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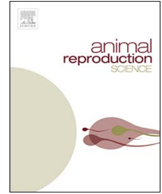

Effects of immunization against bone morphogenetic protein-15 and growth differentiation factor-9 on ovarian function in mares

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Currently there is no contraceptive vaccine that can cause permanent sterility in mares. This study investigates the effect of vaccination against oocyte-specific growth factors, Bone Morphogenetic Protein 15 (BMP-15) and Growth Differentiation Factor 9 (GDF-9), on ovarian function of mares. It was hypothesized that immunization against these growth factors would prevent ovulation and/or accelerate depletion of the oocyte reserve. For this study, 30 mares were randomly assigned to three groups (n = 10/group) and vaccinated with BMP-15 or GDF-9 peptides conjugated to KLH and adjuvant, or a control of phosphate buffered saline and adjuvant. Horses received vaccinations at weeks 0, 6, 12, and 18. Ovarian activity and estrous behavior were evaluated 3 days a week via ultrasonography and interaction with a stallion. The study was initiated on March1, 2016. Upon evaluation of ovulation rate, the GDF-9 group did not have a difference (P = 0.66) in ovulation rate when compared to controls (10.8 and 10.0 ovulations, respectively), but the number of ovulations in the BMP-15 group was less (P = 0.02; 4.9 ovulations). Average follicle size prior to ovulation was less ($P < 0.0001$) in both treatment groups compared to controls. Estrous behavior was altered in both the BMP-15 and GDF-9 groups compared to controls after the second vaccination (P = 0.05 and 0.03, respectively). Although further research is required to determine the continued effects of vaccination against GDF-9 on ovulation rates, these results indicate that vaccination against BMP-15 and GDF-9 could serve as a contraceptive in wild horse populations.

1. Introduction

The current wild horse and burro population in the United States is nearly three times what the rangeland can support, which is detrimental for wild horses, wildlife, and rangeland (BLM.gov, 2017) The Bureau of Land Management (BLM) is investigating the use of contraceptives in decreasing wild horse population growth and a long-term or permanent contraceptive vaccine would be ideal. There, however, is currently no vaccine available for inducing permanent sterility in mares following a single vaccination. One area of interest in contraceptive research is regulation of ovarian follicular growth. By targeting specific factors understood to regulate follicular growth and oocyte development, there is potential to prevent ovulation and/or accelerate the depletion of the oocyte reserve, thereby inducing sterility. This comes as a challenge, however, because the exact mechanisms that control follicular growth, especially in the beginning stages of follicular maturation, are not completely understood. Two known regulators of follicular growth

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are Bone Morphogenetic Protein-15 (BMP-15) and Growth Differentiation Factor-9 (GDF-9; Dong et al., 1996; Galloway et al., 2000). These growth factors are exclusive to the oocyte in most species, making them an ideal target for contraceptive research (Juengel and McNatty, 2005). To date, the roles of BMP-15 and GDF-9 in ovarian function of mares have not been reported. Mutations affecting expression of either the *BMP-15* or *GDF-9* gene induce sterility in homozygous mutant sheep, while heterozygotes have increased fertility rates, indicating that altered gene expression for these growth factors have a dramatic impact on fertility (Galloway et al., 2000; Hanrahan et al., 2004). These studies have been replicated through immunization against BMP-15 and GDF-9 in sheep, deer, and cattle and have produced varying results, with some treatments increasing fertility and others inducing sterility (Juengel, 2002; Juengel et al., 2009; Eckery et al., 2014;). The present study, therefore, is aimed to determine effects of immunization against BMP-15 and GDF-9 on ovarian function in the mare. We hypothesize that immunization against these oocyte-specific growth factors will prevent ovulation and/or accelerate the depletion of the oocyte reserve.

2. Materials and methods

2.1. Horse care

All horse use for this project was approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC #15-5984A) and mares were obtained from Abraham Equine Inc. (Canadian, TX). Mares (n = 30) were housed at Colorado State University Equine Reproduction Laboratory (Fort Collins, CO). Horses ranged between 7 and 14 years of age. The mares were maintained on a dry lot pasture and fed grass-alfalfa mix with free choice salt and mineral supplement. All mares had normal reproductive histories (each having had at least two foals in the past 3 years) and were of good physical health.

2.2. Experimental design

Mares were randomly assigned to one of three treatments (n = 10/group). The three groups were identified as BMP-15, GDF-9, and control. Researchers were blinded to groups until termination of the project as to prevent biases in observation. The observation period spanned February 4th, 2016 through September 13th, 2016.

2.3. Immunization protocol

Horses in BMP-15 and GDF-9 groups were vaccinated with the respective peptides conjugated to keyhole limpet hemocyanin (KLH) in Seppic Montanide™ Pet Gel A adjuvant while horses in the control group were vaccinated with adjuvant and phosphate buffered saline. The BMP-15 peptide consisted of a 24 amino acid sequence (QAGSMGSEVLGPSREREGPESNQC) of the mature protein. The GDF-9 peptide was a 14 amino acid sequence (SEYFKQFLFPQNEC) of the mature protein [Celtek Bioscience, Franklin, TN (1st vaccination); Life Technologies Corporation, Carlsbad, CA (2nd, 3rd, 4th vaccinations)]. Both sequences are 100% homologous to mature protein in horses and 80% or 100% homologous to sequences used in studies in sheep and deer for BMP-15 and GDF-9, respectively. Keyhole limpet hemocyanin was used as a carrier protein to improve immunogenicity of peptides. Each vaccination formulation contained 1000 µg of peptide-KLH conjugate in 2 ml volume. Vaccines were administered intramuscularly in the cervical musculature of the neck using a 20-gauge needle. The mares were vaccinated at weeks 0, 6, 12, and 18 relative to the time of the first vaccination with the first vaccination administered on February 4th, 2016. Injection sites were monitored following each vaccination administration to record evidence of vaccination site reactions including local inflammation and/or abscessing.

2.4. Blood sample collection

Blood samples were obtained every other week for 32 weeks in order to measure individual antibody responses. For each sample, 20 ml of jugular venous blood was obtained from each mare using a 20-gauge x 1.5” blood collection needle and two 10 ml blood collection tubes (Medtronic Animal Health; Minneapolis, MN). Following collection, samples were incubated at room temperature for at least 2 h to allow separation of sera and red blood cells. Samples were centrifuged at 2000*g* for 10 min to separate the blood components. Serum was pooled from collection tubes from each mare and aliquoted into 15 ml conical tubes. Serum was then centrifuged for 30 min at 5250*g* to eliminate debris. Samples were divided into 1 ml aliquots and stored at −80 °C until further processing.

2.5. Antibody responses

Serum was used to identify antibody responses to vaccination with either BMP-15 or GDF-9 using enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Santa Cruz Biotechnology, Inc.; Dallas, TX) were coated using 50 µl of a solution containing 500 ng or 250 ng of BMP-15 peptide or GDF-9 peptide, respectively, in carbonate bicarbonate buffer [Celtek Bioscience, Franklin, TN (1st vaccination); Life Technologies Corporation, Carlsbad, CA (2nd, 3rd, 4th vaccinations)]. Plates were incubated overnight at 4 °C and washed three times with 300 µl PBST (0.01 M phosphate buffered saline (PBS) plus 0.05% Tween 20, pH 7.4) per well at room temperature. In each well, 200 µl of a solution of 20% SeaBlock (Thermo Fisher Scientific; Waltham, MA) and 5% Tween 20 in 0.01 M PBS was applied to block non-specific binding sites and plates were incubated for 1 h at 24 °C, followed by another three washes with PBST. Sera was run in duplicate at a dilution factor of 1:5000 or 1:10,000 for GDF-9 or BMP-15, respectively, in 50 µl of 0.01 M PBS

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