Validation of a fixable stain for assessing viability of stallion sperm
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Most sperm viability assays utilize unfixed sperm. Fixable stains have been developed to evaluate the viability of somatic cells but this methodology has not been validated for equine sperm; hence, the objective was to compare a fixable stain with conventional methods. We hypothesized that sperm viability measures are similar when comparing staining techniques for fixed and unfixed equine sperm. Using a protocol adapted in our laboratory, a fixable staining method was compared with commonly used methods for examining sperm viability in unfixed specimens, i.e, 1) SYBR-14 and propidium iodide (SYPI), 2) Pisum sativum agglutinin and PI (PSAPI), 3) NucleoCounter (NC) which utilizes PI, and 4) eosin nigrosin (EN). Ejaculates (n=34) were obtained from 10 stallions. Initially, sperm concentration and percent viable sperm were determined using an automated cell counter (NucleoCounter®-SP-100™, ChemoMetec A/S, Allerød, Denmark). Aliquots of fresh semen were diluted to 30 x10⁶ sperm/mL in INRA96 extender (INRA 96, IMV, Maple Grove, MN). Semen treatments for analysis were based on different contributions of fresh sperm and flash-frozen sperm (from a single source and diluted to 30 x10⁶ sperm/mL in INRA96 extender). Treatments included: 1) 100% fresh sperm (T100); 2) 50% fresh and 50% frozen-thawed sperm (T50); and 3) 0% fresh sperm (T0). For T100, samples were analyzed using the LIVE/DEAD® Fixable Red Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, MA; FLD), and PSAPI, SYPI, NC, and EN assays. For T50 and T0, samples were analyzed using the FLD, PSAPI, and SYPI assays only. Samples processed for the FLD, PSAPI, and SYPI assays were evaluated by flow cytometry. For the EN assay, semen and stain were mixed on a microscope slide and 100 sperm were scored using microscopy. The relationships between FLD and all unfixed viability assays were determined using correlation statistics (PROC CORR, SAS Institute, Cary, NC) and Bland-Altman analysis. For T100, correlations (r) between FLD and NC, PSAPI, SYPI, and EN assays were 0.90, 0.92, 0.89, and 0.87, respectively (P<0.05). The mean, standard deviation and range of the difference for FLD-NC were 0.4, 6.6, -17 to 13%; for FLD-PSAPI were -2.4, 5.7, -19 to 8%; for FLD-SYPI were -2.3, 7.4, -21 to 14%; and for FLD-EN were -9.4, 7.5, -26 to 2%, respectively. For T50, correlations (r) between FLD and PSAPI or SYPI were 0.82 and 0.77, respectively (P<0.05). The mean, standard deviation and range of the difference for FLD-PSAPI were -5.6, 4.5, -25 to 1% and for FLD-SYPI were -5.3, 5.4, -27 to 7%, respectively. For T0, correlations (r) between FLD and PSAPI or SYPI were 0.93 and 0.89, respectively (P<0.05). The mean, standard deviation and range of the difference for FLD-PSAPI were -2.0, 0.5, -2.8 to -0.9% and for FLD-SYPI were 0.2, 0.2, -0.2 to 0.7%, respectively. This study describes a viability assay in which equine sperm can be fixed immediately following semen collection. Correlations between fixed and unfixed flow cytometric assays were high and absolute values were generally similar. The EN stain tended to overestimate viability, as compared to the FLD assay. The FLD assay allows for assessment of initial sperm viability whereby practitioners can fix semen samples immediately following ejaculation and send to a reference laboratory for analysis.

Keywords: Equine, sperm, viability, assays, fixation