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Abstracts

Opening Session

Relationship between testicular measurements using calipers or ultrasonography with testicular weight in alpacas (*Vicugna pacos*)

N.I. Bott [a], J. Rodriguez [a], S. Sandoval [a], A. Tibary [a,b]
[a] *Department of Veterinary Clinical Sciences, College of Veterinary Medicine, USA*
[b] *Center for Reproductive Biology, Washington State University, Pullman, WA, USA*

Testicular size is an important parameter in breeding soundness examination, as it estimates potential sperm production. Studies on the accuracy of clinical methods for estimation of testicular size and their correlation with testicular weight are lacking in alpacas. The objective of this study was to determine the correlation between *in vivo* testicular measurements and testicular weight (after castration). Forty six (46) male alpacas, 30–128 months old, scheduled for castration in spring (20) or late summer (26), were used. All males underwent a complete physical examination prior to being anesthetized with a combination of ketamine (4 mg/kg), xylazine (0.33 mg/kg) and butorphanol (0.03 mg/kg) given IM. Six males (13%; four from the spring group and two from the summer group) were eliminated from the experiment due to ultrasonographic evidence of cystic rete testis. The long axis (length) and largest diameter (width) of each testis were measured externally using industrial calipers and by ultrasonography. Following castration, testes were dissected to remove the tunica, fascia and epididymis, and then weighed. Correlations amongst various measurements were determined (Statistix®, Analytical software, Tallahassee, FL, USA) in a general linear model with season of castration as a factor, as alpacas are prone to scrotal edema and hydrocele in summer, which could affect external measurements.

Caliper measurements ($r = 0.96$) and ultrasound measurements ($r = 0.98$) were significantly correlated with testicular weight. However, external caliper measurements significantly overestimated testicular length by 9.1 ± 1.3 mm (mean $\pm$ S.E.M.) compared to ultrasonographic measurements. Discrepancies between ultrasonographic measurements and caliper measurements were significantly greater in summer (12.1 ± 2.9 mm). Testicular width did not vary significantly between methods of measurement, and correlated significantly with testicular weight ($r = 0.98$).

We concluded that measuring external testicular dimensions with calipers was useful to evaluate testicular size and estimate testicular weight. However, size should be adjusted for potential overestimation, particularly in summer. The advantages of ultrasonography include more precision, better correlation with testicular weight, and the ability to eliminate variation due to peri-testicular tissue (hydrocele, tissue edema and adipose tissue) from the measurement. In addition, testicular ultrasonography eliminated the risk of measuring testes with cystic changes, which were detected in 13% of the study animals. Additional data are being collected to establish a formula for estimation of testicular weight from various measurements.

Keywords: Testis; Breeding soundness; Alpaca; Camelid.

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0093-691X/$ – see front matter

A quantifiable biochemical marker for cold shock damage to porcine sperm

H. Galantino-Homer, M. Modelski, I. Dobrinski
University of Pennsylvania, School of Veterinary Medicine, Kennett Square, PA, USA

Porcine sperm are extremely sensitive to the damaging effects of cold shock. Accidental cooling below 15–17 °C, the temperature used to ship boar semen for AI, results in cold shock damage and decreased fertility. A method to evaluate the degree of cold shock damage in shipped boar semen would be of great benefit to swine producers. We previously reported a quantifiable increase in protein tyrosine phosphorylation (PY) following cold shock that correlated with temperature and the percentages of spontaneous acrosome reacted (AR) and dead sperm [1]. Therefore, we hypothesized that protein PY is a quantifiable biochemical marker of cold shock damage to porcine sperm. The purpose of this study was to localize cold shock- and capacitation-induced increases in protein PY, and to determine whether or not the same protein undergoes PY in both situations. The sperm-rich fraction was collected from three Yorkshire cross boars, filtered, extended, and washed by centrifugation. The pellet was re-suspended in capacitating medium containing 0.8 mM 2-hydroxypropyl-*b*-cyclodextrin (CD). Sperm in CD medium were washed in PBS, allowed to settle onto coverslips, fixed with 4% paraformaldehyde, permeabilized with methanol (−20 °C), stained with 1:200 antiphosphotyrosine antibody (4G10) and 1:200 Texas Red anti-mouse secondary antibody, and assessed via color fluorescence microscopy (300 cells/slide, 630 × magnification). Data are presented as means ± S.E.M. ($n = 5$, 3 boars used) and were evaluated by ANOVA, followed by Tukey's test (significance set at $P < 0.05$). Indirect immunofluorescence and differential interference contrast microscopy revealed that PY localized to the equatorial region (intact pattern) in intact sperm, to the equatorial and acrosomal regions (AR pattern) in sperm that have recently undergone the AR, but staining of the equatorial region was lost and acrosomal staining decreased (dead pattern) under conditions that promoted cell death (additional incubation following AR induction). The predominant pattern of staining shifted significantly from intact (90.0 ± 1.5% intact, 7.3 ± 1.2% AR, 2.7 ± 0.3% dead) at 0 h before cold shock or incubation to the AR pattern following cold shock (1.3 ± 0.3% intact, 88.0 ± 1.5% AR, 10.7 ± 1.8% dead) or AR induction in capacitated sperm (4.3 ± 3.8% intact, 80.3 ± 3.5% AR, 15.3 ± 5.2% dead). This acrosomal staining could represent proacrosin binding protein PY, as identified previously in capacitated porcine sperm. Sperm proteins extracted before and after cold shock or incubation to promote capacitation were evaluated by two-dimensional gel electrophoresis using the ZOOM™ system and reagents (Invitrogen, Carlsbad, CA, USA). Antiphosphotyrosine immunoblots revealed PY proteins migrating at identical relative molecular mass and isoelectric point after cold shock or capacitation. We inferred that protein tyrosine phosphorylation of an acrosomal protein was a sensitive and quantifiable marker of porcine sperm cold shock damage.

Acknowledgement

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Keywords: Boar sperm; Acrosome reaction; Tyrosine phosphorylation; Cold shock; Cryopreservation

Reference

[1] J Androl 2007;(Suppl.):52

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Effect of cryopreservation medium on post-thaw viability of equine testicular cells

M. Ferrer [a], B. Lutjemeier [b], Y. Lopez [b], M. Sanderson [a], J. Lillich [a], M. Weiss [b]
[a] *Department of Clinical Sciences, Kansas State University, School of Veterinary Medicine, 1800 Denison, Manhattan, KS, USA*
[b] *Department of Anatomy and Physiology, Kansas State University, School of Veterinary Medicine, 1800 Denison, Manhattan, KS, USA*

Although spermatogonial stem cell (SSC) cryopreservation has been proposed for preserving genetics from valuable stallions, a freezing protocol has not been developed for horses. Our objective was to evaluate the effect of three freezing media on post-thaw viability of equine testicular cells. Testes were obtained from 6- to 12-month old colts presented for routine castration ($n = 12$), and testicular cells were isolated by enzymatic digestion. The cell suspension was incubated overnight in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) at 38 °C in 5% CO_2 in air. The following day, non-adherent cells were recovered and divided into three aliquots. Each aliquot was frozen at a final volume and concentration of 1 mL and 3×10^6 cells/mL, respectively, in one of three freezing media: (A) DMEM with

10% FBS, 10% DMSO, and 0.07 M sucrose; (B) DMEM with 10% FBS and 10% DMSO; and (C) FBS with 10% DMSO. The vials were held at −80 °C for 24 h, and then transferred to liquid nitrogen (−196 °C). After 3 weeks, cells were thawed in a water bath at 38 °C for 2 min. After adding 2 mL of DMEM with 10% FBS, cells were centrifuged and resuspended to 0.5 mL in DMEM with 1% BSA. Cell viability was assessed by Trypan blue exclusion immediately after isolation (L_0), before adding the freezing medium (L_1), and after freezing and thawing (L_2). Survival rate (S) was defined as $L_2/L_1 \times 100$. The effect of treatment on L_2 or S was evaluated using one-way ANOVA. Cell viability after isolation, culture, and freezing and thawing (L_0, L_1 and L_2) was compared using ANOVA for repeated measurements (SAS Institute Inc., Cary, NC). There was no effect of treatment on L_2 or S. Overall mean values (±S.D.) of L_0, L_1 and L_2 were 99.1 ± 1.9, 78.5 ± 14.9 and $67.8 \pm 20.5\%$, respectively ($P < 0.05$). Overall survival rate was $86.4 \pm 21.5\%$. We concluded that the three freezing media tested were equally suited for freezing equine testicular cells. Enzymatic digestion yielded a cell suspension with excellent viability, which decreased after incubation and cryopreservation. Freezing equine testicular cells immediately after isolation may result in better post-thaw viability. However, functional assays are still required to positively identify SSCs among testicular cells, and to determine the ability of this cell population to survive freezing and thawing.

Acknowledgments

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Keywords: Spermatogonial stem cells; Spermatogonia; Freezing; Cryopreservation; Stallion

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Separation of viable, motile sperm from red blood cells and dead spermatozoa: A comparison of four density gradient centrifugation media in the dog

T.C. Phillips, G. Dhaliwal, J.P. Verstegen

Small Animal Reproduction Clinic, Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA

Prior to using semen form AI or another reproductive technique, it is essential that ejaculates are devoid of factors such as blood, urine, bacteria, or debris, which could reduce fertility. Our aim was to compare different commercial media [Isolate®, Percoll®, PureCeption®, PureSperm®] for their ability to optimally separate viable, motile sperm from non-motile sperm and red blood cells (RBC). The sperm-rich second fractions of four dogs were pooled. The pooled semen was analyzed with Spermvision (Minitube of America, Verona, WI, USA) for concentration, motility, morphology, and diluted to 300×10^6/mL with CaniPro® chilled extender (Minitube of America). Canine whole blood (10%, v/v) was added to semen to mimic contamination. An aliquot (1 mL) of the blood/sperm admixture was pipetted over 4 mL of a double layered-column of the following gradients: Isolate® (Irvine Scientific, Santa Ana, CA, USA); Percoll® (Pharmacia, Uppsala, Sweden); PureCeption® (SAGE In-Vitro Fertilization, Inc., Trumbull, CT, USA); and PureSperm® (Nidacon International AB, Molndal, Sweden). After centrifugation at $400 \times g$ for 20 min at 23 °C, the gradients were divided into 1 mL fractions (Top, B, C, D, E, and Pellet). The 1-mL fractions were analyzed with Spermvision. The fractions were stained with Dif Quik (Webster Veterinary Supply, Sterling, MA, USA), and assessed with light microscopy (1000X). For the RBC:sperm ratio, 100 cells were counted in three fields in each slide (and the average calculated). The study was repeated three times over a 2-week interval. Data were expressed as means ± S.D. and analyzed with GraphPad Instat3 (GraphPad Software, Inc., San Diego, CA, USA) using one way-ANOVA to compare sperm parameters and efficiency of each media for separation of contaminants. Following $P < 0.05$, two-by-two comparisons were done with the Bonferroni multiple comparisons test. The fraction with the highest concentration of motile sperm and the lowest ratio of RBC for each media was as follows: PureCeption® pellet; PureSperm® E; Percoll® D, pellet; and Isolate® D, pellet. The percent recovery of sperm cells from the fraction with the highest concentration in each media was as follows: PureCeption® ($93.0 \pm 2.3\%$) concentration, ($72.0 \pm 13.0\%$) motility; PureSperm® ($44.8 \pm 25.9\%$) concentration, ($53.5 \pm 17.1\%$) motility; Percoll®

(31.0 ± 22.48%) concentration, (70.39 ± 32.1%) motility; Isolate® (49.67 ± 41.4%) concentration, (59.7 ± 21.4%) motility. PureCeption® had a significantly higher percent recovery of motile sperm cells compared to the other sperm separation media. In this preliminary study, PureCeption® was superior to the other density gradient centrifugation media for separation of viable, motile sperm from non-motile sperm and red blood cells.

Keywords: Spermatozoa; Motility; Blood; Centrifugation; Canine

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Effect of follicular fluid on the developmental competence of dromedary (*Camelus dromedarius*) oocytes obtained from small follicles

H. Khatir [a], A. Anouassi [a], A. Tibary [b]

[a] *Veterinary Research Center, Abu Dhabi, PO Box 44479, United Arab Emirates*

[b] *Veterinary Clinical Sciences and Center for Reproductive Biology, Washington State University, Pullman, WA, USA*

We recently demonstrated that camel oocytes originating from large follicles were more competent than those collected from small follicles [1]. The objective of this study was to evaluate the ability of various concentrations (2.5, 5, and 10%) of follicular fluid from large follicles (LCFF) to support cytoplasmic maturation of camel oocytes derived from small follicles. Cumulus–oocyte complexes (COC) were obtained postmortem from ovaries by dissecting small and large follicles (3–6 and 7–10 mm, respectively). The LCFF was collected from follicles 7 to 10 mm in diameter, pooled, centrifuged (300 × *g* for 10 min), and the supernatant stored (−20 °C) until used for in vitro maturation (IVM). The quality of COC was evaluated after IVM, in vitro fertilization (IVF), and in vitro culture (IVC). All cultures were done in four replicates at 38.5 °C, under 5% CO_2 and > 95% humidity. The COC from small follicles were matured for 28 h in a basic IVM-medium (TCM-199 medium + 10 ng/mL EGF + 250 μm cysteamine; Sigma Chemical Co., St Louis, MO, USA), supplemented with various concentrations of LCFF or with 10% fetal calf serum (FCS; Sigma F24-42). The COC from large follicles (control) were matured with 10% FCS. Fresh semen (0.5×10^6/mL) in modified TALP-solution was used for IVF. Fertilized oocytes were cultured in mKSOMaa, under 5% O_2 and 90% N_2. Statistical analysis was done with Chi square and Fisher's Exact test.

Adding 2.5% LCFF during IVM supported in vitro development of camel oocytes from small follicles. We inferred that LCFF contained factors essential for acquiring developmental competence; however, that higher rates of LCFF suppressed development was unexpected, with no apparent explanation. Further studies are ongoing to determine if LCFF affects viability of hatched embryos transferred to recipients.

Treatment	No. oocytes	No. cleaved (%)	No. blastocysts (%)	No. blastocysts hatching (%)
0% LCFF	104	39 (38)[a]	11 (10)[a]	0 (0)[a]
2.5% LCFF	121	83 (69)[b]	45 (37)[b]	22 (49)[b]
5% LCFF	96	57 (59)[b]	24 (25)[b]	5 (21)[a]
10% LCFF	110	48 (44)[a]	8 (7)[a]	0 (0)[a]
10% FCS	92	51 (55)[b]	21 (23)[b]	6 (29)[ab]
Control	107	76 (71)[b]	36 (34)[b]	18 (50)[b]

[a,b]Within a column, values without a common letter differed ($P < 0.05$).

Reference

[1] Khatir H, Anouassi A, Tibary A. Effect of follicular size on in vitro developmental competence of oocytes and viability of embryos after transfer in the dromedary (*Camelus dromedarius*). Anim Reprod Sci 2007;99:413–20.

Keywords: Follicular fluid effect; IVM; IVF; IVC; Oocyte competence

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Effects of recovery technique, freezing extender and antioxidants on motility parameters of cryopreserved stallion epididymal sperm

A.E.M. Johnson, M.A. Coutinho da Silva

Department of Clinical Sciences, Cornell University, Ithaca, NY, USA

The objectives were to determine: (1) the best technique to recover sperm from the cauda epididymis; and (2) the effects of freezing extenders and presence of antioxidants on post-thaw motility of cryopreserved epididymal sperm. Testes and epididymides were obtained from six stallions (2–25 y old) presented for elective castration. Each cauda epididymis were isolated and randomly assigned to one of two recovery techniques: floating or retrograde flushing. Sperm were recovered using 20 mL of skim milk extender (EZ-Mixin "CST", Animal Reproduction Systems, Chino, CA,

USA) and diluted to 100 mL with the same media. Total number of sperm was determined using a hemacytometer, and motility was determined by CASA (Hamilton Thorne Biosciences, Beverly, MA, USA). Samples were pooled and centrifuged ($600 \times g$, 10 min). The supernatant was removed and sperm were diluted to 100×10^6 sperm/mL with either Lactose EDTA (LAC) or BotuCrio® (BC; BioTech-Botucatu, Brazil), containing 0, 2, or 6 mM of Tempol (Sigma Aldrich Co., St. Louis, MO, USA) or 0, 2, 6, or 18 mM of L-Ergothioneine (Oxis International Inc., Foster City, CA, USA). Samples were frozen in 0.5 mL straws using the IceCube® (Minitube of America, Verona, WI, USA). Motility was determined prior to cryopreservation and post-thaw (after 0, 30, 60, and 90 min at 37 °C). Sperm recovery data were analyzed by ANOVA and paired *t*-test, whereas motility data were evaluated by repeated measures ANOVA and Tukey's HSD test ($P < 0.05$ was significant). There was no difference between floating and flushing for mean (±S.E.M.) total number of sperm recovered ($8.4 \pm 3 \times 10^9$ and $8.9 \pm 3 \times 10^9$, respectively), or for percentage of progressively motile sperm (46 ± 4 vs $33 \pm 7\%$). Total motility tended ($P = 0.087$) to be higher for floating than flushing (86 ± 2 vs $77 \pm 4\%$). The addition of antioxidants did not affect sperm parameters. The interaction of extender*time was significant for total motility, and tended ($P = 0.093$) to be significant for progressive motility. Post-thaw total motility was higher for BC versus LAC at 0, 30, 60 and 90 min (67 ± 2 vs 35 ± 4; 58 ± 3 vs 19 ± 3; 55 ± 2 vs 21 ± 3; and 44 ± 3 vs 19 ± 3). Post-thaw progressive motility was also higher for BC versus LAC at 0, 30, and 60 min (41 ± 2 vs 12 ± 1; 35 ± 2 vs 8 ± 1; 32 ± 2 vs 9 ± 1), but was not significantly different at 90 min (24 ± 2 vs 10 ± 2). In summary, recovery of sperm from cauda epididymis by either floating or flushing resulted in similar numbers of sperm and similar motility. Antioxidants in the freezing media did not affect post-thaw motility. Cryopreservation of epididymal sperm in BC resulted in higher post-thaw motility than LAC.

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Keywords: Stallion; Epididymis; Sperm; Antioxidants; Cryopreservation

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Post-thaw quality of canine semen cryopreserved with commercial canine and equine semen extenders

S.A. Layne, C.R.F. Pinto, D.M. Kozink, B.E. Holland
College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA

Two commercial canine semen extenders, CaniPRO (Minitube of America, Verona, WI, USA) and CanFreeze (Partnar Animal Health, Port Huron, MI, USA), and a one-step semen extender (LE, Animal Reproduction Systems, Chino, CA, USA), originally designed to cryopreserve equine semen, were used to cryopreserve canine semen. The main objective was to investigate whether simplified semen cryopreservation protocols (CanFreeze and LE) would be as efficacious as a proven protocol (CaniPRO). We hypothesized that post-thaw semen quality would not differ among extenders.

Ten pooled ejaculates were obtained from proven stud dogs. Concentration, motility, viability, morphology, and integrity of the sperm membrane and acrosome were determined, and the pooled semen was used to test the three cryopreservation media (using their respective protocols). Processed semen was loaded into 0.5 mL plastic straws and cryopreserved in liquid nitrogen vapor, 2 cm above the liquid nitrogen (LN) for 10 min, before being plunged into the LN. For each extender, one straw was randomly thawed by immersion in a water bath (37 °C for $\geq$ 30 s). Post-thaw semen samples cryopreserved with the two canine semen extenders were diluted with thaw media before analysis (as recommended by the manufacturer), whereas, samples cryopreserved with LE equine extender (one-step protocol) were not. An ANOVA ($P = 0.05$) was used to determine the effect of treatment groups.

For post-thaw semen, the mean (±S.E.M.) percentage of total motility for CanFreeze (74 ± 4.5), CaniPRO (71 ± 4.8), and LE (70.2 ± 5.1) did not differ significantly. However, post-thaw progressive motility of semen cryopreserved with LE extender (12.6 ± 3.4) was significantly lower than that of semen cryopreserved with CanFreeze (35.5 ± 2.7) or CaniPRO (31.7 ± 2.9). In contrast, the percentage of membrane integrity of post-thaw semen samples cryopreserved with LE extender (81.9 ± 1.3) was greater ($P = 0.018$) than that obtained with CanFreeze (74.3 ± 2.1) or CaniPRO (72.4 ± 3.2). Acrosome integrity and sperm viability did not differ among extenders ($P > 0.1$).

Overall, the three semen-freezing extenders successfully cryopreserved canine semen. The only differences

in post-thaw semen quality were the percentage of progressively motile sperm and membrane integrity, with LE extender having the lowest mean progressive motility, but greatest mean membrane integrity. Differences in thawing protocols resulted in LE samples being analyzed for motility at a relatively high concentration, which may have affected the accuracy of the computerized semen analyzer for assessing progressive motility. Unlike the CaniPRO, the CanFreeze and the LE protocols do not require the addition of fresh egg yolk. CanFreeze and LE are simplified freezing protocols that may be of benefit for semen freezing laboratories with limited technical assistance.

Keywords: Semen; Sperm; Cryopreservation; Extender; Dog

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Use of a vital fluorescent stain to identify apoptosis in bovine oocytes

S.L. Ayres
Tufts Cummings School of Veterinary Medicine, North Grafton, MA, USA

The objective was to determine if a vital fluorescent stain, Yo Pro-1 (Invitrogen, Carlsbad, CA, USA) could be used to identify early apoptosis in bovine oocytes. To accomplish this, bovine oocytes were stained with both Yo Pro-1 and Annexin V (Invitrogen), a fluorescent stain previously used to identify early apoptosis in oocytes.

Two shipments of cow oocytes were obtained from BoMed (Madison, WI, USA). Each shipment contained approximately 50 oocytes that were ''good'' quality, and 50 oocytes that were ''poor'' quality, as determined by the vendor. Oocytes were shipped overnight in an incubator in maturation medium at 39 °C. The oocytes were first exposed to Yo Pro-1, then to Annexin V. The two stains can be used together; Annexin V indicates phosphatidylserine translocation and uses a blue fluorescent marker, whereas the Yo Pro-1 indicates early loss of plasma membrane integrity and uses a green fluorescent marker. Propridium iodide (Invitrogen) was used to stain dead oocytes (red fluorescent stain). A total of 164 oocytes were stained and individually evaluated using a Zeiss Axiovert 200 fluorescent microscope with Axiovision software. The percentage of apoptotic oocytes identified by the two fluorescent stains was the same in both the ''good'' and ''poor'' groups of oocytes, approximately 80%. Of the 132-apoptotic oocytes, 130 (98.5%) took up both stains, whereas two oocytes were stained with Yo Pro-1 only. The percentage of live oocytes was significantly greater in the ''good'' group (12.6 versus 1.3% in the ''poor group'', $P < 0.0001$), whereas the percentage of dead oocytes was greater in the ''poor'' group (18.2 versus 7.0% in the ''good'' group, $P < 0.0001$; two-tailed Fisher's Exact test).

	Good (%)	Poor (%)	Total
Live	11 (12.6)[a]	1 (1.3)[a]	12
Apoptotic	70 (80.4)[b]	62 (80.5)[b]	132
Dead	6 (7.0)[c]	14 (18.2)[c]	20
Total	87	77	164

[a–c]Within a column, values without a common superscript differed ($P < 0.05$).

The Yo Pro-1 stained the same population of cells stained by Annexin V; therefore, Yo Pro-1 has potential as a vital stain to identify early apoptosis in bovine oocytes. In addition, it appeared that a substantial number of cow oocytes shipped overnight may have been undergoing apoptosis, regardless of their initial morphologically classification as ''good'' or ''poor''. Further studies are ongoing to assess the viability and developmental potential of oocytes and embryos sorted into apoptotic and non-apoptotic populations using this vital stain.

Keywords: Oocytes; Apoptosis; Fluorescent stains; Bovine; Cattle

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Species Abstracts

Equine

Can vaginal electrical impedance be used to characterize estrus and ovulation in the mare?

J.E. Larsen, S.T. Norman
School of Animal and Veterinary Science, Charles Sturt University, Wagga Wagga, New South Wales, Australia

The Ovatec® (Key Business Services, Batavia, NY, USA) intravaginal probe measures vaginal electrical impedance (VEI) of cervical mucus. This study investigated whether this device could be used to characterize the mare's estrous cycle, and in particular, to predict ovulation. The hypothesis tested was whether VEI of mare's cervical mucus is cycle- and ovulation-

dependent. A preliminary study was conducted to determine whether VEI readings were repeatable and whether regular use of the probe was deleterious to the mare. Mare behaviour was compared to an ethogram and analysed with Chi-square. The objective of the second experiment was to correlate VEI with concurrent observations of reproductive status, as assessed by transrectal palpation and ultrasonography, steroid hormone concentrations, and cervical mucus characteristics. To standardise and focus VEI measurements on the endocrinological events of estrus and ovulation, 10 sexually mature and proven mares were synchronized with controlled intravaginal drug release (CIDR) devices, estradiol benzoate and prostaglandin $F_{2\alpha}$ [1]. Every 8 h during the peri- and post-ovulation periods, VEI was determined, plasma and cervical mucus samples were collected, and mares were observed for estrous behaviour, to determine the relationship between these variables and to ascertain whether VEI could predict ovulation. The Spearman correlation coefficient for ranked data was used. In this study, the ability to safely use the probe was quickly attained by a novice operator. Furthermore, the Ovatec® probe was less stressful to the mare than repeated transrectal palpation. When used once or thrice daily (in accordance with the manufacturer's instruction), the Ovatec® probe could be used to detect estrus in the mare. Over the preovulatory period, there was a positive correlation of VEI with time ($r = 0.76$, $P < 0.0001$) and follicle size ($r = 0.72$, $P < 0.0001$). There was a significant, negative correlation between VEI readings and plasma estradiol concentrations ($r = -0.52$, $P = 0.006$), but no significant correlations between VEI and either plasma progesterone concentrations or characteristics of cervical mucus. Although the Ovatec® probe appeared to be a repeatable and relatively reliable non-invasive method of detecting estrus, it was not suitable for determining time of ovulation, neither predicting it prospectively nor detecting it retrospectively.

Keywords: Estrus; Vaginal electrical impedence; Ovatec®; Ovulation; Mare

Reference

[1] Norman ST, Larsen JE, Morton JM. Oestrous response and follicular development in mares after treatment with an intravaginal progesterone releasing device in association with single injections of oestradiol benzoate and $PGF_{2\alpha}$. Aust Vet J 2006;84:47–9.

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Centrifugation has minimal effects on motility, viability, and acrosome integrity of equine sperm

J.A. Len [a], J.A. Jenkins [b], B.E. Eilts [a], D.L. Paccamonti [a], S.K. Lyle [a], G. Hosgood [a]

[a] *Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, USA*

[b] *US Geological Survey, National Wetlands Research Center, Lafayette, LA, USA*

The objectives were to determine the effects of centrifugation on progressive motility, plasma membrane integrity (viability), and acrosome integrity of equine sperm. We hypothesized that high centrifugation forces will be detrimental to equine sperm, but will increase recovery rates. Ejaculates from six stallions were collected, extended (INRA96, IMV Technologies, Maple Grove, MN, USA) to a concentration of 25×10^6 cells/mL, and subjected (10 min) to: (1) no centrifugation (NC); (2) $400 \times g$ (400); (3) $900 \times g$ (900); and (4) $4500 \times g$ (4500). Before and after centrifugation (Day 0), and after 24 h of cooling (Day 1), sperm motility was assessed (Sperm Vision®, Minitube, Verona, WI, USA), and samples were stained with SYBR-14/propidium iodide (PI) and PI/FITC-PNA (*Arachis Hypogaea*; Molecular Probes, Eugene, OR, USA), and assessed by flow cytometry. Data were analyzed using Shapiro–Wilk's statistics, and a mixed linear model was used to determine effects of treatment and day. The 4500 treatment group had reduced ($P < 0.05$) motility, viability, and intact acrosomes as compared with the other groups (Table 1). The 400 and 900 treatment groups had lower recovery rates than the

Table 1
Mean (±S.D.) equine sperm progressive motility, viability, and acrosome integrity after centrifugation (Day 0) and 24 h after cooling (Day 1)

Treatment	Progressive motility (%)		Viability (%)		Acrosome (%)	
	Day 0	Day 1	Day 0	Day 1	Day 0	Day 1
NC	82.6 ± 6.7^{a}	76.4 ± 7.1^{a}	81.1 ± 6.2^{a}	76.4 ± 5.2^{ab}	81.9 ± 4.6^{a}	76.0 ± 5.7^{ab}
400	85.6 ± 8.7^{a}	74.9 ± 12.2^{a}	85.5 ± 5.6^{a}	81.1 ± 2.8^{a}	83.7 ± 2.4^{a}	80.3 ± 5.8^{a}
900	84.2 ± 7.4^{a}	73.4 ± 10.8^{a}	81.7 ± 5.2^{a}	79.0 ± 4.6^{a}	81.3 ± 5.0^{a}	79.6 ± 5.3^{a}
4500	74.2 ± 6.2^{b}	66.5 ± 11.5^{b}	72.8 ± 6.6^{b}	70.7 ± 7.9^{ab}	72.5 ± 6.1^{b}	69.5 ± 8.5^{ab}

Within a column, values without a common letter (a and b) differed ($P < 0.05$).

4500 treatment group (NC = 100.0 ± 0.0%, 400 = 54.4 ± 8.6%, 900 = 75.0 ± 7.1%, and 4500 = 97.9 ± 2.8%; $P < 0.05$). Centrifugation at 400 or 900 × *g* did not damage equine sperm. Further studies of centrifugation forces between 900 and 4500 × *g* are warranted to find optimal forces that maximize recovery rate, minimize sperm damage and do not affect fertility.

Keywords: Centrifugation; Spermatozoa; Plasma membrane; Acrosome; Recovery rate

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Serum concentrations of ergovaline/ergot alkaloids in late-term pregnant mares grazing endophyte-infected tall fescue pastures: A preliminary report

A.F. Lehner [a], B.P. Fitzgerald [a], C.G. Hughes [a], T. Tobin [a], F.C. Camargo [b], J. May [c], L. Dirikolu [d], D.L. Christiansen [e], P.L. Ryan [e]

[a] *Department of Veterinary Sciences, University of Kentucky, Lexington, KY, USA*

[b] *Department of Animal Sciences, University of Kentucky, Lexington, KY, USA*

[c] *Environmental Research Training Laboratory, University of Kentucky, Lexington, KY, USA*

[d] *Department of Veterinary Biosciences, University of Illinois, Urbana, IL, USA*

[e] *Department of Pathobiology and Population Medicine, Mississippi State University, Mississippi State, MS, USA*

Ergot alkaloid toxicities such as tall fescue toxicosis from *Neotyphodium coenophialum*-endophyte-infected [E+] tall fescue pastures are important veterinary and economic problems. Tall fescue toxicosis apparently results from ingestion of vasoconstrictive ergot alkaloids produced by symbiotic fungal endophytes; ergovaline is generally considered the critical toxin. To date, ELISA and HPLC with UV/fluorescence detection have been the predominant means of ergot alkaloid determination. These techniques, however, lacked sufficient sensitivity and/or specificity, to detect serum concentrations of specific ergot alkaloids. Thus, the objective of this study was to employ highly sensitive analytical techniques that included the application of liquid-liquid extraction, HPLC and Electrospray(+) ionization mass spectrometry (ESI-MS) with multiple reaction monitoring (MRM), to detect specific ergot alkaloids in equine serum with a limit of detection estimated at 1 pg/mL. To this end, weekly blood samples were collected by venipuncture from late-term pregnant mares (>290 d) exposed to toxic endophyte-infected (>90% contaminated) tall fescue pastures. Serum samples were analysed from four mares that showed clinical signs of ergot alkaloid reproductive toxicity (i.e., agalactia, prolonged gestation, placental thickening, mare and neonatal mortality). We now report that serum concentrations of ergovaline in mares grazing E+ tall fescue pastures ranged from 0.7 to 3.8 pg/mL, and its 8-positon epimer ergovalinine, from 1.5 to 5.5 pg/mL. Conversely, concentrations of ergocryptine and its epimer, ergocryptinine, ranged from 1.2 to 6.8 pg/mL and 2.2 to 7.5 pg/mL, respectively. These very low serum concentrations of ergovaline were consistent with a relatively small daily dose (approximately 1 mg/d on E+ pastures), and with ergovaline being an exceptionally potent xenobiotic/toxin. However, it is likely that these serum samples also contained an apparent mixture of ergot-related compounds, each of which may contribute to the overall clinical fescue toxicosis syndrome, and the specific identification of which will require broadening the scope of this highly sensitive and specific analytical method.

Acknowledgments

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Keywords: Equine; Fescue toxicosis; Endophyte-positive; Ergovaline; Ergovalinine

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Safety and efficacy against uterine infections of an equine in-tranasal *Salmonella* vector vaccine

R.C. Causey [a], S.C. Artiushin [b], I.F. Crowley [a], A.D. Homola [a], A. Kelley [a], J.A. Weber [a], H.M. Opitz [a], L.A. Stephenson [a], S. Guilmain [a], J.F. Timoney [b]

[a] *Department of Animal and Veterinary Sciences and the Maine Agriculture and Forestry Experiment Station, University of Maine, Orono, ME, USA*

[b] *Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY, USA*

Attenuated *Salmonella enterica* serotype Typhimurium (*S. typhimurium*), expressing the SzP protective protein of the MB 9 serovar of *Streptococcus zooepidemicus* (SzP-MB9) was tested for its safety and efficacy as a vaccine against streptococcal uterine infections in mares. A laboratory-prepared Δ*cya* Δ*crp-pabA* mutant, attenuated strain of *S. typhimurium* (MGN 707), originally isolated from a case of equine

salmonellosis, was electroporated with plasmid pGP1-2 containing T7 RNA polymerase, and pET20-b containing the gene encoding SzP-MB9. It was previously demonstrated that mares vaccinated with this construct had serum, nasal and uterine antibody responses to *Salmonella* lipopolysaccharide (St-LPS) and to SzP-MB9. In the present study, responses to SzP-MB9 in reproductively healthy, vaccinated mares were compared to similar mares vaccinated with the *Salmonella* vector only. Serum, uterine and nasal washings were collected during estrus on two successive estrous cycles pre-vaccination, and at the first estrus $\geq$3 weeks post-vaccination. Antibody levels were assessed by an SzP-MB9-specific ELISA; cross-reacting antibodies were absorbed out using a heterologous streptococcal strain. Data were reported as optical density (OD), and analyzed by linear regression, with post-vaccination data as a reference (intercept) to which pre-vaccination values (regression coefficients) were compared. Vaccine vaccinated mares (expressor group, $n = 7$, 3–7 years old) had significant increases in SzP-MB9-specific uterine IgA (0.77 ± 0.54 vs. 2.13 ± 0.38), nasal IgA (0.84 ± 0.48 vs. 2.26 ± 0.32) and serum IgG (1.16 ± 0.23 vs. 2.06 ± 0.22); combined pre-vaccination data versus post-vaccination, mean OD $\pm$ S.E.M., $P < 0.05$). However, vector vaccinated mares (control group, $n = 7$, 3–16 years old) did not have significant changes in anti-SzP-MB9-specific antibody. Both groups had significant responses to St-LPS in serum, nasal and uterine secretions. When expressor ($n = 5$, age 4–17 years) and control ($n = 5$, age 3–8 years) groups were challenged intrauterinely with 6.3×10^9 cfu of *S. zooepidemicus*, significantly less *S. zooepidemicus* was cultured from uterine flushings of the expressor group (0.69 ± 0.41) compared to the control group (3.20 ± 0.65; $\log_{10}$ cfu, mean $\pm$ S.E.M., $P < 0.01$). There were no adverse reactions to vaccination. Most mares developed clinically apparent, transient endometritis after challenge. Since protective levels of SzP-MB9 antibodies have not been defined in the mare, it cannot be claimed that the reported antibody increases are protective *per se*. Based on the challenge study, vaccinated mares eliminated an inoculum typical of natural infection more quickly than controls; however, follow-up studies are necessary for confirmation. It was particularly noteworthy that uterine IgA mucosal responses were induced in the mare by intranasal vaccination.

Keywords: Uterus; Vaccination; Intranasal; *Salmonella*; Horse

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Food Animal

An estimate of the normal variation in the sperm DNA fragmentation index of Holstein bulls, and its association with serum testosterone and prolactin, over two spermatogenic cycles

R. Kasimanickam [a], W.H. Eyestone [a], J.M. DeJarnette [b], W.D. Whittier [a]

[a] *Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA*

[b] *Select Sires Inc., Plain City, OH, USA*

Numerous factors affect sperm DNA stability in bulls including age, breed, frequency of semen collection, sperm concentration, freezing and laboratory procedural differences, toxins, and infections. The objective of this study was to determine the variation in the sperm DNA fragmentation in bi-weekly ejaculates collected via artificial vagina over two spermatogenic cycles (19 wk) from 10 Holstein bulls (14–18 mo of age) housed in a commercial AI center. On sampling day, two ejaculates were collected in succession from each sire and pooled (within sire) for processing and cryopreservation. The frozen semen samples were thawed at 35 °C and subjected to flow cytometry analysis of sperm chromatin structure to estimate the DNA fragmentation index (DFI). Blood samples (for testosterone and prolactin) were collected bi-weekly (on sampling day). Correlations between the DFI and hormone concentrations were determined using Pearson's correlation coefficient. The DFI were analyzed using repeated measures ANOVA. Differences in the sperm DFI were compared both within and across bulls and weeks of collection. There was no significant correlation between serum testosterone and sperm DFI ($r = 0.03$; $P > 0.1$) and between serum prolactin and DFI ($r = 0.04$; $P > 0.1$). For DFI, there were no differences for bulls between weeks ($P > 0.1$), but there were differences among bulls within weeks ($P < 0.001$). In conclusion, within bulls, the sperm DNA fragmentation index was similar during the study period (two spermatogenic cycles). However, observed variations in the DFI among bulls within bi-weekly samples may contribute to differences in sire fertility potential. In this population of reproductively sound AI sires, there were no correlations among bi-weekly testosterone, prolactin, and DFI levels.

Keywords: Sperm; Semen; Chromatin; DNA fragmentation index; Bull

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Comparison of one versus two doses of prostaglandin F2 alpha in a 5-day, progesterone-based synchronization protocol in Angus-cross beef cows

R. Kasimanickam [a], M.L. Day [c], J.S. Rudolph [a], J.B. Hall [b], W.D. Whittier [a]

[a] *Department of Large Animal Clinical Sciences, The Ohio State University, Columbus, OH, USA*

[b] *Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA*

[c] *Department of Animal Sciences, The Ohio State University, Columbus, OH, USA*

The objective was to compare reproductive performance of Angus-cross beef cows synchronized with GnRH, a progesterone-releasing intravaginal device (CIDR) for 5 d, and one dose of either dinoprost tromethamine (PGF) or cloprostenol sodium (CLP), or two doses of PGF on the day of CIDR withdrawal. Angus-cross beef cows ($N = 830$) at six locations received 100 μg of GnRH and a CIDR on Day 0; the CIDR was subsequently removed on Day 5 (in the morning). Cows were fitted with a pressure-sensitive estrus detection device (Kamar) at CIDR withdrawal. Within farm, cows were randomly allocated to receive: 25 mg of PGF at CIDR removal (1XPGF; $n = 277$); or 25 mg of PGF at CIDR removal, and again 7 h later (2XPGF; $n = 282$); or 500 mg of CLP at CIDR removal (1XCLP; $N = 271$). On Day 8 (72 h after CIDR removal), all cows were given 100 μg of GnRH and concurrently inseminated.

Data were analyzed using a Glimmix model (SAS 9.1; SAS Institute, Cary, NC, USA). Accounting for other significant variables such as AI sire ($P < 0.05$) and cows that exhibited estrus at or prior to the time of AI ($P < 0.0001$), timed-AI pregnancy rates were greater ($P < 0.0001$) in the 2XPGF (69.0%) than the 1XPGF (52.0%) and 1XCLP (54.3%) treatments. Accounting for body condition score at the initiation of synchronization protocols ($P < 0.05$), breeding-season pregnancy rates were not different among 2XPGF, 1XPGF and 1XCLP treatment groups, 92.9, 87.0, and 87.5%, respectively ($P > 0.1$). The treatment × location interaction for pregnancy rate approached significance for timed-AI ($P = 0.09$) and was significant ($P < 0.01$) for breeding season pregnancy rate.

In conclusion, cows that received two doses of PGF on the day of CIDR removal in a 5-d CO-Synch + CIDR synchronization protocol had higher timed-AI pregnancy rates than cows receiving a single treatment with either PGF or CLP (at CIDR removal).

Keywords: Estrus synchronization; CIDR; $PGF2_a$; Pregnancy rate; Beef cattle

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Performance evaluation of the SQA-Vb automated sperm quality analyzer for bulls

U. Shalit [a,c], L. Rabinovitch [a], S. Greenberg [a], Y. Zeron [b]

[a] *Medical Electronic Systems, Caesarea, Israel*

[b] *Sion A.I. Company, Israel*

[c] *Medisoos Veterinary Practice, Israel*

The objective was to compare the automated SQA-Vb (Medical Electronic Systems, Caesarea Industrial Park, Israel) test results to manual semen analysis data, to determine if the automated system is a viable alternative to the manual method. The SQA-Vb is an analytical sperm quality analyzer for bulls that performs a 45-s automated quantitative evaluation of fresh and frozen-thawed bull semen, including sperm concentration, motility, progressive motility, normal morphology, and velocity. The study was conducted at Sion AI laboratory (Israel). For fresh and frozen-thawed bull semen samples ($n = 101$ and $n = 30$, respectively), automated semen analysis results were compared to manual test data analyzed microscopically. Sperm concentration was measured using a Makler sperm counting chamber (Sefi-Medical Ltd., Haifa, Israel).

Table 1
Performance characteristics of SQA-Vb automated sperm quality analyzer

Semen sample	Semen parameter	CV (%)	Correlation to manual method (r)	Range
Fresh $n = 101$	Concentration ($\times 10^6$/mL)	2.4	0.93	205.7–1900.0
	Motility (%)	4.1	0.81	4.2–99.1
	Progressive motility (%)	8.0	0.75	0.1–97.2
	Velocity (μ/s)	9.9	0.81	0–70
Frozen $n = 30$	Concentration ($\times 10^6$/mL)	4.7	0.99	5.7–129.6
	Motility (%)	5.6	0.90	32.1–76.1
	Progressive motility (%)	6.0	0.71	15.2–42.9
	Velocity (μ/s)	3.6	0.86	0–70

Abbreviations: CV: coefficient of variation; r: correlation coefficient; n: no. samples.

Sperm motility and progressive motility were evaluated with B-Sperm™ computerized video software (Medical Electronic Systems, Ltd. Caesarea Industrial Park, Israel). Bull semen was visualized ''live'' on a PC monitor using the video image mode; non-motile and non-progressively motile spermatozoa were counted first, then the ''live'' image was ''frozen'' and all sperm were counted. Percentages of motility and progressive motility were calculated. Each semen sample was counted twice (minimum of 100 spermatozoa per replicate). For precision, coefficients of variation (CV) were calculated based on duplicate samples. For analytical accuracy, correlations were determined between the automated versus the manual method. Data are summarized below (Table 1).

Coefficients of variation $\leq$10% demonstrated high repeatability of the SQA-Vb. Furthermore, SQA-Vb and manual data were highly correlated. Therefore, we concluded that the SQA-Vb was a viable alternative to manual analysis of bull semen.

Keywords: SQA-Vb; Semen analysis; Motility; Sperm; Cattle

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Assessing cryocapacitation in frozen-thawed bovine sperm from a commercial bull stud

M. Knobbe [a], M. Reese [a], M. Kaproth [b], I. Dobrinski [a], H. Galantino-Homer [a]

[a] *University of Pennsylvania School of Veterinary Medicine, Kennett Square, PA, USA*

[b] *Genex Cooperative, Inc., Ithaca, NY, USA*

Precocious sperm capacitation or 'cryocapacitation' may contribute to reduced fertility for cryopreserved semen, but there is no quantifiable, biochemical assay for this functional change. We hypothesized that if frozen-thawed bovine sperm undergo cryocapacitation, it would be detected by protein tyrosine phosphorylation, a quantifiable biochemical marker of fresh bovine sperm capacitation, and an increase in the rate of lysophosphatidylcholine-induced acrosome reaction (LC AR), a standard sperm capacitation assay. For this study, commercially processed cryopreserved semen (milk-glycerol extender) from single ejaculates of 19 Holstein bulls was evaluated twice for each bull. Samples were evaluated post-thaw (0 h) or after a 4-h incubation in TALP medium with: no additives (NA); or 10 μg/mL heparin (H); or 10 μg/mL heparin and 1 mM dibutyryl-cAMP plus 100 μM IBMX (HcA; known to maximize bovine sperm capacitation). Sperm were assessed for viability using CFDA/Propidium Iodide staining, for spontaneous (sAR) and LC AR (Coomassie G-250 stain), and for protein tyrosine phosphorylation, as assessed by both antiphosphotyrosine slot blot analysis and standard one-dimensional SDS-PAGE immunoblot analysis. Protein tyrosine phosphorylation of slots and one band (p48) were quantified by image analysis (Gel-Pro 4.5, Media Cybernetics, Silver Spring, MD, USA). Data are presented as means $\pm$ S.D. S.D. and were evaluated by three-way ANOVA, followed by Tukey's test for multiple comparisons, with significance set at $P < 0.05$ (Sigma-Stat 3.0; SPSS Inc., Chicago, IL, USA). Except for two bulls with low viability (A, B), there were no significant inter-bull differences in sperm viability, but viability declined in significant increments for NA, H, and HcA (0 h: 60.7 $\pm$ 8.9%; NA: 53.0 $\pm$ 8.2%; H: 47.8 $\pm$ 10.3; and HcA: 33.3 $\pm$ 8.9%). The rates of sAR at 0 h were lower than the 4 h incubated samples (0 h: 15.7 $\pm$ 6.6%; NA: 40.6 $\pm$ 21.3%; H: 42.7 $\pm$ 19.8; HcA: 39.6 $\pm$ 16.9%) and only two bulls (B, C) had significantly higher sAR rates. Rates of LC AR at 0 h were highly variable, did not differ significantly between bulls or between experiments for the same bull, and were consistent with some cryocapacitation (14.8 $\pm$ 17.1%). LC AR at 4 h was significantly reduced compared to 0 h, highly variable, and not consistently repeatable (NA: 1.7 $\pm$ 17.7%; H: $-$1.3 $\pm$ 13.8; HcA: 1.4 $\pm$ 10.2%). Slot blot analysis was consistently repeatable for 0 h samples, but not for incubated samples, and detected significantly higher protein tyrosine phosphorylation for one bull (A). Tyrosine phosphorylation of p48 relative to positive control (HcA), differed significantly among treatments (0 h: 17.8 $\pm$ 7.8%; NA: 27.5 $\pm$ 10.7%; H: 33.3 $\pm$ 13.5%), was consistently repeatable, and detected two bulls (D, E) with reduced, and one with increased (F) p48 tyrosine phosphorylation. The relative lack of inter-bull differences detected with these assays was not unexpected, as all bulls were proven sires. Tyrosine phosphorylation of p48 provided data similar to LC AR at 0 h, but not following incubation, perhaps due to the high rate of sAR. Protein tyrosine phosphorylation is therefore a good candidate biochemical marker for bovine sperm cryocapacitation, but should be characterized in bulls with more variable responses to freezing.

Keywords: Semen analysis; Sperm; Cryocapacitation; Tyrosine phosphorylation; Bovine

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Competitive Session

Anti-luteogenic and luteolytic effects of $PGF_{2\alpha}$ during the post-ovulatory period in mares

C. Rubio [a], C.R. Pinto [a], B.E. Holland [a], B.L. da Silva Jr.[a], S.A. Layne [a], L.H. Heaton [a], C.S. Whisnant [b]
[a] *Department of Population, Health and Pathobiology, College of Veterinary Medicine, Raleigh, NC, USA*
[b] *Department of Animal Science, North Carolina State University, Raleigh, NC, USA*

In the present study, it was hypothesized that mares receiving multiple doses of $PGF_{2\alpha}$ during the post-ovulatory period would undergo luteolysis similar to control mares given a single injection of $PGF_{2\alpha}$ during mid-diestrus. The specific objectives were to document the effects of $PGF_{2\alpha}$ treatment on concentrations of plasma progesterone (P_4) and interval to ovulation. Cycling mares were examined using transrectal ultrasonography once daily (when in estrus) and were allocated to treatment groups as follows: Group I ($n = 10$) received 2.5 mg of $PGF_{2\alpha}$ IM (Lutalyse®, Pfizer Animal Health, New York, NY, USA), on Day 10 (ovulation = Day 0); Group II ($n = 10$) received 2.5 mg of $PGF_{2\alpha}$ once daily on Days 2, 3, and 4; Group III ($n = 7$) received 2.5 mg of $PGF_{2\alpha}$ twice daily on Days 0, 1, and 2; and Group IV ($n = 6$) received 10 mg of $PGF_{2\alpha}$ twice daily on Days 0, 1, and 2. Plasma samples were collected daily and stored at -20 °C pending determination of P_4 (RIA). An ANOVA was used to compare plasma P_4 concentrations and intervals from $PGF_{2\alpha}$ treatment to ovulation. Data are presented as mean (±S.E.M.) and intervals from treatment to ovulation were relative to the first $PGF_{2\alpha}$ treatment. For Groups I, II, III, and IV, plasma P_4 concentrations (ng/mL) before treatment were 12.05 ± 1.6, 5.3 ± 0.7, 0.2 ± 0.04, and 0.5 ± 0.3, respectively ($P < 0.05$), 3 d after the start of treatment they were 0.6 ± 0.1, 1.3 ± 0.14, 1.3 ± 0.5, and 0.48 ± 0.2 ($P > 0.05$), and 5 d after the start of treatment they were 0.5 ± 0.07, 1.8 ± 0.4, 2.9 ± 1.0, and 0.5 ± 0.4 ($P < 0.05$). All mares in Group I ovulated 8.2 ± 0.7 d after treatment. In Group II, six of 10 mares underwent complete luteolysis and ovulated 9.4 ± 1.4 d after treatment; the remaining four mares underwent partial luteolysis, followed by a resurgence in luteal function ($P_4 > 1.0$ ng/mL), and ovulated 14.7 ± 2.1 d following treatment. For all mares in Group III, $PGF_{2\alpha}$ treatment markedly suppressed the rise in plasma P_4, followed by resurgence in luteal function. Six Group III mares ovulated 14–26 d after treatment and one remained anovulatory. In Group IV, $PGF_{2\alpha}$ treatment also had an anti-luteogenic effect; mean plasma P_4 on Day 5 remained < 1.0 ng/mL, and four of six mares ovulated 7.0 ± 1.8 d after treatment.

In summary, luteal function was affected by $PGF_{2\alpha}$ treatment in mares during early diestrus (Group II); the immature CL was responsive to multiple $PGF_{2\alpha}$ injections. Serial administration of $PGF_{2\alpha}$, beginning on the day of ovulation, had an anti-luteogenic effect, manifested as low plasma P_4 concentrations for several days after treatment, especially in mares of Group IV (that received a higher dose of $PGF_{2\alpha}$ than those in Group III). Future adjustments in $PGF_{2\alpha}$ treatment (frequency and dosage) may increase the percentage of mares ovulating in Groups II and IV.

Keywords: Corpus luteum; $PGF_{2\alpha}$; Anti-luteogenic; Luteolysis; Horse

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Comparison of three doses of reFSH for super-ovulation of mares

C.A. DeLuca, P.M. McCue, M.L. Patten, E.L. Squires
Colorado State University, Fort Collins, CO, USA

Superovulation using purified equine follicle stimulating hormone (eFSH) increased the efficiency of embryo recovery in mares. In preliminary studies, recombinant equine FSH (reFSH) stimulated ovarian follicular development in transitional mares and increased ovulation rates of cycling mares. The objective of this study was to compare ovulation and embryo recovery rates among three doses of reFSH. Our hypothesis was that reFSH would have a dose-dependent increase in ovulation and embryo recovery rates.

Twenty-eight mares were randomly assigned to one of five treatment groups (total of 67 estrous cycles). Mares in Group 1 served as untreated controls. Mares in Group 2 received 12.5 mg eFSH twice daily IM, whereas those in Groups 3, 4, and 5 received 0.35, 0.5, or 0.65 mg reFSH, twice daily IM, respectively. Mares were allowed to go through one estrous cycle (to determine day of ovulation) and treatment was initiated when a follicle 22–25 mm in diameter was detected. Mares received cloprostenol sodium (250 μg IM) on the second day of treatment; eFSH or reFSH was given until ≥50% of the developing follicles reached 35 mm in diameter. Mares were allowed to 'coast' for 36 h before

receiving hCG (2500 units IV). Mares were inseminated 12 h later with 500 million progressively motile sperm, and rebred with cooled semen the following day. Ovulation was confirmed via daily transrectal ultrasonography and embryo recovery was attempted 7 or 8 d post-ovulation. Data were analyzed with Fisher's Exact Test and ANOVA, and results are tabulated below:

Group	No. of cycles	Ovulated (%)	Ovulation rate*	Embryos recovered per flush**
Control	19	100	1.31 ± 0.51^{a}	0.77 ± 0.38^{a}
eFSH, 12.5 mg	11	81.8	$3.04 \pm 0.64^{b,c}$	2.38 ± 0.57^{b}
reFSH, 0.35 mg	14	78.6	$2.11 \pm 0.59^{a,c}$	$1.41 \pm 0.49^{a,b}$
reFSH, 0.5 mg	11	100	4.59 ± 0.64^{b}	$1.48 \pm 0.48^{a,b}$
reFSH, 0.65 mg	12	91.7	$3.34 \pm 0.62^{b,c}$	2.69 ± 0.48^{b}

Within a column, values without a common superscript (a, b, c) differed ($P < 0.05$).

* Includes data from all mares treated.

** Only mares that ovulated were flushed.

In summary, for the three reFSH doses evaluated, ovulation rates per cycle were highest in mares given 0.5 or 0.65 mg twice daily. However, there was no significant difference among doses in embryo recovery rates per flush.

Keywords: Superovulation; FSH; Embryo; Equine; Horse

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Efficacy of medroxyprogesterone acetate in suppression of estrous behavior and follicular activity in cycling mares

E.K. Gee, P.M. McCue, C.A. DeLuca, J.L. Stylski

Equine Reproduction Laboratory, Colorado State University, Fort Collins, CO, USA

Various progestins are used in performance mares to pharmacologically suppress estrous behavior, including injectable medroxyprogestone acetate (MPA). However, there have been no scientific studies to evaluate the efficacy of MPA in suppressing estrus in the mare. The purpose of our study was to evaluate the effects of MPA on ovarian follicular activity and estrous behavior. Eighteen cycling Quarter Horse-type mares were randomly assigned to one of three treatment groups: MPA (Wedgewood Pharmacy, Swedesboro, NJ, USA), saline, or altrenogest (Regu-Mate®, Intervet, Millsboro, DE, USA). Treatments started 7 d after ovulation. Mares in the MPA treatment group ($n = 6$) were treated with 1600 mg MPA IM initially, then 400 mg once weekly for 5 weeks. Saline-treated mares ($n = 6$) were given saline IM once weekly for 6 weeks. Altrenogest-treated mares ($n = 6$) received 10 mL altrenogest (22 mg) orally daily for 48 d. During treatment and 18 d after treatment ceased, mares were teased daily by an experienced stallion and were categorized as displaying behavior characteristic of estrus or diestrus. Transrectal ultrasonographic examinations were performed thrice weekly, or daily beginning when a 35 mm follicle was identified until ovulation. Blood samples were collected weekly and serum frozen for analysis of progesterone. Assessments of behavior, follicular activity, and serum progesterone concentrations were done in the blind. Data were analyzed by Chi-square and ANOVA, with pair-wise comparisons.

Mares given saline versus MPA had no significant differences in duration of diestrous behavior (mean $\pm$ S.D., 14 ± 2 and 16 ± 4 d, respectively) or estrous behavior (8 ± 2 and 7 ± 1 d, respectively) for the duration of the study. No altrenogest-treated mares displayed signs of estrous behavior during the treatment period, but four resumed estrous behavior 6–18 d after cessation of treatment. All mares in the saline and MPA treatment groups had normal follicular development and ovulations throughout the study. No mares given altrenogest ovulated during the treatment period; four resumed normal follicular development after treatment ceased. Least squares means showed significant differences in the interovulatory interval of mares given altrenogest versus saline and those given altrenogest versus MPA ($P < 0.0001$ and $P < 0.0001$, respectively), with no differences in interovulatory interval between saline versus MPA mares ($P = 0.7$). Serum progesterone concentrations were consistent with follicular and behavioral activity for all mares. In conclusion, MPA did not effectively suppress estrous behavior or follicular activity in normal cycling mares; therefore, it is not recommended for this purpose.

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Keywords: Estrus suppression; Estrous behavior; Medroxyprogesterone acetate; Equine; Horse

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Soluble adenylate cyclase generated cAMP acts via protein kinase-A and not Epac 1/2 to direct capacitation-associated protein tyrosine phosphorylation in stallion sperm

L.A. McPartlin, S.J. Bedford-Guaus
Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

Capacitation is a complex process that encompasses the molecular changes sperm must undergo to successfully fertilize an oocyte. Our laboratory recently defined for the first time *in vitro* incubation conditions that yielded significant time-dependent increases in protein tyrosine phosphorylation, coupled with significant rates of progesterone-induced acrosomal exocytosis, suggesting that sperm are undergoing changes consistent with capacitation. Using pharmacological inhibitors and activators, our objectives were to investigate the roles of soluble adenylate cyclase (sAC), cAMP-dependent protein kinase-A (PKA) and the recently identified cAMP-dependent guanine nucleotide exchange factors, Epac 1 and 2, in capacitation-associated protein tyrosine phosphorylation in stallion sperm. Semen was collected with an artificial vagina from six adult stallions of proven fertility. Seminal plasma was removed by centrifugation at 37 °C and sperm were resuspended at 10×10^6 sperm/mL in modified Whitten's medium (MW: 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose (anhydrous), 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt, and 1.0 mM pyruvic acid, 25 mM $NaHCO_3$ and 7 mg/mL BSA; pH 7.25) and incubated at 37 °C in a humidified atmosphere for 0, 2, 4, and 6 h. In all experiments, mouse sperm were used as a positive control; cauda epididymal sperm were isolated using the swim-out method into 37 °C MW (pH 7.35). Sperm were centrifuged, resuspended at 2×10^6 sperm per 300 μL MW, and incubated in a 37 °C water bath for 0, 30, and 60 min. Immunoblot analysis using anti-phosphotyrosine antibodies was used to determine the effects of treatment on protein tyrosine phosphorylation. The sAC specific inhibitor KH7 prevented the time-dependent increase in protein tyrosine phosphorylation in both stallion (60 μM) and mouse sperm (30 μM). Because PKA is a downstream target of cAMP, we tested two different PKA inhibitors: (1) H89, which inactivates the active catalytic subunit of PKA; and (2) PKI, which binds to the regulatory subunits and prevents disassociation of the holoenzyme. In stallion sperm, incubation in the presence of H89 caused a dose-dependent increase in tyrosine phosphorylation, whereas PKI (60 μM) effectively inhibited tyrosine phosphorylation. In mouse sperm, as expected, both H89 (30 μM) and PKI (40 μM) inhibited tyrosine phosphorylation. Next, we wanted to determine whether Epac 1/2 are present in equine sperm, and their plausible role in promoting protein tyrosine phosphorylation. Using indirect immunofluorescence, we localized Epac 1 to the acrosome and Epac 2 to the midpiece in both stallion and mouse sperm. However, incubation of stallion or mouse sperm in the presence of 8-pCPT-2′-O-Me-cAMP (100 μM), an Epac-specific cAMP analogue that does not activate PKA, had no effect in the pattern or levels of time-dependent protein tyrosine phosphorylation. Collectively, these experiments demonstrated for the first time the requirement for sAC generated cAMP, and that cAMP is acting through PKA and not Epac 1/2, to direct these capacitation-associated phosphorylation events. We are presently investigating the potential role of Epac 1/2 in other capacitation-associated events in stallion sperm.

Keywords: Stallion; Sperm; Capacitation; Protein kinase-A; Epac

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Luteinizing hormone-induced release by kisspeptide in primary cultures of equine pituitary cells

C. Magee, J.A. Arreguin-Arevalo, J.D. Cantlon, M.M. Mrdutt, T.M. Nett, C.M. Clay
Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, USA

Kisspeptins, in association with their receptor GPR54, are believed to have a ''gatekeeper''-like effect on reproductive function by mediating GnRH release at the level of the hypothalamus. Recently we demonstrated that in the horse, as in many other species, IV administration of the decapeptide KiSS-10 increased serum concentrations of both LH and FSH. Although the main role of kisspeptide occurred at the hypothalamus, some reports indicated a secondary effect at the level of the pituitary gland. The objective of this series of experiments was to determine if KiSS-10 was able to release LH by a direct effect at the level of the pituitary. Primary cultures of gelding (age, 2 to 5 y) pituitary cells were conducted in October ($n = 2$) and January ($n = 2$). In October, cultures (6×10^5 cells/well, $n = 3$ wells/treatment) were treated for 60 min with 100 nM KiSS-10 (AnaSpec, Inc., San Jose, CA, USA), or control (no treatment), or 10 nM GnRH (Bachem, Fine Chemicals,

Torrance, CA, USA) to serve as a positive control. Media was then collected for detection of equine LH via RIA. There was a nine-fold increase in LH in GnRH vs control cells (mean ± S.E.M., 997.5 ± 114.8 vs 169.0 ± 59.2 ng/mL, $P < 0.006$), but there was no significant difference in the control vs KiSS-10 (182.4 ± 32.9 ng/mL) cells. To determine if cell density or KiSS-10 dose affected the response to KiSS-10, cells in January were also plated at two concentrations (6×10^5 vs 1.2×10^6/well), and we used three doses of KiSS-10 (1×10^2, 1×10^3, and 1×10^4 nM; $n = 3$ wells/treatment). In the absence of a significant trend in response using the two cell densities, we inferred that increasing cell density did not have an effect on gonadotrope cell function nor LH release. Nonetheless, there was a two-fold increase in LH following GnRH vs control (78.1 ± 9.3 vs 41.5 ± 6.2 ng/mL, $P < 0.005$). Interestingly, KiSS-10 also elicited a two-fold increase in LH vs control cells, regardless of dose ($P < 0.02$ for all treatments). Both the reduced LH response to GnRH and the reduced mean LH concentration obtained from non-treated control cells in January were consistent with decreased GnRH receptor number and LH content in the equine pituitary at the depth of the non-breeding season. The direct pituitary response to KiSS-10 in January may be explained by increased pituitary sensitivity to KiSS-10, mediating the ''gatekeeper'' effect in seasonal transition. Assessing seasonal changes in equine pituitary GPR54 may yield a more precise evaluation of pituitary sensitivity to KiSS-10 and delineate the hierarchal role of kisspeptins.

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Keywords: Kisspeptide; LH; GnRH; Pituitary culture; Horse

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Evaluation of cryopreserved-thawed stallion sperm before and after density gradient centrifugation with silane-coated silica particles (EquiPure®)

A. Stoll, B.L. Stewart, A.M. Brum, I.K. Liu, B.A. Ball
School of Veterinary Medicine, University of California, Davis, CA, USA

By enhancing the sperm population used for AI, fewer cells can be used and dead or damaged sperm, which may have deleterious effects on the normal sperm, can be removed. Specifically, we hypothesized that the removal of dead or damaged sperm will increase the quality of live sperm after thawing of frozen semen, as measured by mitochondrial membrane potential, motility, and morphology of live sperm.

Semen from five mature stallions (two ejaculates each) was cryopreserved (in 0.5-mL straws) using a standard protocol, and stored in liquid nitrogen at −196 °C. For each ejaculate, four straws were thawed (37 °C for 30 s), diluted in a modified Tyrode's medium containing 0.1% polyvinyl alcohol (TALP-PVA), layered over an 80% EquiPure® gradient, and centrifuged at 200 × *g* for 35 min (EquiPure® sample). After centrifugation, sperm were collected from the bottom of the tube, resuspended in TALP-PVA, and washed once. Four additional straws were similarly thawed, diluted with TALP-PVA, and washed once to remove freezing extender (untreated sample). Sperm motility was determined by computerized semen analysis (CASA) and concentrations of both sperm preparations were adjusted to 5×10^6 sperm/mL. Aliquots of both preparations were analyzed for changes in membrane structure by staining with YoPro-I and propidium iodide (DNA stains with differential permeability across the plasma membrane). Mitochondrial function was assessed by staining with JC-1, a potentiometric probe which can be used to quantify mitochondrial membrane potential in equine sperm. Following incubation with YoPro-I, propidium iodide, and JC-1, samples were assessed with flow cytometry. Sperm morphology was assessed with differential interference contrast (DIC) microscopy (1000×) by an observer blinded to treatment group.

The separation of frozen-thawed equine sperm by centrifugation through the EquiPure® gradient increased ($P < 0.001$) total (80 ± 2.4% vs 41.7 ± 2.4%) and progressive sperm motility (69.5 ± 2.9% vs 31.5 ± 2.9%), as well as the percentage of morphologically normal sperm (45 ± 3.9% vs 27.7 ± 3.9%) compared to the control. In addition, the proportion of sperm in the treated sample with high mitochondrial membrane potential increased (81.6 ± 1.8% vs 42.1 ± 1.8%), as

did sperm viability (71.1 ± 2.4% vs 39.5 ± 2.4%) compared to the control. The proportion of sperm with altered membrane permeability was decreased in the EquiPure® sample (6.4 ± 1.1% vs 18.9 ± 1.1%).

In conclusion separation of frozen-thawed equine sperm via centrifugation through an EquiPure® density gradient increased total and progressive motility, normal morphology, mitochondrial membrane potential, and plasma membrane alteration of the separated sperm population (compared to the initial control). Although fertility was not examined in the present study, in specific situations, this separation procedure may improve fertility of frozen-thawed equine sperm.

Keywords: EquiPure®; Density gradient separation; Equine spermatozoa; Flow cytometry; Horse

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