Bachem, Inc., Torrance, CA, USA) in diestrous mares ($n = 12$ per treatment). Preliminary RIA data suggested that 0.5 mg KiSS-10 elicited a LH response similar to that of 25 μg LHRH. To determine if a single intravenous injection of the decapeptide formulation was sufficient to induce ovulation in the estrous mare, 1.0 mg KiSS-10 was compared to a known ovulation inducing agent (2500 IU hCG, iv; Chorulon, Intervet Inc., Milisboro, DE, USA) and a negative control (1.0 mL saline, iv). There was no difference ($P > 0.3$, t-test) in the time (mean ± S.E.M.) from treatment to ovulation in the saline ($n = 11$, 64.5 ± 9.5 h) and kisspeptide ($n = 11$, 69.1 ± 10.1 h) mares. When compared with kisspeptide-treated mares, hCG-treated mares consistently ovulated within a predicted and shorter ($n = 12$, 41.4 ± 0.5 h) interval ($P < 0.005$, t-test). No mare ovulated within 12 h of treatment in any group. Five mares failed to ovulate within 4 days of treatment, three of which were in the saline group and two were in the kisspeptide group. Given the duration of the equine pre-ovulatory LH surge, it is not surprising that ovulation was not induced with a single intravenous dose of the decapeptide. Using immunohistochemistry, individual GnRH and kisspeptide neurons in the diestrous mare hypothalamus ($n = 6$) have been identified. Characterization and distribution mapping of these neuronal structures is ongoing. In conclusion, we have demonstrated that the estrous mare can respond to exogenous administration of kisspeptide by releasing LH. Upon further investigation of the decapeptide and its receptor, a dose regimen of kisspeptide sufficient to induce ovulation will be determined. Evaluation of the role of kisspeptin in the hypothalamic-pituitary-gonadal axis in the mare is essential for future applications for ovulation induction in the estrous mare or management of seasonal transition.

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Keywords: Mare; Kisspeptin; Hypothalamic-pituitary-gonadal axis; Ovulation

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ENRICHMENT OF EQUINE SPERM BY MAGNETIC ACTIVATED CELL SORTING

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Exposure of phosphatidylserine (PS) in the outer membrane leaflet of the plasma membrane represents disturbed membrane integrity, an early phase of apoptosis or cell death. Annexin V binds to exposed PS and enables the identification of cells with deteriorated membrane integrity at an earlier stage than staining with supravital stains. Miltenyi Biotec (Auburn, CA, USA) manufactures paramagnetic annexin V-microbeads that can be combined with their magnetic activated cell sorting (MACS) system to eliminate dead or membrane damaged cells from a population. This method has been used successfully to enrich human sperm populations. The objective of this study was to investigate its use with equine sperm.

A test population (50×10^6/mL) containing a mixture of annexin-positive and -negative cells was obtained by mixing (at a ratio of 1:1) freshly ejaculated equine sperm extended in BWW with positive control sperm (generated by incubating sperm for 30 min on ice in 3% formalin). A 1 mL aliquot was centrifuged and resuspended with 400 μL of binding buffer plus 100 μL of annexin-microbeads and incubated for 15 min at room temperature (in the dark). The LD MACS column was prepared by flushing with 3 mL of binding buffer. After incubation, sperm were centrifuged and resuspended in 1 mL of binding buffer and applied to the LD column. The negative fraction was collected together with an additional 1 mL wash with binding buffer. The column was removed from the magnet and the positive fraction was collected by pushing 1 mL of binding buffer through the column with the syringe component. An aliquot (500 μL) of positive- and negative-fractions and unsorted sperm were labeled (8 min, room temperature, in the dark) with Annexin V (0.25 μg/mL) and propidium iodide (4.8 μg/mL), and then analyzed by flow cytometry. MACS ($n = 7$) significantly increased live annexin-negative sperm in the negative fraction (81 ± 2.9%; mean ± S.E.M.) when compared to the unsorted sample (44 ± 3.0%). In a subsequent preliminary experiment ($n = 3$), cryopreserved equine sperm were thawed (30 s at 37 °C), diluted to 50×10^6 mL^{-1} in BWW, centrifuged, incubated with

annexin microbeads, and subjected to column separation as described above. MACS resulted in a significant increase in live annexin-negative sperm in the negative fraction (64 ± 6.5%) when compared to the unsorted sample (34 ± 2.0%).

In conclusion, magnetic activated cell sorting of equine sperm resulted in an enriched population of live, membrane-intact sperm. However, there are a number of obstacles with the methodology that remain to be overcome, including the binding buffer which negatively affects sperm motility, the cost of the reagents that potentially limits routine clinical application and adaptation of the protocol to handle higher volumes and cell concentrations.

Keywords: Equine sperm; Cell separation; Annexin; MACS

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EFFECT OF ARTIFICIAL INSEMINATION PROTOCOL AND SPERM DOSE ON PREGNANCY RESULTS IN MARES

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Objectives: The objectives of this study were to evaluate whether: (1) deep horn insemination with a low sperm dose is feasible with commercially available semen doses; (2) those low doses require deep horn insemination instead of conventional insemination into the corpus uteri; (3) the device used for deep horn insemination [Ghent device (Beldico, Belgium) versus Minitüb pipette (Minitüb, Germany)] affects pregnancy rates.

Materials and methods: Twenty-five cyclic, non-lactating French trotter mares with an average age of 5 years (range, 3–7) were housed in pens with access to pasture. Twenty-one were maiden, three had foaled once and one had foaled twice. The mares had no history of reproductive failure and were clinically normal. During spontaneous estrous cycles, the dominant follicle was measured by ultrasonography every 6 h; mares were inseminated with frozen/thawed semen [(high dose: 138×10^6 pms in five straws (2.5 mL) or low dose: 27×10^6 pms in one straw (0.5 mL)] within 6 h post ovulation. Only mares that had no intrauterine fluid at that time were included. For each ovulation, mares were assigned randomly to one of four artificial insemination (AI) protocols (cross-over design): (1) HUB = high dose AI into the uterine body; (2) LUB = low dose AI into the uterine body; (3) LDHgd = low dose AI deep into the horn with the Ghent Device; (4) LDHmp = low dose AI deep into the horn with the Minitüb pipette. Semen of two stallions with a known fertility status (first: 'good fertilizer' and second: 'bad fertilizer') was used. Fourteen days after insemination, pregnancy diagnosis was made by ultrasonography (5 MHz, Pie Medical, Falco). Data were analysed using logistic regression with pregnancy result as outcome variable, AI protocol as predictor variable of main interest, and mare as random effect to account for repeated measurements.

Results and conclusions: In total, 143 inseminations were performed, resulting in 40 pregnancies (27.9%). In the HUB and LUB groups, 14 out of 35 and 5 out of 35 inseminations respectively, were successful. The average difference in pregnancy result between HUB and LUB was 26.4% in favour of HUB (95% CI: 0.05–46.8, $P = 0.01$).

LDHgd insemination resulted in 12 pregnancies out of 48 inseminations and LDHmp in 10 pregnancies out of 25 inseminations. The average difference in pregnancy results between LDHmp and LUB was 30.6% (95% CI: 7–54, $P = 0.01$).

Pregnancy results were not different between the HUB and LDHmp protocols (4.2% difference in favour of LDHmp, 95% CI: −30.7 to 0.22, $P = 0.74$) and between the HUB and the LDHgd group (12.4% difference in favour of HUB, 95% CI: −8 to 33.8, $P = 0.24$).

The conclusion is that deep horn insemination with a reduced sperm dose using the Minitüb pipette resulted in the same pregnancy rate as uterine body insemination with a full sperm dose.

Keywords: Deep horn insemination; Low sperm dose; Frozen-thawed semen

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