

Equine

THE PATHWAY OF EQUINE GROWTH HORMONE ON EQUINE OOCYTE MATURATION AND RECEPTOR LOCALIZATION BY IMMUNOCHEMISTRY AND REAL-TIME PCR

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In previous studies, equine growth hormone (eGH) had a positive effect on equine oocyte maturation. The objective of this study was to determine the location of eGH-receptor (eGH-R) on equine ovarian structures, including follicular wall, oocyte and cumulus oocyte complexes. Additionally, we observed if the eGH influences on oocyte maturation is removed when specific inhibitors against growth hormones are added to the culture in vitro. Real-time reverse transcription-PCR (RT-PCR) and immunocytochemistry methods were used to localize eGH-R in equine follicles and oocytes. Paraffin sections comprising the equine oocyte attached to the follicular wall were treated by streptavidin-biotin method for immunochemical detection. Briefly, primary antibodies (α -growth hormone; 1:20 dilution) were incubated overnight at 4 °C in a humidified chamber, later incubated with biotinylated secondary antibodies (α -mouse IgG; 1:200 dilution), streptavidin complex (1:400 dilution) and counterstained with haematoxylin. Quantification of the relative transcript levels of eGH-R was made by *Taq-Man* PCR Reagent kit and performed using an ABI Prism 7700 Sequence Detection System. Primers for eGH receptor precursor were based on cDNA sequence published at Genbank (AF097588). Pools of 15 cumulus oocyte complexes, denuded oocytes and cumulus cells alone were used for RT-PCR experiment. Addition of a specific adenylate cyclase inhibitor (DDA) and a specific inhibitor against cyclic AMP-dependent protein kinase (H-89) were used during culture in vitro to observe whether this inhibitory effect can block the eGH action on oocyte maturation. Results of different treatments groups

were compared by χ^2 -test for trend (significance was set at $P < 0.05$). Cumulus oocytes complexes incubated with eGH, 29/61 oocytes (47.5%), were classified as mature versus 18/64 oocytes (28.1%) in the control group ($P = 0.02$). The H-89 inhibitor used at 10×10^{-6} $\mu\text{M}/\text{mL}$ decreased the number of oocytes reaching maturity when compared with control (5/13 versus 9/10; respectively; $P = 0.05$). The DDA inhibitor also reduced the number of oocytes reaching maturity (4/19 versus 9/12; $P = 0.05$). Results showed that: (1) significant positive immunostaining for eGH-R in cumulus cells, oocyte and granulosa cells of the follicle; (2) quantification by *Taq-Man* PCR showed that relative gene transcription for eGH-R was higher in cumulus cells and cumulus oocyte complexes at the start of in vitro maturation; (3) H-89 and DDA can be used in vitro to block the action of eGH on equine oocyte maturation. We inferred that the presence of eGH-R in equine ovarian follicular structures might mediate a positive effect in response to eGH in equine in vitro oocyte maturation.

Keywords: Equine growth hormone; Growth hormone receptor; Oocyte maturation

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EVALUATION OF KISSPEPTIN IN THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS OF THE MARE

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Kisspeptin and its receptor, GPR54, are crucial elements in the onset of pubertal development and may play a role in seasonal estrus and induction of ovulation via a mechanism of signaling GnRH release at the level of the hypothalamus. The purpose of this series of experiments was to evaluate the role of kisspeptide and GPR54 in the seasonally estrous mare. In a preliminary study, no difference was observed in LH response profiles following intravenous administration of 1.0 and 10.0 mg of rat kisspeptide (KiSS-10, AnaSpec, Inc., San Jose, CA, USA) in diestrous mares ($n = 3$ per treatment). To further elucidate a physiologic LH response to kisspeptide administration, three dose rates of kisspeptide (1.0 μg , 0.5 mg, 1.0 mg, iv) were compared to that of native GnRH (25 μg LHRH, iv;

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 $\pm$ 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 ($50 \times 10^6/\text{mL}$) 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 BWB 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 $\mu\text{g}/\text{mL}$) and propidium iodide (4.8 $\mu\text{g}/\text{mL}$), and then analyzed by flow cytometry. MACS ($n = 7$) significantly increased live annexin-negative sperm in the negative fraction ($81 \pm 2.9\%$; mean $\pm$ S.E.M.) when compared to the unsorted sample ($44 \pm 3.0\%$). In a subsequent preliminary experiment ($n = 3$), cryopreserved equine sperm were thawed (30 s at 37°C), diluted to $50 \times 10^6 \text{ mL}^{-1}$ in BWB, centrifuged, incubated with