Relationships among morphologic characteristics and membrane integrity of stallion sperm[*]

Steven P. Brinsko, Jennifer A. Spooner,[†] Terry L. Blanchard, Charles C. Love, Dickson D. Varner
Department of Large Animal Clinical Sciences, Texas A&M University,
College Station, TX

Abstract

Various measures of semen quality and fertility often yield either nonexistent or weak correlations. While it is generally assumed that morphologically normal sperm are progressively motile and capable of fertilization, some sperm may have membrane damage that could limit their fertilizing capacity. This study examined the relationship between morphology and membrane integrity of stallion sperm. Semen from 22 sexually active stallions underwent standard evaluation as part of a breeding soundness examination. Aliquots of gel-free semen were diluted to 200×10^6 sperm/mL in semen extender then stained with SYBR-14 and propidium iodide (PI). Random fields were observed and digitally captured under 1250x magnification using epifluorescence, followed by differential interference contrast (DIC) microscopy to assess morphologic characteristics of sperm in the same microscopic field. Captured images were evaluated side by side to compare fluorescent staining to sperm morphology. Membrane integrity of sperm was based on SYBR-14 (green = membrane-intact) or PI (red = membrane-damaged) labeling of the sperm head. Bivariate analyses were performed to determine the odds ratios (OR) of sperm having intact membranes within each morphologic classification compared to sperm without that morphologic characteristic. Morphologically normal sperm were equally likely to be membrane-damaged as membrane-intact (OR = 1.015). While sperm with abnormal heads (OR = 3.26, P<0.0001) were more likely to be membrane-damaged, 39% of sperm with abnormal heads were membrane-intact. Sperm with proximal droplets (OR = 4.95) and distal droplets (OR = 5.75) were more likely to be membrane-intact than sperm without droplets.

Keywords: Stallion, sperm, morphology, membrane integrity

Introduction

Evaluation of semen quality is integral to stallion breeding soundness examinations and in the calculation of insemination doses. Studies attempting to correlate various measures of semen quality with fertility have yielded mixed results, with some of these studies yielding either nonexistent or weak correlations.[1,2] Early on, it was noted that poor fertility can exist even in the presence of sufficient numbers of morphologically normal sperm and it was suggested that additional tests might more accurately predict fertility.[1] However, total sperm numbers, percentage of progressively motile sperm and percentage of morphologically normal sperm remain significant, albeit weak, predictors of fertility as well as principal components of semen evaluation.[2-4]

Integrity of the plasma membrane is essential to proper sperm functions which lead to capacitation, the acrosome reaction and fertilization.[5] However, Samper found that membrane integrity was poorly correlated with motility.[6] While it is generally assumed that morphologically normal sperm will be both progressively motile and capable of fertilization, it is conceivable that some morphologically normal and motile sperm may have some degree of membrane damage that could limit their survivability or otherwise render them incapable of fertilization. In the boar for example, sperm membrane integrity is reported to be a more accurate predictor of fertility than motility.[7] To date, there have been no published reports of the association between membrane integrity and morphology in equine sperm. The purpose of this study was to determine if relationships exist among sperm morphologic characteristics and membrane integrity in fresh ejaculated semen of sexually active stallions.

Materials and methods

Semen was obtained from 22 stallions of varying fertility (10 to 79% per-cycle pregnancy rate) and semen quality (22 to 83% morphologically normal sperm) using a Missouri model artificial vagina (Nasco, Ft. Atkinson, WI) equipped with an in-line nylon micromesh filter (Animal Reproduction Systems, Chino, CA). An ovariectomized mare was used for sexual stimulation and breeding phantom was used as a mount source. Following at least seven days of sexual rest, two ejaculates were collected one hour apart. A third ejaculate was collected the following day to examine the association between sperm morphologic characteristics and membrane integrity. Ejaculates underwent standard evaluation as described by Kenney et al.[8] Sperm concentration in ejaculates was determined photometrically using a densimeter (Model 543A, Animal Reproduction Systems). An aliquot of raw semen was diluted to 25 x 10^6 sperm/mL in semen extender (EZ-Mixin CST®, Animal Reproduction Systems) and sperm motion characteristics were determined using a computer-assisted sperm motion analysis system (CASMA; IVOS Version 12.2L, Hamilton Thorne Biosciences, Beverly, MA).

Separate aliquots of gel-free semen were extended to 200 x 10^6 sperm/mL in semen extender prior to fluorescent staining with PI and SYBR-14 (LIVE/DEAD® Sperm Viability Kit, Molecular Probes, Eugene, OR), and JC-1. Stock solutions of SYBR-14, PI and JC-1 were prepared as previously described by Garner and Johnson.[9] Although included in protocol, JC-1 staining characteristics were not included in the analyses. Samples were incubated at 37° C for 20-30 minutes then, immediately prior to analysis, 0.5 µL of 2% parafomaldehyde solution was added to 100 µL of fluorescently labeled samples to immobilize the cells and facilitate microscopic imaging and digital capture without adversely affecting membrane integrity.[10] However, because the sperm were immobilized to facilitate imaging and capture, JC-1 staining characteristics of the mitochondrial membrane potentials in the midpiece were not included in the analyses. Two µL of the stained sample was placed on a microscope slide and covered with a 22 x 22 mm coverslip. Random fields were observed under 1250x magnification using epifluorescence to assess fluorescent staining patterns of 100 sperm followed by DIC microscopy of the same sperm to assess morphologic characteristics. Each microscopic field was captured under both epifluorescence and DIC using a digital camera (Optronics, Goleta, CA) interfaced to an Olympus BX3 microscope (Olympus America, Inc., Center Valley, PA). Time required to capture images encompassed approximately one hour per sample. Preliminary data indicated that addition of paraformaldehyde did not alter membrane staining characteristics over time (P > 0.1). Captured images were later evaluated side by side on a computer screen to compare the fluorescent staining patterns to the morphologic characteristics of individual sperm cells (Figure). Morphologic classification was based on the system established by Kenney et al,[8] and all abnormalities present on an individual sperm were recorded. The order in which the sperm images were captured was recorded to determine if time to evaluation affected membrane integrity. Only sperm cells visualized throughout their entire length were included for analysis. Premature germ cells were not enumerated or evaluated. Membrane integrity of sperm was based on the labeling of the sperm head with either SYBR-14 (green = membrane-intact) or PI (red = membrane-damaged). Sperm cells considered membrane-intact had heads that fluoresced entirely green, while heads of sperm with some mixture of red and green or entirely red were considered membrane-damaged.

Bivariate analyses were performed to determine the odds ratios of sperm having intact membranes within each morphologic classification by comparing them to sperm not having that morphologic characteristic (e.g. proportion of membrane-intact sperm with an abnormal head compared to sperm not having an abnormal head). A P-value of < 0.05 was used to determine if significant odds ratios existed. Effect of time to evaluation (approximately one hour to capture images of 100 sperm) on membrane integrity was examined using paired t-tests to determine if overall sperm membrane integrity and membrane integrity of sperm within each morphologic classification differed (P< 0.05) between the first 50 and last 50 sperm evaluated.

Results

Percentages of membrane-intact and membrane-damaged sperm, and odds ratios for sperm within morphologic classifications, are presented in the table. More of the first 50 sperm per sample evaluated were membrane-intact (66.6%) compared to the second 50 (56.7%; P < 0.01). However, pair wise comparisons within morphologic classifications demonstrated that this difference was only due to a reduction in membrane integrity of morphologically normal sperm (68% vs 54.5%; P < 0.01) and coiled tails (68% vs 44%; P = 0.014) over time. Of the 2200 sperm evaluated, 58% were morphologically normal, and 5% had coiled tails. Surprisingly, morphologically normal sperm were just as likely to be membrane-damaged as they were to be membrane-intact. As expected, all sperm with detached heads allowed permeation of PI and were considered membrane-damaged. Sperm with abnormal heads were 3.26 times more likely to be membrane-damaged than sperm without abnormal heads; yet, 39% of sperm with abnormal heads were membrane-intact. Only 18% sperm with abnormal acrosomes had damaged plasma membranes. Sperm with abnormal acrosomes were almost three times more likely to be membrane-intact than sperm with normal appearing acrosomes. Sperm with proximal droplets were almost five times more likely, and sperm with distal droplets were almost six times more likely, to be membrane-intact than sperm without droplets.

Discussion

These data demonstrate that morphologically normal sperm are equally likely to be membrane-damaged as membrane-intact. Associations with fertility were not assessed in the present study because insufficient data were available to make valid comparisons[11] and any attempt to do so would only add to the confusion that already exists in the literature regarding semen quality and fertility. Assessment of stallion fertility is a multifactorial process that encompasses far more than simply correlating semen quality with pregnancy rates. In addition to semen characteristics, valid comparisons would also require not only data regarding how the stallions were bred, i.e. natural cover, artificial insemination, fresh semen, cooled semen, frozen semen, but also a multitude of other factors which are difficult if not impossible to control retrospectively, including, but certainly not limited to, the inherent fertility of the mares and their breeding management. Even so, the data presented here may help to explain results of earlier studies where correlations between fertility and motility,[2] or fertility and morphology,[1,12] were poor or nonexistent. These findings also raise concerns over using only the number of progressively motile sperm or number of morphologically normal sperm in an ejaculate as an assessment of semen quality. This would be especially worrisome if these methods were employed on the first ejaculate obtained after sexual rest to determine its suitability for cooling or freezing as the discrepancy between normal morphology and membrane integrity is magnified in ejaculates from sexually rested stallions.[13] Stresses associated with cooling or freezing could amplify any membrane damage that existed prior to processing. When this occurs, fertility could be much lower than would be expected based upon the morphologic and motion characteristics of the sample. Approximately 14% of the normal sperm in this study appeared to lose membrane integrity within one hour, suggesting that longevity tests should be performed on stallion semen, especially when initial assessments of sperm motility and morphology do not coincide with a stallion's reduced fertility.

Of particular interest is the finding that sperm with droplets were almost five to six times more likely to be membrane-intact than sperm without droplets. The biological significance of this is unclear, but may be related to biochemical or structural properties of droplets that are not found in non-droplet bearing sperm.[14] Cytoplasmic droplets are usually shed in the cauda epididymis or upon ejaculation,[15,16] and ejaculates containing a high proportion of spermatozoa with attached droplets have been associated with altered epididymal function and reduced fertility,[17-19] although the exact mechanism by which cytoplasmic droplets adversely affect fertility has yet to be elucidated.

In contrast, using dismount samples and logistic regression to examine the relationship of specific sperm morphologic characteristics on the pregnancy outcome, Love et al., found that the presence of proximal or distal droplets in Thoroughbred ejaculates did not have significant influence on the odds of pregnancy.[3] Though not confirmed in the stallion, in other species sperm with droplets have been shown

to be incapable of capacitation, binding to the zona pellucida and undergoing the membrane fusion events of the acrosome reaction.[20] It would seem unlikely that the cytoplasmic droplets of stallion sperm would be that much different than those of other species.

Rathi et al., reported that bicarbonate-induced merocyanin responses associated with capacitation were absent in equine sperm with cytoplasmic droplets.[21] It is interesting to note that in the current study, sperm with abnormal acrosomes were almost three times more likely to be membrane-intact than sperm with normal appearing acrosomes. Both the acrosome and cytoplasmic droplets contain lysosomal enzymes and have an intimate association with the Golgi apparatus during their formation. In addition, the acrosomal membrane, plasma membrane and cytoplasmic droplets all undergo significant modifications as sperm traverse the length of the epididymis.[13,22-24] In light of this and the above findings, determining whether or not the retention of cytoplasmic droplets and altered acrosomal function share common mechanistic influences on fertility, as well as the significance of cytoplasmic droplets in equine ejaculates, warrant further investigation.

References

1. Kenney RM, Kingston RS, Rajamannon AH: Stallion semen characteristics for predicting fertility. Proc Annu Conv Am Assoc Equine Pract 1971.p 53-67.
2. Jasko DJ, Little TV, Lein DH, et al: Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). J Am Vet Med Assoc 1992;200:979-985.
3. 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.
4. Blanchard TL, Brinsko SP, Varner DD, et al: Relationships between stallion age, book size, number of matings (covers), breeding soundness examination findings, and fertility parameters in 15 Thoroughbred stallions (34 stallion years). Clin Therio 2010;2:78-89.
5. Brito LFC, Barth AD, Bilodeau-Goeseels S, et al: Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. Theriogenology 2003;60:1539-1551.
6. Samper JC: Evaluation of cryopreserved semen: an alternative assay. Acta Vet Scand 1992;88:59-65.
7. Fraser L, Gorszczaruk K, Strzezek J: Relationship between motility and membrane integrity of boar spermatozoa in media varying in osmolality. Reprod Domest Anim 2001;36: 325-329.
8. Kenney RM, Hurtgen J, Pierson R, et al: Clinical fertility evaluation of the stallion. Society for Theriogenology Manual. J Soc Therio 1983;Vol IX, Part II.
9. Garner D, Johnson LA: Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995;53:276-284.
10. Haas GG, DeBault LE, D'Cruz O, et al.: The effect of fixatives and/or air-drying on the plasma and acrosomal membranes of human sperm. Fertil Steril 1988;50:487-492.
11. Amann RP: Weaknesses in reports of "fertility" for horses and other species. Theriogenology 2005;63:698-715.
12. Voss JL, Pickett BW, Squires EL: Stallion spermatozoal morphology and motility and their relationship to fertility. J Am Vet Med Assoc 1981;178:287-289.
13. Brinsko SP, Spooner JA, Blanchard TL, et al: Relationships among sperm membrane integrity, motility and morphology in first and third ejaculates of sexually rested stallions. Proc Annu Conv Am Assoc Equine Pract 2004. p. 502-504.
14. Coratadellas N, Durfort M: Fate and composition of cytoplasmic droplet of hamster epididymal spermatozoa. J Morphol 1994;221:199-210.
15. Dott HM, Dingle JT: Distribution of lysosomal enzymes in the spermatozoa and cytoplasmic droplets of bull and ram. Exp Cell Res 1968;52:523-540.
16. Amann, RP, Hay SR, Hammersted RH: Yield, characteristics, motility and CAMP content of sperm isolated from seven regions of ram epididymis. Biol Reprod 1982;27:723-733.
17. Cummins JM: The effects of artificial cryptorchidism in the rabbit on the transport and survival of spermatozoa in the female reproductive tract. J Reprod Fertil 1973;33:469-479.
18. Bedford JM: Maturation, transport and fate of spermatozoa in the epididymis. In: Hamilton DW, Greep RO, editors. Handbook of physiology, Section 7, Endocrinology, Vol 5. Male reproductive system. Washington, DC: American Physiological Society, 1975. p. 303-317.
19. Amann RP, Seidel GE, Mortimer RG: Fertilizing potential of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. Theriogenology 2000;54:1499-1515.
20. Peña AI, Barrio M, Becerra JJ, et al: Infertility in a dog due to proximal cytoplasmic droplets in the ejaculate: investigation of the significance for sperm functionality in vitro. Reprod Domest Anim 2007;42:471-478.
21. Rathi R, Colenbrander B, Bevers MM, et al: Evaluation of in vitro capacitation of stallion spermatozoa. Biol Reprod 2001;65:462-470.

22.   Jones R: Plasma membrane structure and remodeling during sperm maturation in the epididymis. J Reprod Fertil Suppl 1998;53:73-81.
23.   Cooper TG, Yeung C-H: Acquisition of volume regulatory response of sperm upon maturation in the epididymis and the role of the cytoplasmic droplet. Microsc Res Tech 2003;61:28-38.
24.   Toshimori K: Biology of spermatozoa maturation: An overview with an introduction to this issue. Microsc Res Tech 2003;61:1-6.
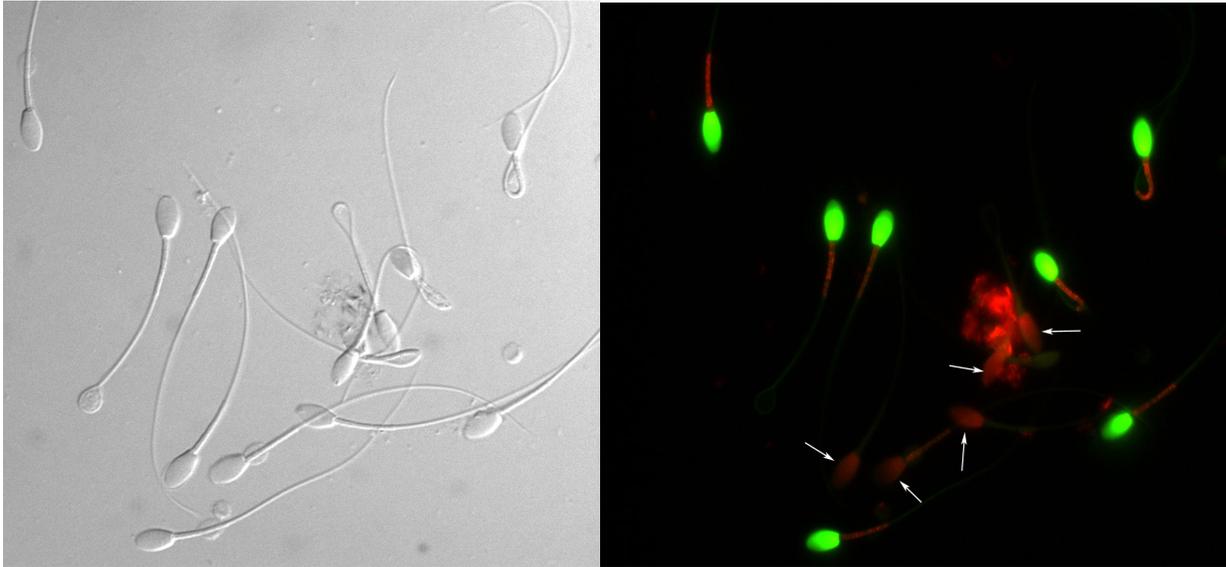
Figure. Digital images of the same sperm viewed under DIC and epiflourescence microscopy for side-by-side comparison of morphologic and membrane integrity staining characteristics using SYBR-14 and PI. Sperm with heads fluorescing green (bright in black and white image) are considered membrane-intact and sperm with heads fluorescing red (arrows; pale in black and white image) are considered membrane-damaged. (For color rendition of the image, please consult the on-line version of Clinical Theriogenology at http://www.therio.org)

Table. Mean percentages of and odds ratios for sperm (n = 2200) with intact or damaged membranes within morphologic classifications in the third ejaculate of 22 stallions (n = 100 sperm evaluated per ejaculate).

Morphologic Characteristic	% of Total Sperm Evaluated	% Membrane-Intact	% Membrane-Damaged	P-value	Odds Ratio to be (i = intact; d =damaged)
Normal	57.9	61	39	0.60	1.015$_i$
Non-normal	42.1	63	37		
Proximal Droplet	5.8	89	11	<0.0001	4.95$_i$
no Proximal Droplet	94.2	60	40		
Distal Droplet	5.2	89	11	<0.0001	5.75$_i$
no Distal Droplet	94.8	60	40		
Abnormal Head	8.3	39	61	<0.0001	3.26$_d$
no Abnormal Head	91.7	64	36		
Bent Midpiece	13	60	40	0.40	1.15$_d$
no Bent Midpiece	87	62	38		
Abnormal Midpiece	5.4	57	43	0.29	1.51$_d$
no Abnormal Midpiece	94.6	62	38		
Bent Tail	6	65	35	0.43	1.13$_i$
no Bent Tail	94	62	38		
Detached Head	1.5	0	100	<0.0001	--
no Detached Head	98.5	63	37		
Abnormal Acrosome	2.5	82	18	0.002	2.80$_i$
no Abnormal Acrosome	97.5	61	39		
Coiled Tail	5	53	47	0.06	1.36$_d$
no Coiled Tail	95	62	38		