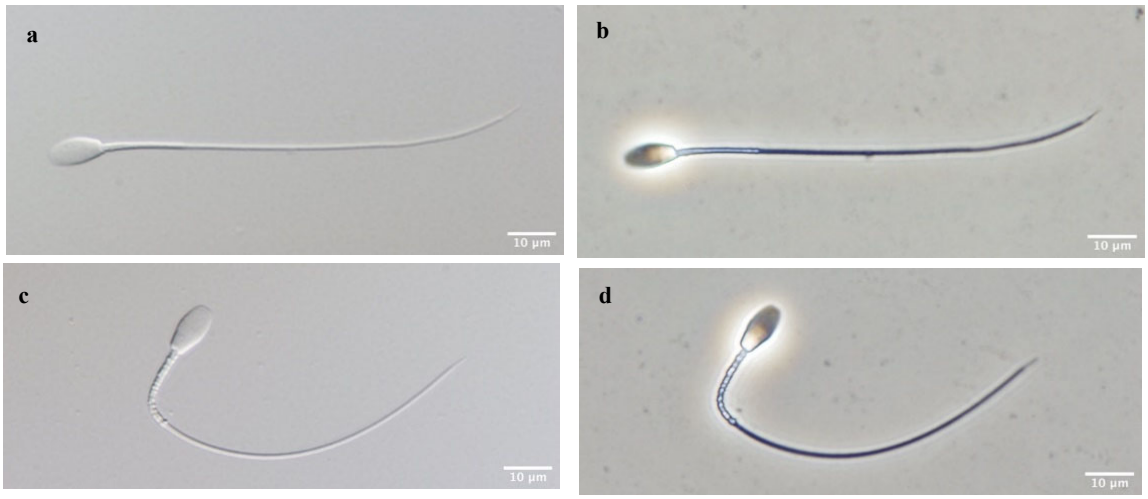
Microscopic examination of stallion sperm morphology provides a useful criterion for assessing semen quality and reproductive capabilities of a stallion. In previous investigations, personnel in our laboratory noted an apparent artifactual defect of the sperm midpiece when stallion semen was mixed with various commercial extenders prior to formalin fixation for sperm morphologic analysis. We coined this defect "stippled midpiece." Objective was to determine the etiology of this previously unreported defect by investigating the induced morphologic change using light and electron microscopic techniques. Sixteen diluents/extenders in 2 sperm exposure times were compared prior to fixation for their effect on the incidence of stippled midpieces. Raw semen was used as a control. The abnormality was most prevalent ($p < 0.05$) when semen was mixed with diluents containing casein, or milk and egg yolk. In contrast, diluents containing albumin or no protein were similar ($p > 0.05$) and exhibited the lowest ($p < 0.05$) incidence of stippled midpieces among diluent groups. Diluents containing protein exhibited a higher ($p < 0.05$) incidence of stippled midpieces compared to raw semen. Intrinsic sperm quality was not correlated with the incidence of stippled midpieces when semen was formalin-preserved in raw form. However, sperm quality was correlated with the incidence of stippled midpieces when semen was mixed with certain diluents prior to formalin fixation. This relationship was most pronounced when diluent contained milk or casein. Transmission electron microscopic findings corroborated that of light microscopy for the location and structural features of the stippled midpieces.

Keywords: Stallion, sperm, midpiece, morphology, fixation

Introduction

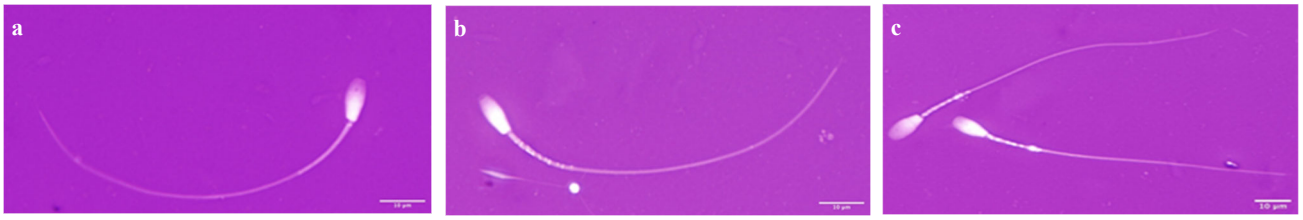
Comprehensive semen evaluation is imperative to understand the reproductive capability of a stallion and for any projected diagnoses of subfertility or infertility.[1,2] Microscopic examination of sperm morphology provides a useful criterion for assessing the quality of semen.[3-5] Semen samples may be sent to a reference laboratory for evaluation of semen characteristics and to provide insight into the reproductive capabilities of a stallion. To protect sperm function for cooled or frozen transport prior to analysis or artificial insemination, semen is diluted with a semen extender.[6-8] Semen can also be subjected to standard centrifugation techniques, or to centrifugal fractionation techniques, to select an enriched population of viable and morphologically normal sperm in an ejaculate.[9-16] Through morphologic evaluation of several semen samples sent to our laboratory, an apparent artifactual disruption of the sperm midpiece was observed on an inconsistent basis. The observed defect was readily detectable when examining wet mounts of sperm using differential interference contrast (DIC) or phase contrast microscopy (Figure 1), or by bright field microscopic evaluation of stained (eosin-nigrosin) smears of sperm (Figure 2). The severity of the change was variable (Figure 3). This disruption was initially attributed to improper semen handling or semen processing techniques while considering that it could also be a reflection of a stallion's intrinsic sperm quality. This same midpiece disruption was observed in our laboratory following centrifugal fractionation of stallion semen through a silanated silica-particle solution (EquiPure™, Nidacon, Mölndal, Sweden) and resuspension of sperm in a milk-based extender, followed by fixation in buffered formol saline. Objective was to further investigate this novel disruption in sperm morphologic structure, an abnormality we have termed "stippled midpiece."

Figure 1. Normal sperm and sperm with stippled midpiece (phase contrast or differential interference contrast microscopy)



a. Normal sperm, as viewed with differential interference contrast microscopy b. with phase contrast microscopy
c. Sperm with stippled midpiece, as viewed with differential interference contrast microscopy d. with phase contrast microscopy

Figure 2: Normal sperm compared to sperm exhibiting stippled midpieces (bright-field microscopy eosin-nigrosin staining)



a. Normal sperm b. Sperm with stippled midpiece c. Two sperm with stippled midpieces

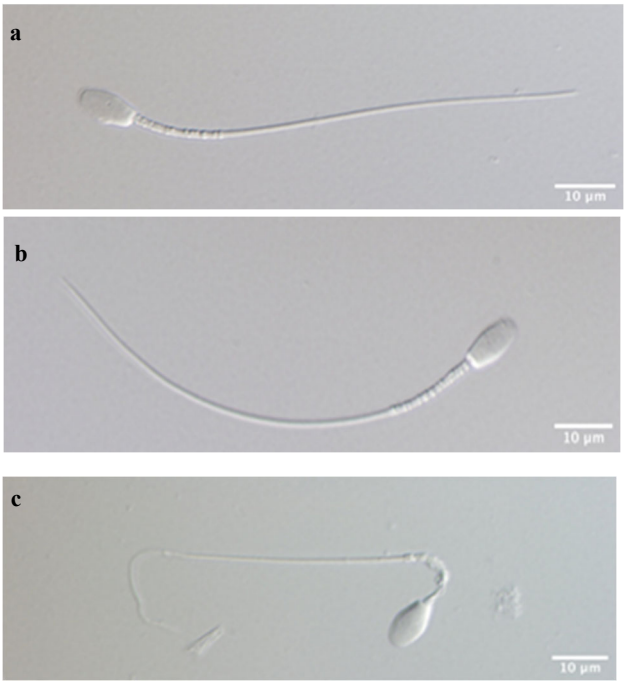


Figure 3. Severity of stippled midpieces as viewed with differential interference contrast microscopy

a. Mild
b. Moderate
c. Marked

Materials and methods

Stallions and semen collection

All animal procedures were conducted following Institutional Animal Ethics Committee guidelines. Stallions were of light breed, healthy, sexually active, and mature (aged 7 - 24 years). Ejaculates were collected using an artificial vagina (Missouri-model; Nasco, Ft. Atkinson, WI) equipped with an in-line nylon micromesh filter (Animal Reproduction Systems, Chino, CA) to permit collection of gel-free semen. Immediately prior to semen collection, the artificial vagina was lubricated with approximately 5 ml of nonspermicidal lubricant (Clarity® A.I. Lubricating Jelly; Aurora Pharmaceutical, LLC, Northfield, MN). An insulated thermal jacket was used to protect semen in the semen receptacle (Animal Reproduction Systems) from light and temperature fluctuations during transport to the adjacent laboratory. Semen was collected using a breeding phantom in the presence of an ovariectomized mare. Prior to each semen collection attempt, stallion's penis was rinsed with warm water and dried thoroughly with paper towels.

Semen processing and analysis

Following semen collection, gel-free semen volume was measured by weight and immediately placed in an incubator (37ºC) to protect sperm from light and changes in temperature. Sperm concentration and viability were measured[17,18] using a fluorescence-based cell counter (NucleoCounter-SP100™, Chemometec, A/S, Allerød, Denmark). Sperm motion characteristics were analyzed using a computer-assisted sperm motion analysis system (CASMA, IVOS II Version 1.9.1, Hamilton Thorne Biosciences, Beverly, MA,) and measured.[18] Motility endpoints were percentage of total sperm motility, progressive sperm motility, curvilinear and straightness, and velocity (μm/s).

Sperm morphology was evaluated[9] in wet mounts using differential interference contrast microscopy (1563 x magnification, Olympus BX60, Olympus America, Inc., Melville, NY). For analysis, aliquots (1.25 μl) of each sample were applied to a glass microscope slide (VWR® Micro Slides, VWR International, LLC, West Chester, PA) and overlain with a 22 x 22 mm No. 1.5 cover glass. A total of 100 sperm per sample were evaluated for morphology and evidence of midpiece stippling. Each sperm was scored for morphologic characteristics and for presence or absence of a stippled midpiece. Sperm morphology was classified[19] and sperm were assigned to the following categories: normal, abnormal head, abnormal acrosome, proximal droplet, proximal droplet only (with no other defects), distal droplet, distal droplet only (with no other defects), abnormal midpiece, bent midpiece, bent tail, coiled tail, and broken tail. Percentages of normal sperm and each morphologic defect were recorded, along with percentage of stippled midpieces.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to provide further information about alterations in ultrastructural morphology of sperm induced by sperm manipulation prior to fixation. Raw semen (negative control), semen diluted 1:1 (semen:extender) with extender (INRA-96® [INRA96]; IMV Technologies, L'Aigle, France), and semen diluted 1:1 with phosphate buffered saline (PBS; Corning Cellgro, Manassas, VA) were processed for TEM imaging. Two ejaculates from a stallion that exhibited a high incidence of stippled midpieces when exposed to semen extender prior to fixation were used to identify the ultrastructural details of sperm with stippled midpieces. For TEM processing, 0.5 ml of either raw or diluted semen was added to 15 ml tubes containing 6 ml of 2% glutaraldehyde in 0.1 M cacodylate and exposed for 1 hour. Tubes were centrifuged at 500 x g for 10 minutes, and the supernatant was removed. Pelleted sperm were resuspended and layered over a 0.1 M cacodylate buffer. Sperm were washed by centrifugation at 500 x g for 10 minutes. Pelleted sperm were transported to the Image Analysis Laboratory, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University for further processing and analysis.

At the Image Analysis Laboratory, samples were incubated with 3% (v/v) glutaraldehyde in

0.1 M cacodylate buffer at room temperature for 1 hour. Following incubation, samples were washed 3 times for 10 minutes each time, in water at room temperature. Samples were then fixed and stained for 30 minutes in 1% osmium tetroxide in 0.1 M cacodylate buffer at room temperature for 30 minutes, washed 3 times for 10 minutes each time, in water on a shaker at room temperature, and stained for 2 hours in saturated aqueous uranyl acetate. Following fixation and staining, a final wash was performed for 10 minutes, 3 times, in water on a shaker at room temperature. Samples were dehydrated using increasing concentrations of ethanol (30, 50, 80, 95, and 100%) and then incubated for 15 minutes with 100% propylene oxide at room temperature. Using propylene oxide:resin (1:1; epon 812), samples were incubated for 30 minutes at room temperature and then were set for 16 hours in 100% resin at room temperature. Finally, the samples were transferred to BEEM® (Better Equipment for Electron Microscopy, Bronx, NY) specimen embedding capsules on a glass slide and baked at 65ºC for 48 hours. Ultrathin sections (100 nm thick) were cut using a ultramicrotome (Leica EM UC6, Leica Microsystems Inc., Buffalo Grove, IL) equipped with a DiATOME diamond knife. Transmission electron microscope used was a FEI Morgagni™ 268 (FEI Company Hillsboro, OR) equipped with a MegaView III CCD (Olympus, Tokyo, Japan) camera. Each sample was scanned to capture sagittal views of sperm midpieces at appropriate magnification to view the ultrastructural detail (~ 7,000 - 40,000 x magnification). The TEM imaging was used to augment our findings using DIC microscopy, which were viewed as an artifactual disruption of the sperm midpiece. Sperm were examined to obtain sagittal views in order to evaluate the integrity of the sperm plasma membrane and alignment of mitochondria. Sperm were compared to previous reports describing the accepted ultrastructural detail of stallion sperm midpieces in order to distinguish normal and abnormal midpiece structure.[22-27] Sperm were classified as normal or abnormal in regard to midpiece structure.

Experimental design

Three ejaculates from 5 stallions (n = 15 ejaculates) were collected. Semen aliquots were mixed with 16 diluents at a 1:1 (v/v) dilution ratio: EquiPure™ 80% silica particle solution (EQUIPURE; protein-free); PureSperm™ 80% silica particle solution (PURESPERM; protein-free); Redigrad™ 80% silica particle solution (REDIGRAD; GE Healthcare Bio-Sciences AB, Uppsala, Sweden; contained albumin); INRA96 extender (contains a fractionated milk component, native phosphocaseinate); BotuSemen™ extender (BOTU-S; Botupharma USA, Phoenix, AZ [contains milk]); BotuSemen™ Special extender (BOTU-SP; Botupharma USA [contains milk with added cholesterol]); BotuSemen™ Turbo extender (BOTU-T; Botupharma USA [contains milk and added pentoxifyline]); BotuSemen™ Gold extender (BOTU-G; Botupharma USA [contains casein with added cholesterol]); TAMU extender (TAMU; prepared in laboratory [contains milk]), Tyrode's albumin lactate pyruvate media (TALP; prepared in laboratory [contains albumin]); E-Z-Freezin® MFR5 (MFR5; Animal Reproduction Systems [contains both milk and egg yolk, and glycerol as a permeable cryoprotectant]), E-Z Freezin® Lactose-EDTA (LE; Animal Reproduction Systems [contains egg yolk and glycerol as a permeable cryoprotectant without milk]), E-Z Freezin® CryoMax™ LE (C-LE; Animal Reproduction Systems [contains egg yolk and glycerol and methylformamide as permeable cryoprotectants without milk]); E-Z Freezin® CryoMax™ MFR5 (C-MFR5; Animal Reproduction Systems [contains milk, egg yolk, glycerol, and methylformamide]); and HEPES-glucose-lactose media (HGLL; prepared in laboratory [protein-free]);[28] commercial PBS (PBS; Corning Cellgro, Manassas, VA [protein-free]). Raw semen (RAW) was used as a negative control. To determine if there was an influence of semen exposure time to extender prior to fixation, subsets of all diluent treatments were exposed to semen for 1 hour prior to fixation (T1h), or immediately fixed following dilution in diluent (T0h). All samples were fixed at a 1:9 dilution ratio (v:v; semen:fixative) with 4.75% buffered formalin saline (Hancock formula).[20] Morphologic analysis using DIC microscopy was conducted following a 24-hour fixation period. In total, 34 samples per ejaculate were evaluated (17 experimental groups, including 16 diluents and raw semen as a control group, and 2 exposure times per group prior to fixation). In total, 510 fixed semen samples were evaluated using DIC microscopy. Sperm morphologic analysis was conducted by an evaluator who was blinded to treatments, but not stallion identification.

Data analyses

Data were analyzed using proprietary software (SAS 9.4, SAS Institute Inc., Cary, NC). Distribution of data was determined using the Shapiro-Wilk test. Most of the data were not normally distributed; therefore, a rank-transformation of data was conducted (PROC RANK) prior to analysis using a general linear model (PROC GLM). A LS-Means Tukey adjustment test was used for mean separation when treatment F-ratios were significant ($p < 0.05$). Linear relationships between stippled midpieces and various features of sperm quality were analyzed using a Spearman correlation method (PROC CORR). Untransformed data are provided in text and tables for ease of interpretation.

Results

Variability among individual stallions for certain endpoints was detected (Tables 1 and 2). Percent morphologically normal sperm and progressive sperm motility were lower ($p < 0.05$) for 1 stallion (Stallion E) compared to 4 remaining stallions. One stallion (Stallion A) exhibited a higher ($p < 0.05$) percentage of morphologically normal sperm compared to other stallions and 2 stallions (Stallions A and B) had a higher ($p < 0.05$) percentage of progressively motile sperm. Stallion A had higher ($p < 0.05$) percentages of total sperm motility and viability compared to Stallion E.

Main effects ($p < 0.05$) of diluent and time were detected for percent stippled midpieces. However, diluent-by-time interaction ($p > 0.05$) was not detected. Effects of diluent type on the percentage of stippled midpieces are presented (Table 3). INRA96 yielded a higher ($p < 0.05$) incidence of stippled midpieces compared to other diluents except MFR5 and C-MFR5. Percent stippled midpieces was lowest ($p < 0.05$) for diluents PBS, HGLL, REDIGRAD, EQUIPURE, PURESPERM, and RAW. Type of permeable cryoprotectant did not impact percentage of stippled midpieces, as values were similar ($p > 0.05$) between diluents MFR5 and C-MFR5, and between diluents LE and C-LE. Exposure of semen to various diluents for 1 hour prior to fixation yielded a slightly higher ($p > 0.05$) incidence of stippled midpieces (5.5 ± 6.15) compared to immediate fixation (4.2 ± 4.9).

When combining diluents that contained common protein sources, diluents containing no protein (EQUIPURE, PURESPERM, HGLL, PBS) or albumin only (REDIGRAD, TALP) yielded a lower ($p < 0.05$) percent of stippled midpieces compared with diluents that had milk or egg-yolk (Table 4). Diluents containing egg yolk only as a protein source (LE and C-LE) yielded lower ($p < 0.05$) percent stippled midpieces than diluents containing casein (INRA96, BOTU-G), milk only (TAMU, BOTU-S, BOTU-SP, BOTU-T), or a combination of milk and egg yolk (MFR5, C-MFR5). Raw semen (control) yielded the lowest ($p < 0.05$) percentage of stippled midpieces except for semen diluted in media containing no protein (EQUIPURE, PURESPERM, HGLL, PBS) prior to fixation

A stallion-by-diluent interaction ($p < 0.05$) was detected. Effect of 6 representative diluents on the percentage of stippled midpieces by stallion is illustrated (Table 5). Percentage of stippled midpieces was low and similar ($p > 0.05$) among all stallions for Groups RAW and C-LE. For diluents containing casein (INRA96), milk (TAMU), or milk and egg yolk (C-MFR5) the percentage of stippled midpieces was higher ($p < 0.05$) for Stallion E (lowest sperm quality) compared to Stallion A (highest sperm quality). Measures of semen quality summarized (Tables 1 and 2). Within Group INRA96, Stallions A and D had a lower ($p < 0.05$) percentage of stippled midpieces than Stallion E Stallions B, C, and E exhibited a similar ($p > 0.05$) incidence of stippled midpieces. In Group EQUIPURE, percentages of stippled midpieces were low for all stallions (range 0 - 2%) but were slightly lower ($p < 0.05$) in Stallion D than Stallion E.

Spearman correlation coefficients, as depicted in Table 6, revealed that no correlations ($p > 0.05$) were detected between percentage of stippled midpieces and various measures of sperm quality in raw semen. Negative correlations ($p < 0.05$) between percent stippled midpieces and percent morphologically normal sperm, total motility, and progressive motility were detected in all other treatment groups). The correlations between percent stippled midpieces and percentages of normal sperm,

Table 1. Percentage of specific sperm morphologic characteristics for 3 ejaculates from 5 stallions (mean ± SD, range in parentheses)

Stallion	NRM	AH	AA	DH	PD	PD ONLY	DD	DD ONLY	AMP	BMP	BT	CT	BK
A	79 ± 8 (70-84)^{a}	1 ± 1 (0-2)^{b}	3 ± 1 (2-4)^{a}	1 ± 1 (0-1)^{c}	7 ± 5 (2-11)^{b}	6 ± 4 (2-9)^{a}	7 ± 4 (4-12)^{b}	2 ± 1 (1-3)^{b}	3 ± 2 (1-5)^{d}	3 ± 2 (1-4)^{a}	5 ± 3 (2-8)^{b}	3 ± 1 (2-4)^{b}	1 ± 2 (0-3)^{a}
B	56 ± 8 (51-65)^{b}	6 ± 3 (3-8)^{ab}	3 ± 1 (2-4)^{a}	3 ± 2 (2-5)^{abc}	15 ± 2 (13-16)^{ab}	11 ± 1 (11-12)^{a}	5 ± 5 (1-10)^{b}	3 ± 4 (0-7)^{b}	19 ± 4 (16-24)^{a}	9 ± 4 (6-13)^{a}	3 ± 3 (1-6)^{b}	4 ± 1 (4-5)^{b}	1 ± 1 (0-1)^{a}
C	58 ± 8 (50-66)^{b}	7 ± 1 (7-8)^{ab}	5 ± 6 (1-11)^{a}	4 ± 1 (3-5)^{ab}	10 ± 4 (7-15)^{b}	8 ± 6 (2-14)^{a}	17 ± 6 (12-24)^{ab}	8 ± 4 (6-13)^{ab}	5 ± 3 (3-8)^{cd}	3 ± 2 (1-5)^{a}	8 ± 2 (6-10)^{ab}	6 ± 2 (4-8)^{ab}	1 ± 2 (0-3)^{a}
D	57 ± 6 (52-64)^{b}	14 ± 2 (12-15)^{a}	2 ± 2 (1-4)^{a}	3 ± 2 (1-4)^{bc}	18 ± 2 (16-20)^{a}	13 ± 4 (8-16)^{a}	9 ± 7 (2-15)^{b}	7 ± 6 (1-12)^{ab}	10 ± 4 (7-15)^{bc}	6 ± 2 (4-8)^{a}	3 ± 1 (2-3)^{b}	3 ± 1 (2-4)^{b}	1 ± 1 (0-1)^{a}
E	17 ± 2 (16-19)^{c}	11 ± 8 (5-20)^{ab}	11 ± 2 (10-13)^{a}	8 ± 3 (5-10)^{a}	18 ± 6 (12-24)^{a}	6 ± 4 (3-10)^{a}	38 ± 3 (34-40)^{a}	14 ± 1 (13-15)^{a}	12 ± 2 (11-14)^{b}	8 ± 3 (6-11)^{a}	27 ± 3 (24-30)^{a}	12 ± 4 (8-16)^{a}	1 ± 1 (0-2)^{a}

$^{a-d}$ Within columns, means without a common superscript differed ($p < 0.05$)
NRM = morphologically normal sperm; AH = abnormal heads (%); AA = abnormal acrosomes (%); DH = detached heads (%); PD = proximal droplets (%); PD ONLY = proximal droplets only (with no other sperm defect) (%); DD = distal droplets only (with no other sperm defect) (%); AMP = abnormal midpieces (%); BMP = bent midpieces (%); BT = bent tails (%); CT = coiled tails (%); BK = broken tails (%); PGC = premature germ cells (%)

total motility, and progressive motility were stronger in diluents containing milk proteins (Groups Casein, Milk and Egg Yolk, and Milk) than in Group No Protein. Positive correlations ($p < 0.05$) were observed between stippled midpieces and percent bent tails and broken tails in all treatment groups except the raw semen control. Positive correlations ($p < 0.05$) were observed between percent coiled tails and percentage stippled midpieces in all treatment groups, including raw semen. Of all sperm morphologic defects examined, only percent proximal droplets were negatively correlated ($p < 0.05$) to percent stippled midpieces. This was observed consistently in Groups Casein, Milk and Egg Yolk, and Milk.

Transmission electron microscopy

Similar to DIC microscopy studies, ultrastructural morphologic alterations of the sperm midpiece was observed following induction by exposure to a milk-based extender (INRA96) prior to fixation. Only 1 stallion was used in this trial, as we selected the stallion with the highest incidence of stippled midpieces based on DIC microscopy in prior experiments. In this TEM experiment, we confirmed presence of stippled midpieces by DIC microscopy in semen exposed to extender prior to formalin fixation. Normal sperm midpieces were observed within all samples (Figure 4), consistent with what was observed using DIC microscopy. However, sperm exhibiting disruption within the midpiece were only evident in samples diluted with INRA96 extender prior to formalin fixation (Figure 5). Disrupted midpieces (Figure 5), exhibited a loss of the plasma membrane, disorder or misalignment of mitochondria, and a serrated appearance. A clear distinction was observed when comparing normal sperm (Figure 4) to those exhibiting an apparent disruption of the sperm midpiece (Figure 5).

Table 2. Sperm output, motion characteristics, and viability for 3 ejaculates from 5 stallions (mean ± SD, range in parenthesis)

Stallion	TSN	CONC	TMOT	PMOT	VCL	STR	VIAB	TPMSN
A	4 ± 1 (4-5)^{b}	140 ± 46 (100-191)^{bc}	85 ± 2 (84-87)^{a}	63 ± 5 (57-66)^{a}	220 ± 31 (184-243)^{a}	68 ± 6 (62-72)^{ab}	85 ± 3 (82-87)^{a}	3 ± 1 (2-3)^{ab}
B	10 ± 1 (9-12)^{a}	194 ± 17 (179-213)^{abc}	77 ± 3 (75-81)^{ab}	55 ± 2 (53-57)^{a}	241 ± 10 (233-253)^{a}	67 ± 2 (65-69)^{ab}	78 ± 8 (72-87)^{ab}	5 ± 1 (5-6)^{a}
C	10 ± 4 (7-14)^{a}	326 ± 114 (252-458)^{a}	77 ± 8 (69-85)^{ab}	38 ± 6 (32-42)^{b}	199 ± 79 (107-245)^{a}	58 ± 13 (49-73)^{b}	79 ± 5 (74-84)^{ab}	4 ± 2 (2-6)^{a}
D	6 ± 1 (5-7)^{ab}	108 ± 40 (63-140)^{c}	78 ± 2 (76-80)^{ab}	45 ± 5 (40-49) ^{b}	223 ± 3 (220-225)^{a}	58 ± 2 (56-60)^{b}	79 ± 1 (78-80)^{ab}	3 ± 1 (2-3)^{ab}
E	5 ± 1 (4-6)^{b}	246 ± 73 (182-326) ^{ab}	25 ± 9 (15-32)^{b}	18 ± 9 (9-26)^{c}	149 ± 3 (146-152)^{a}	77 ± 2 (76-80)^{a}	58 ± 12 (45-69)^{b}	1 ± 0 (0-1)^{b}

$^{a-c}$Within columns, means without a common superscript differed ($p < 0.05$)

TSN = total sperm number (x 10^9); CONC = concentration (x 10^6/mL); TMOT = total motility (%); PMOT = progressive motility (%); VCL = curvilinear velocity (μm/s); STR = straightness ([VAP/VCL]100; %); VIAB = sperm viability (%); TPMSN = total progressively motile sperm in the ejaculate (total sperm number (x 10^9) x progressive motility (%)).

Table 3. Effect of diluent type on the percentage of stippled midpieces in stallion sperm following fixation in buffered formol saline (3 ejaculates from 5 stallions; mean ± SD)

Treatment*	Stippled midpieces (%)
INRA96	12 ± 1^{a}
MFR5	12 ± 1^{ab}
C-MFR5	11 ± 1^{ab}
BOTU-G	8 ± 1^{bc}
TAMU	6 ± 1^{cd}
BOTU-T	5 ± 1^{d}
BOTU-SP	5 ± 1^{d}
BOTU-S	5 ± 1^{d}
LE	3 ± 1^{e}
C-LE	3 ± 0.4^{e}
TALP	2 ± 0.3^{e}
PBS	2 ± 0.4^{f}
HGLL	1 ± 0.2^{f}
REDIGRAD	1 ± 0.3^{f}
EQUIPURE	1 ± 0.2^{f}
PURESPERM	1 ± 0.2^{f}
RAW	1 ± 0.2^{f}

$^{a-f}$ Means without a common superscript differed ($p < 0.05$)

*INRA96 = INRA-96® Extender; MFR5 = E-Z Freezin® MFR5 Equine Semen Extender; C-MFR5 = E-Z Freezin® CryoMax™ MFR5 Equine Semen Extender; BOTU-G = BotuSemen™ Gold extender; TAMU = milk-glucose-sucrose-based extender; BOTU-T = BotuSemen™ Turbo extender; BOTU-SP = BotuSemen™ Special extender; BOTU-S = BotuSemen™ extender; LE = E-Z Freezin® LE Equine Semen Extender; C-LE = E-Z Freezin® CryoMax™ LE Equine Semen Extender; TALP = Tyrode's albumin lactate pyruvate; PBS = Corning® Phosphate-Buffered Saline; HGLL = Hepes-glucose-lactose media; REDIGRAD = Redigrad™ 80% silica particle solution; EQUIPURE = EquiPure™ 80% silica particle solution; PURESPERM = PureSperm™ 80% silica particle solution; RAW = raw semen.

Table 4. Effect of diluent composition on the percentage of stippled midpieces in stallion sperm following fixation in buffered-formol saline (3 ejaculates from each of 5 stallions; mean ± SD)

Diluent Type*	Stippled midpieces (%)
Casein only	10 ± 7^{a}
Milk and egg yolk	11 ± 8^{a}
Milk only	6 ± 4^{b}
Egg yolk only	3 ± 3^{c}
Albumin only	2 ± 2^{d}
No protein	1 ± 2^{de}
Raw semen	1 ± 1^{e}

[a-e] Means without a common superscript differed ($p < 0.05$)

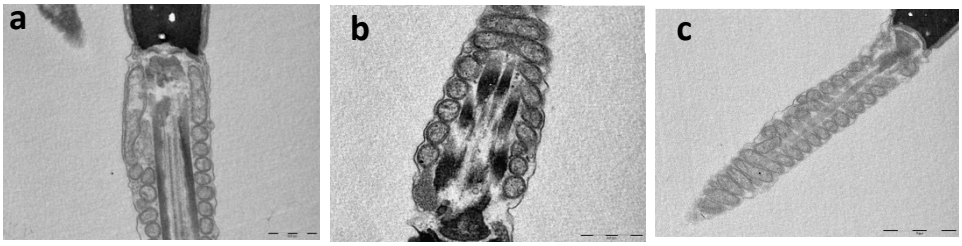
*Casein only = INRA-96® extender and BotuSemen™ Gold extender; Milk and egg yolk = E-Z-Freezin® MFR5 and E-Z Freezin® CryoMax™ MFR5; Milk only = BotuSemen™ extender, BotuSemen™ Special extender, BotuSemen™ Turbo extender, and TAMU extender; Egg yolk only = E-Z-Freezin® Lactose-EDTA and E-Z Freezin® CryoMax™ LE; Albumin only = Redigrad™ 80% silica particle solution and TALP (Tyrode's albumin lactate pyruvate) media; No protein = EquiPure™ 80% silica particle solution, PureSperm™ 80% silica particle solution, HEPES-glucose-lactose media (HGLL), and Corning® (commercial) PBS.

Table 5. Percentage of stippled midpieces by stallion following dilution in selected commercially available semen diluents. (3 ejaculates from each of 5 stallions; mean ± SD)

Stallion	RAW	INRA96	C-MFR5	C-LE	TAMU	EQUIPURE
A	1 ± 1^{a}	6 ± 3^{c}	5 ± 3^{c}	2 ± 2^{a}	3 ± 3^{b}	1 ± 1^{ab}
B	1 ± 1^{a}	11 ± 3^{ab}	8 ± 4^{bc}	3 ± 1^{a}	5 ± 2^{a}	2 ± 1^{ab}
C	1 ± 1^{a}	11 ± 2^{ab}	11 ± 6^{ab}	2 ± 1^{a}	8 ± 5^{a}	1 ± 1^{ab}
D	0 ± 0^{a}	8 ± 3^{bc}	7 ± 3^{bc}	2 ± 2^{a}	6 ± 1^{a}	0 ± 0^{b}
E	2 ± 2^{a}	23 ± 6^{a}	24 ± 6^{a}	5 ± 3^{a}	11 ± 4^{a}	2 ± 1^{a}

[a-c]Within columns, means without a common superscript differed ($p < 0.05$)
RAW = Raw Semen; INRA96 = INRA-96® extender; C-MFR5 = E-Z Freezin® CryoMax™ MFR5; C-LE = E-Z Freezin® CryoMax™ LE; TAMU = TAMU extender; EQUIPURE = EquiPure™ 80% silica particle solution

Figure 1. Three images of normal sperm midpieces, as viewed with transmission electron microscopy



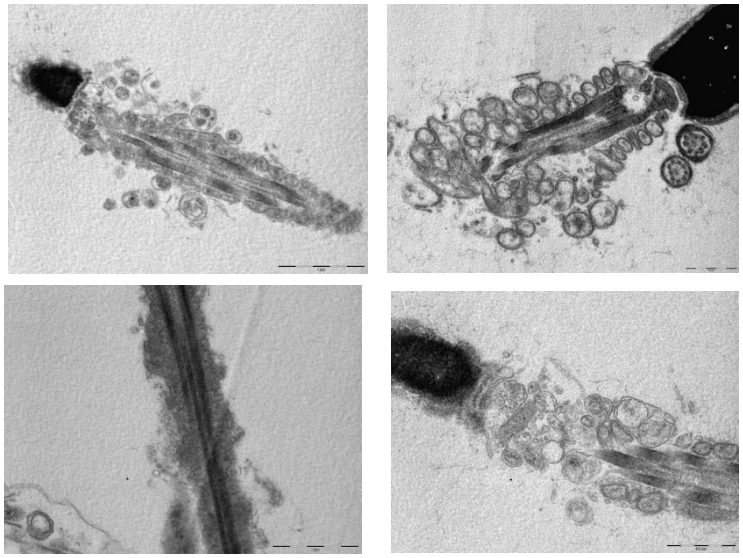
a. Raw semen sample; b. Raw semen sample; c. Semen sample in INRA96 extender

Table 6. Spearman correlation coefficients (r value and p value) between semen diluent type and various sperm morphologic or motion characteristics for the percentage of stippled midpieces (3 ejaculates from 5 stallions)

	Raw Semen	Casein	Milk and egg yolk	Milk	Egg yolk	Albumin	No protein
NRM	NS	-0.6344 0.0027	-0.8530 <.0001	-0.5722 0.0001	-0.6846 0.0009	-0.6584 0.0016	-0.4705 0.0022
AH	NS	NS	0.54409 0.0131	0.3681 0.0194	NS	NS	NS
KA	NS	0.59603 0.0055	NS	0.3651 0.0205	NS	NS	NS
DH	NS	0.54581 0.0128	0.64378 0.0022	0.5920 <.0001	0.5032 0.0237	NS	NS
PD	NS	-0.4785 0.0328	-0.5587 0.0104	-0.4161 0.0076	NS	NS	NS
PD only	NS	-0.5770 0.0077	-0.5730 0.0083	-0.5111 0.0008	NS	-0.4994 0.0250	-0.3681 0.0194
DD	NS	0.6165 0.0038	0.5068 0.0226	0.5772 <.0001	NS	0.4832 0.0309	0.3251 0.0406
DD only	NS	0.6149 0.0039	0.5646 0.0095	0.6399 <.0001	NS	NS	0.3789 0.0159
AMP	NS	NS	0.4537 0.0445	NS	NS	NS	NS
BMP	NS	NS	0.4689 0.0370	NS	NS	NS	NS
BT	NS	0.5461 0.0127	0.5092 0.0218	0.48660 0.0015	0.5722 0.0084	0.6482 0.0020	0.4824 0.0016
CT	0.7865 0.0070	0.6466 0.0021	0.7943 <.0001	0.5277 0.0005	0.6259 0.0032	0.7770 <.0001	0.6287 <.0001
BK	NS	0.6222 0.0034	0.6877 0.0008	0.7218 <.0001	0.5513 0.0118	0.7308 0.0003	0.5299 0.0004
PGC	- -	NS	NS	- -	NS	NS	NS
TMOT	NS	-0.7977 <.0001	-0.9109 <.0001	-0.7496 <.0001	-0.6531 0.0018	-0.6536 0.0018	-0.5955 <.0001
PMOT	NS	-0.7350 0.0002	-0.8066 <.0001	-0.7879 <.0001	-0.4590 0.0418	-0.4810 0.0318	-0.3854 0.0140
VCL	NS	-0.4718 0.0357	-0.4876 0.0292	-0.4979 0.0011	NS	NS	NS
STR	NS	NS	NS	NS	NS	0.5889 0.0063	0.3877 0.0134

NS = not significant
NRM = Normal; AH = abnormal heads (%); AA = abnormal acrosomes (%); DH = detached heads (%); PD = proximal droplets (%); PD ONLY = proximal droplets only (with no other sperm defect) (%); DD = distal droplets only (with no other sperm defect) (%); AMP = abnormal midpieces (%); BMP = bent midpieces (%); BT = bent tails (%); CT = coiled tails (%); BK = broken tails (%); PGC = premature germ cells (%); TMOT = total motility (%); PMOT = progressive motility (%); VCL = curvilinear velocity (μm/s); STR = straightness ([VAP/VCL]100; %); Casein = INRA-96® extender and BotuSemen™ Gold extender; Milk and egg yolk = E-Z-Freezin® MFR5 and E-Z Freezin® CryoMax™ MFR5; Milk = BotuSemen™ extender, BotuSemen™ Special extender, BotuSemen™ Turbo extender, and TAMU extender; Egg yolk = E-Z-Freezin® Lactose-EDTA and E-Z Freezin® CryoMax™ LE; Albumin = Redigrad™ 80% silica particle solution and TALP (Tyrode's albumin lactate pyruvate) media; No protein = EquiPure™ 80% silica particle solution, PureSperm™ 80% silica particle solution, HEPES-glucose-lactose media (HGLL), and Corning® (commercial)

Figure 2. Four images of disrupted sperm midpieces observed in sperm extended in INRA96 extender sample prior to formalin fixation, as viewed with transmission electron microscopy



Discussion

Evaluation of sperm morphology is one of the standard procedures used to assess the breeding potential of a stallion and evaluation of formalin-fixed wet mount specimens is commonplace for this purpose. Evaluation of wet mount samples allows for a more detailed analysis of sperm with enhanced ability to better detect acrosome defects, nuclear vacuoles, and midpiece abnormalities when compared to stained semen smears. Furthermore, the use of wet mount samples for evaluation of sperm morphology reduces the likelihood of artifactual abnormalities, such as detached heads, bent tails, broken tails, and coiled tails.[29,30]

To our knowledge, artifactual disruption of the sperm midpiece following previous exposure of sperm to semen diluents has not been reported. Microscopically, this iatrogenic structural abnormality is characterized by a distinct disruption in the mitochondrial region that is revealed by a stippling appearance at the level of midpiece.

Stippled-midpiece abnormality was first observed in our laboratory following morphologic analysis of semen samples processed using density-gradient centrifugation and resuspension of sperm pellets in a milk-based extender. The apparent disruption of the sperm midpiece was initially attributed to improper semen handling or semen processing techniques. However, we also considered it to be a possible reflection of a stallion's intrinsic sperm quality. A preliminary experiment was conducted to provide a more detailed description of the midpiece abnormality. A higher incidence of stippled midpieces was detected following density-gradient centrifugation protocol and resuspension of sperm in milk-based media as compared to raw semen, following formalin fixation and examination by differential interference contrast microscopy. Based on these results, it appeared that the defect was a result of semen processing. However, it remained unclear if the disruption was due to improper semen handling, centrifugation, prefixation diluent type, time of fixation, or fixation method. Further preliminary experiments were conducted to evaluate the various effects of semen-diluent type, fixative type and concentration, time of exposure to diluent prior to fixation, and time of fixation on the incidence of stippled midpieces. Results suggested an increased incidence of stippled sperm midpieces following prior exposure of semen to diluents containing protein. A higher percentage of stippled midpieces was particularly evident in diluents containing milk. The stippled midpiece abnormality was detected when semen was exposed to diluents prior to fixation without a centrifugation step. Therefore, centrifugation was determined not to be a contributing factor of the disruption.

The present study allowed for a more thorough analysis of the effects of diluent type and time of exposure to diluent prior to fixation on the incidence of stippled midpieces and incorporated an expanded sample size. Five stallions with varying semen quality were used in this experiment to determine if stallion influenced the incidence of stippled midpieces. A clear distinction of sperm quality between 2 stallions (Stallion E - lowest semen quality and Stallion A - highest semen quality) was observed, based on sperm morphologic and motion characteristics.

Initial analysis of 16 semen diluents revealed that semen exposure to diluents containing milk or milk products, or, to a lesser extent egg yolk, exhibited a higher incidence of stippled midpieces, as compared to diluents containing no protein, or to raw semen. Diluents were then grouped according to those containing milk only, casein only, milk and egg yolk, egg yolk only, or albumin only as exogenous sources of protein in order to compare with diluents containing no protein or raw semen (control). This categorization of semen diluents revealed an effect of diluent protein type on incidence of stippled midpieces. This distinction was most pronounced in semen diluents containing casein or a combination of milk and egg yolk, where the highest incidence of stippled midpieces was detected following formalin fixation. However, presence of any exogenous protein in diluents resulted in a higher incidence of stippled midpieces compared to the raw semen control. Diluents containing no protein yielded similar results to the raw semen control.

In addition to diluent composition, intrinsic semen quality had an impact in the incidence of stippled midpieces. A stallion effect was not detected in fixed raw semen but was detected in semen diluted in extender containing casein, milk, or a combination of milk and egg yolk, prior to formalin fixation. Under these conditions, an increased incidence of stippled midpieces was noted in the stallion with lower semen quality as compared to the stallion with higher semen quality. To further support this finding, significant correlations were observed between the percentage of stippled midpieces and percent morphologically normal sperm (negative correlation), or percent total or progressive sperm motility (negative correlations) for all diluent groups except the raw semen control. Therefore, we inferred that intrinsic sperm quality did not impact the incidence of stippled midpieces when raw semen was formalin-preserved. However, sperm quality did impact the incidence of stippled midpieces when semen was mixed with certain diluents prior to formalin-fixation. The effect appeared to be most pronounced when the diluent contained milk, casein, or a combination of milk and egg yolk.

An extender-fixative interaction was responsible for the stippled midpiece abnormality and there were correlations between stallion semen quality and incidence of stippled midpieces. The stallion with lowest semen quality had a higher incidence of the stippled midpieces when sperm were exposed to fixative following dilution in extender. Inherently, such stallions have reduced fertility, so an extender-fixative assay could conceivably be used as a laboratory test of stallion fertility. We consider it questionable, however, that such an ancillary test in a BSE would be of practical value.

An effect of diluent type on the incidence of stippled midpieces was observed throughout this study. Semen extenders are used to protect sperm during in vitro storage; however, the specific mechanism(s) of the protective effects of milk and egg yolk are largely unknown.[31] These animal-based biological products consist of a variety of substances that cannot be accurately standardized, allowing for variation in consistency among batches. This variation may be responsible for the discrepancies observed within diluent types containing milk or egg yolk and the incidence of stippled midpieces observed. In order to reduce inconsistencies within milk, fractionation of milk through microfiltration, ultrafiltration, diafiltration, and freeze-drying has been performed to prepare purified extracts.[35] Among the fractions tested, French workers determined that native phosphocaseinate, composed of total micellar caseins, to be the milk component responsible for most of the protective benefits on cool-stored equine sperm.[36] In the present study, a relatively high incidence of stippled midpieces was observed in diluents containing casein. Therefore, simple standardization of extender composition did not resolve the issue of stippled midpieces when semen was mixed with extender prior to formalin fixation.

Use of an extender (Beltsville F3) containing casein disabled evaluation of sperm morphology, possibly due to casein precipitation.[37] This observation provides possible explanation as to why a higher incidence of stippled midpieces was observed in casein-based extenders in the current study. Possibly,

extensive postfixation protein precipitation occurred within all extenders containing protein, thereby leading to the stippling pattern in the sperm mitochondrial region. Sperm midpiece stippling was rarely detected in raw semen following formalin fixation.

Stippled midpiece appearance that was quite evident in this study was also detected with phase contrast or DIC microscopy and bright-field microscopy of eosin-nigrosin stained sperm. Distinction between normal sperm midpieces and stippled sperm midpieces was pronounced (Figures 1 - 3). Although sperm morphologic evaluation was conducted using DIC microscopy in the present study, images were also captured using phase contrast optics and eosin-nigrosin staining to illustrate the abnormality using various methods commonly used for evaluating sperm morphology. Transmission electron microscopy allowed for ultrastructural examination of extended semen prior to fixation and corroborated the abnormality viewed using DIC microscopy, revealing loss of the plasma membrane, disorder or misalignment of mitochondria, and a serrated appearance of the midpiece region of some affected sperm. Semen from only 1 stallion was used for the TEM experiment. As such, it is possible that this stallion may not be representative of a population of stallions. Further studies are needed to address this point. We selected this stallion simply because its sperm had the highest incidence of stippled midpieces when exposed to extender prior to fixation. We were interested in examining this defect at the ultrastructural level.

Several sperm midpiece defects have been previously described, including midpiece reflex (simple bent or folded midpiece), segmental aplasia of the mitochondrial sheath, fractured or swollen midpiece (thick, pseudodroplet), roughened midpiece (corkscrew), Dag-like defect, disrupted sheath (filamentous), duplicated midpiece, and stump tail.[3,4,22,29] Abnormalities of sperm midpiece have been well documented and are considered to have a deleterious effect on stallion fertility.[3,4] Midpiece abnormalities are most commonly associated with impaired spermatogenesis as well as iatrogenic or artifactual defects due to temperature shock during semen processing.[38,39] These abnormalities are viewed as intrinsic in nature, whereas the stippled midpiece abnormality described in the present study is iatrogenically induced. Stippled midpieces can have some structural similarities to other abnormalities, such as segmental aplasia of the mitochondrial sheath, disrupted sheath, or swollen or roughened midpiece, some of which characterize sperm damage after cooling or a freeze-thaw cycle.[22,24,29,32,40]

Reports comparing extender types that include morphologic analysis have been published, although these reports differ from the present study due to the method of evaluation or method of classification. One study did not subject sperm to fixation prior to morphologic analysis nor did these investigators evaluate or enumerate abnormal sperm.[41] Sperm morphology of semen extended in either milk or egg yolk-based extender prior to fixation was evaluated.[42] However, the stippled midpiece abnormality was not reported and abnormal midpieces were not among the most prevalent abnormalities observed.[42] One stallion in that study exhibited 25% midpiece defects. However, this stallion was reported to have low fertility, and no images of midpiece defects were provided. In a study evaluating morphology of human sperm, wet preparations yielded the highest percentage of midpiece morphologic defects (48.6 ± 11. l).[43] Although not explicitly indicated, it is presumed that raw semen samples were evaluated and no images of the midpiece defects were provided. It is then difficult to discern if the same artifactual midpiece disruptions were observed. The results of the present study suggest that the defect is a result of a reaction involving semen diluents and fixative, and possibly seminal plasma, and can be considered to be an artifactual change.

Due to the significance of sperm morphology for evaluation of the stallion for breeding soundness, it is important to the industry to determine an optimal method for preserving extended semen prior to sperm morphologic evaluation to avoid such artifactual changes. To address this, future studies could be directed at optimizing evaluation of flash frozen extended semen samples to avoid the addition of buffered formal saline for analysis of sperm morphology. However, caution is required because acrosome disruption can be caused by freezing and thawing of raw semen (unpublished results). The incidence of stippled midpieces observed was highest among diluents containing animal-sourced protein, as compared to diluents containing no protein or to raw semen. It would be beneficial to further examine protein types and concentrations in such diluents to deduce any potential relationships between protein

type/concentration and incidence of stippled midpieces. Evaluation of plant-based products as an additive to semen extenders could also be conducted. Future investigations could be directed at evaluating susceptibility of individual sperm to midpiece stippling to determine why certain sperm appear more susceptible to acquisition of this artifactual defect. This notion could be further assessed by dual fluorescent/DIC imaging of individual sperm to compare mitochondrial function and presence or absence of midpiece stippling. Effect of seminal plasma on the incidence of stippled midpieces has also not been studied. As such, it might be prudent to examine sperm morphology of extended-fixed epididymal sperm. It is also perplexing that the stippling appearance was only observed in the midpiece region of sperm and not in the head or principal-piece regions. Isolation of membranes from these various segments of sperm prior to dilution in extender and fixation may allow for closer scrutiny of the relative susceptibilities of regionalized sperm membranes to artifactual stippling.

Conclusion

Mixing of ejaculated semen with certain extender types produced an artifactual midpiece defect in sperm. We have coined this defect "stippled midpiece." This defect can confound interpretation of sperm morphology but can be largely avoided by fixation of raw semen for sperm morphologic analysis.

Conflict of interest

Authors declare no conflicts of interest

Acknowledgement

Authors thank Dr. Joseph Szule, Image Analysis Laboratory, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University for his help with processing and analysis by transmission electron microscopy used for this study.

Funding

Legends Premier Stallion Season Auction Fund, Texas A&M University

References

1. Griffin PG: The breeding soundness examination in the stallion. J Equine Vet Sci 2000;20:168-171.
2. Love CC: Sperm quality assays: How good are they? The horse perspective. Anim Reprod Sci 2018;194:63-70.
3. Love CC: Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 2011;76: 547-557.
4. Love CC, Varner DD, Thompson JA: Intra-and inter-stallion variation in sperm morphology and their relationship with fertility. J Reprod Fertil (suppl) 2000;56:93-100.
5. Aitken RJ: Sperm function tests and fertility. Int J Androl 2006;29:69-75.
6. Aurich C: Semen extenders for cooled semen (Europe). In: McKinnon AO, Squires EL, Vaala WE, et al: editors. Equine Reproduction. 2nd edition,Ames; Wiley-Blackwell: 2011. p. 1336-1340.
7. Brinsko SP: Semen extenders for cooled semen (North America). In: McKinnon AO, Squires EL, Vaala WE, et al: editors. Equine reproduction. 2nd edition,Ames; Wiley-Blackwell: 2011. p. 1341-1343.
8. Sieme H: Semen extenders for frozen semen. In: McKinnon AO, Squires EL, Vaala WE, et al: editors. Equine Reproduction. 2nd edition,Ames; Wiley-Blackwell: 2011. p. 2964-2971.
9. Edmond AJ, Brinsko SP, Love CC, et al: Effect of centrifugal fractionation protocols on quality and recovery rate of equine sperm. Theriogenology 2012;77:959-966.
10. Macpherson ML, Blanchard TL, Love CC, et al: Use of a silane-coated silica particle solution to enhance the quality of ejaculated semen in stallions. Theriogenology 2002;58:317-320.
11. Varner DD, Love CC, Blanchard TL, et al: Breeding-management strategies and semen-handling techniques for stallions–case scenarios. Proc Am Assoc Equine Pract 2010; p. 215-226.
12. Varner DD, Love CC, Brinsko SP, et al: Semen processing for the subfertile stallion. J Equine Vet Sci 2008.
13. Johannisson A, Morrell JM, Thorén J, et al: Colloidal centrifugation with Androcoll-E™ prolongs stallion sperm motility, viability and chromatin integrity. Anim Reprod Sci 2009;116:119-128.
14. Morrell JM, Dalin AM, Rodriguez-Martinez H: Comparison of density gradient and single layer centrifugation of stallion spermatozoa: yield, motility and survival. Equine Vet J 2009;41:53-58.
15. Morrell JM, Mari G, Kútvölgyi G, et al: Pregnancies following artificial insemination with spermatozoa from problem stallion ejaculates processed by single layer centrifugation with Androcoll-E. Reprod Domest Anim 2011;46:642-645.

16. Morrell JM, Rodriguez-Martinez H, Johannisson A: Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size. Equine Vet J 2010;42:579-585.
17. Foster ML, Varner DD, Hinrichs K, et al: Agreement between measures of total motility and membrane integrity in stallion sperm. Theriogenology 2011;75:1499-1505.
18. Waite JA, Love CC, Brinsko SP, et al: Factors impacting equine sperm recovery rate and quality following cushioned centrifugation. Theriogenology 2008;70:704-714.
19. Kenney RM, Hurtgen J, Pierson R, et al: Clinical fertility evaluation of the stallion. J Soc Theriogenol 1983;IX, Part II:3-100 .
20. Hancock JL: The morphology of boar spermatozoa. J R Microsc Soc 1956;76:84-97.
21. Foster ML, Love CC, Varner DD, et al: Comparison of methods for assessing integrity of equine sperm membranes. Theriogenology 2011;76:334-341.
22. Varner DD, Johnson L: From a sperm's eye view-revisiting our perception of this intriguing cell. Proc Am Assoc Equine Pract 2007; p. 104-177.
23. Fawcett DW: The mammalian spermatozoon. Dev Biol 1975;44:394-436.
24. Eddy EM: The spermatozoon. In: Knobil E, Neill JD: editors. The Physiology of Reproduction, San Diego; Elsevier Academic Press: 2006. p. 3–54.
25. Amann RP, Graham JK: Spermatozoal function. In: McKinnon AO, Voss JL: editors. Equine Reproduction. Philadelphia; Lea & Febiger: 1993. p. 715-745.
26. Pesch S, Bostedt H, Failing K, et al: Advanced fertility diagnosis in stallion semen using transmission electron microscopy. Anim Reprod Sci 2006;91:285-298.
27. Veeramachaneni DN, Moeller CL, Sawyer HR: Sperm morphology in stallions: ultrastructure as a functional and diagnostic tool. Vet Clin North Am Equine Pract 2006;22:683-692.
28. Magistrini M, Couty I, Palmer E: Interactions between sperm packaging, gas environment, temperature and diluent on fresh stallion sperm survival. Acta Vet Scand (suppl) 1992;88:97-110.
29. Brito LF: Evaluation of stallion sperm morphology. Clin Tech Equine Pract 2007;6:249-264.
30. Brito LF, Greene LM, Kelleman A, et al: Effect of method and clinician on stallion sperm morphology evaluation. Theriogenology 2011;76:745-750.
31. Aurich C: Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. Anim Reprod Sci 2005;89:65-75.
32. Aitken RJ, Baker MA: Oxidative stress and male reproductive biology. Reprod Fertil Dev 2004;16:581-588.
33. Eddy EM, O´Brien DA: The spermatozoon. In: Knobil E, Neill JD: editors. The Physiology of Reproduction, New York; Raven Press: 1994. p. 29-77.
34. Kankofer M, Kolm G, Aurich J, et al: Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5 °C. Theriogenology 2005;63:1354-1365.
35. Pierre A, Fauquant J, Le Graet Y, et al: Préparation de phosphocaséinate natif par microfiltration sur membrane. Le lait 1992;72:461-474.
36. Batellier F, Magistrini M, Fauquant J, et al: Effect of milk fractions on survival of equine spermatozoa. Theriogenology 1997;48:391-410.
37. Pursel VG, Johnson LA: Glutaraldehyde fixation of boar spermatozoa for acrosome evaluation. Theriogenology 1974;1:63-68.
38. Hurtgen JP: Evaluation of the stallion for breeding soundness. Vet Clin North Am Equine Pract 1992;8:149-165.
39. Baumber-Skaife J: Evaluation of semen. In: McKinnon AO, Squires EL, Vaala WE, et al: editors. Equine Reproduction. 2nd edition,Ames; Wiley-Blackwell: 2011. p. 1278-1291.
40. Barth AD, Oko RJ: Abnormal morphology of bovine spermatozoa, Ames; Iowa State University Press: 1989. p. 214-228.
41. Stanic P, Tandara M, Sonicki Z, et al: Comparison of protective media and freezing techniques for cryopreservation of human semen. Eur J Obstet Gynecol Reprod Biol 2000;91:65-70.
42. Morrell JM, Johannisson A, Dalin A-M, et al: Sperm morphology and chromatin integrity in Swedish warmblood stallions and their relationship to pregnancy rates. Acta veterinaria Scandinavica 2008;50:2.
43. Meschede D, Keck C, Zander M, et al: Influence of three different preparation techniques on the results of human sperm morphology analysis. Int J Androl 1993;16:362-369.