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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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ABSTRACT

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

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 2000g 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 5250g 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

and incubated for 1 h at 25 °C. Plates were then washed three times with PBST. Secondary antibody, rabbit anti-horse IgG (Sigma-Aldrich; Saint Louis, MO) diluted 1:5000 in 0.01 M PBS (50 μl) was applied and incubated for 1 h at 25 °C, followed by three washes with PBST. Bound anti-BMP-15 or anti-GDF-9 antibody was detected using 50 μl of horseradish peroxidase conjugated goat-anti-rabbit IgG (Sigma-Aldrich; Saint Louis, MO) diluted 1:5000 in 0.01 M PBS. Samples were incubated for 1 h at 25 °C and washed three times with PBST. Enzyme substrate (3,3',5,5'-tetramethylbenzidine (TMB) dihydrochloride in phosphate citrate buffer with urea H_2O_2; Sigma-Aldrich) was added to each well. After 6 min, 50 μl of 2 M sulfuric acid was added to terminate the reaction. Absorbance of each well was measured at 450 nm and plate background was corrected for by subtracting mean absorbance of all PBS wells from all assay plates. Antibody responses were reported as optical densities. Antibody response thresholds for respective antigens were calculated as the pre-vaccination sample mean plus three standard deviations. Samples with values above threshold value were classified as positive for BMP-15 or GDF-9 antibodies. Samples from control animals were processed on plates coated with either BMP 15 or GDF9. No values from control samples were above the respective thresholds. In addition, samples from BMP15 animals were run on plates coated with GDF9 and samples from GDF9 animals were run on plates coated with BMP15. No values were greater than the respective thresholds indicating there was no cross reactivity between peptide antigens.

2.6. Ovarian activity records

Transrectal ultrasonography was performed three times a week for 32 weeks using a Sonosite M-Turbo ultrasonic system with a 5.0 MHz linear array transducer (Sonosite, Bothell, WA). During each exam, ovarian follicle diameters and appearances were recorded and any abnormal ovarian structures were noted, including hemorrhagic anovulatory follicles, persistent anovulatory follicles, and hemorrhagic corpora lutea. A hemorrhagic anovulatory follicle was defined as a follicle containing echogenic strands within the antral space, with no thickened follicular wall present initially (Ginther et al., 2007; Cuervo-Arango and Newcombe, 2010, 2013). A persistent anovulatory follicle was defined as a follicle with a thickened, highly echogenic wall, with or without echogenic strands within the antrum. A hemorrhagic corpus luteum was noted by an incomplete luteinization of a corpus luteum, resulting in anechoic pockets within the echogenic luteinized body (reviewed by Cuervo-Arango and Newcombe, 2013). Representations of each follicle or corpus luteum type are depicted in Fig. 1. Uterine edema and fluid accumulation were recorded to monitor stage of estrous cycle and incidence of abnormal estrous cycles. Edema was based on a 4-point scale, with 0 representing no uterine folds and a score of 3 describing the presence of very edematous uterine folds (Hayes et al., 1985). Fluid presence within the uterus was described by both quantity and echogenicity. Quantity of fluid was described by either trace, small, medium, or large accumulation within the uterus. Echogenicity was measured on a scale of 0–4, with 0 representing clear fluid with minimal echogenicity, and a score of 4 representing cloudy fluid with high echogenicity (reviewed by Traub-Dargatz and McKinnon, 1988).

2.7. Estrous cycle behavior records

Throughout the breeding season, mares were monitored three times a week to record their sexual receptivity to a stallion, potentially indicating stage in the estrous cycle. Several stallions owned by the Colorado State University Equine Reproduction
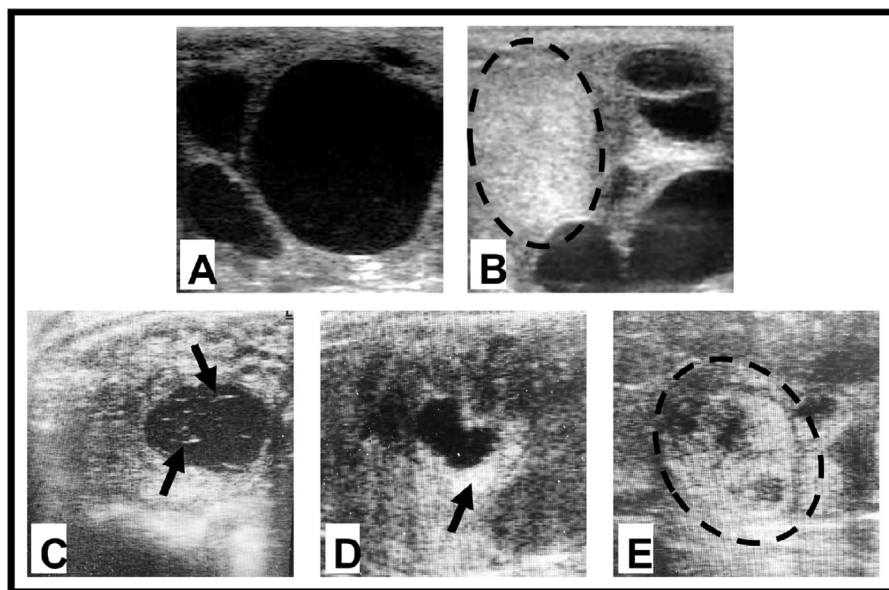

Fig. 1. Identification of follicle types and luteal structures observed during ultrasonic examination of mare ovaries. A) Appearance of a normal follicle. B) Appearance of a normal corpus luteum. C) Hemorrhagic anovulatory follicle, noted by the white strands (↑) within the black antral space. D) Persistent anovulatory follicle, noted by the thickened, highly echogenic wall (↑). E) Hemorrhagic corpus luteum, denoted by the inconsistent echogenicity within the structure.

Table 1

Descriptions of "tease" scores as indicated by the mare's behavior upon interaction with a stallion. Teasing scores are correlated to a status of either "in estrus" or "not in estrus" at each observation.

Status	Tease Score	Description
Not in estrus	0	Combative with pinned ears, a swishing tail, bared teeth, and general discontent
Not in estrus	1	Indifferent toward stallion, neither combative or receptive
In estrus	2	Slightly receptive with raised tail but no urination or squatting posture
In estrus	3	Delayed teasing behavior, with signs of estrus (urination, squatting, raised tail, etc.) occurring only after individual interaction has ended
In estrus	4	Teasing behavior upon individual interaction, including urination, squatting, clitoral winking, posturing, and raised tail
In estrus	5	Intense teasing behavior (urination, squatting, raised tail, etc.) beginning prior to individual interaction with the stallion

Laboratory were utilized in a "rail-teasing" scenario. For "rail-teasing", the mares were arranged in a single file chute and the stallion interacted with each mare individually. Each mare was observed for her behavior and scored on a six-point sexual receptivity scale. A score of 0 indicated that the mare was combative toward the stallion, which was indicated by pinned back ears, swishing tail, bared teeth and general discontent in the stallion's presence. A score of 5 indicated that the mare was very sexually receptive and would show all signs of estrus including squatting, posturing, urinating, and expressing an intense interest prior to interaction with the stallion. Descriptions of scores 0 through 5 are outlined in Table 1. Estrous cycle behavior was analyzed using the "tease" scores recorded throughout the season. To compare days in estrus between groups, a score of 2 or greater was considered an "in estrus" observation.

2.8. Statistical analysis

Ovulation rates and occurrence of abnormal follicles between any two groups were compared using two-tailed, student's t-tests (Microsoft Excel; Microsoft Office Software, Redmond, WA). All other statistical analyses were completed using SAS 9.4 (SAS Institute Inc., Cary, NC). Pre-ovulation follicle sizes were compared using Proc GLM with a Tukey-Kramer adjustment, with days to ovulation as a covariable. Total days observed in estrus was compared across the entire observation period and observations following the first booster vaccination. Both analyses were completed using Proc GENMOD using a Poisson distribution. A Wald Chi-Squared test (F-test) was used for evaluation. Statistical significance for all analyses was denoted as $P \leq 0.05$.

3. Results

3.1. Antibody responses

The mares vaccinated with BMP-15 elicited a consistent positive antibody response according to an average optical density measurement that was greater than the antibody response threshold value following administration of the second vaccination. Average response continued to be greater than the positive threshold for the remainder of the study (Fig. 2A). Average response of GDF-9 vaccinated mares was only greater than the positive threshold after the third and fourth vaccination, decreasing to below the threshold in subsequent observations (Fig. 2B). Local inflammation at the injection site was common following vaccination; however, swelling subsided less than a week post injection. There was no incidence of vaccination site abscesses following any vaccination.

3.2. Ovarian function

Mares in the BMP-15 treatment had fewer ($n = 5.0$) ovulations than controls ($n = 10.0$; $P = 0.024$; Fig. 3). Four of ten mares in the BMP-15 treatment group had one or no ovulations. When evaluating incidence of ovulations in relation to antibody responses in each group, 92% of ovulations in the BMP-15 group occurred after the final vaccination. Ovulation occurrence in mares in the GDF-9 treatment group ($n = 10.8$) was not different from control group ($n = 10.0$; $P = 0.66$; Fig. 3). In control and GDF-9 groups, ovulations occurred in the same frequency throughout the study. The last recorded follicle size before ovulation was different in all treatment groups with an average measurement before ovulation of 21.3 mm for BMP-15 group, 27.8 mm for GDF-9 group, and 35.7 mm for control group ($P < 0.0001$ for both treatments, Fig. 4). Of the mares in the BMP-15 group that had more than three ovulations, 50% of ovulations resulted from follicles measuring less than 30 mm in diameter compared to 9% of control ovulations and 5% of GDF-9 ovulations (Fig. 5). There was a greater incidence of abnormal follicles, hemorrhagic or anovulatory, in both BMP-15 ($P = 0.010$) and GDF-9 ($P = 0.017$) treatment groups compared to control group. Mares with fewer than two ovulations were excluded from this evaluation because there was a lesser presence of adequately sized follicles capable of becoming hemorrhagic or anovulatory. There was no difference in presence of hemorrhagic corpus lutea between groups (Fig. 6).

3.3. Estrous behavior

Upon evaluation of total days observed in estrus, neither the BMP-15 group nor GDF-9 group (21.4 days, $P = 0.09$, and 22.2 days, $P = 0.12$, respectively) differed from controls (25.7 days). When days observed in estrus were evaluated following the second
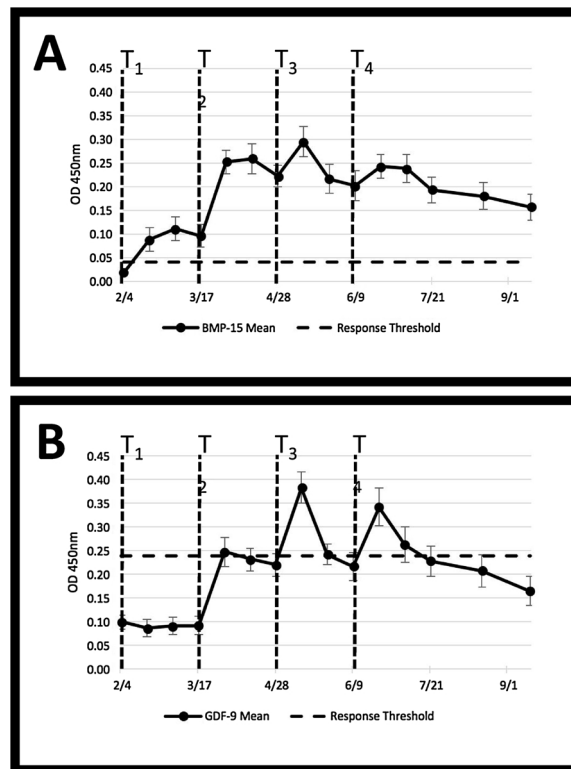
Fig. 2. Mean antibody responses of treatment mares compared to controls, measured by optical density (OD) at 450 nm. The response threshold, represented by the horizontal dashed line, is the pre-vaccination average plus three standard deviations. Vertical dashed lines indicate vaccination dates (T_1, T_2, T_3, T_4). Error bars represent standard error of the mean. A) BMP-15 vaccinated mares and controls. B) GDF-9 vaccinated mares and controls.
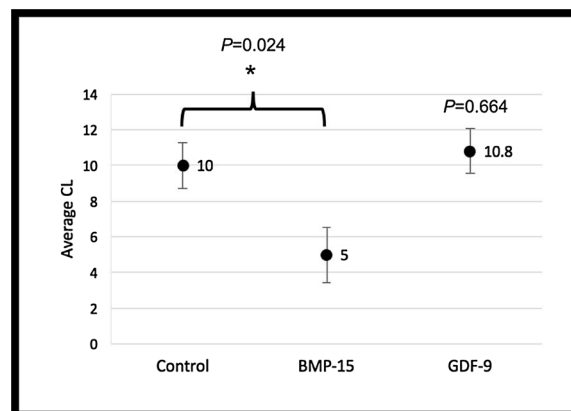

Fig. 3. Average number of ovulations indicated by number of corpora lutea in each treatment group. Error bars indicate standard error of the mean and significance at $P < 0.05$ when compared to controls is denoted by an asterisk.

immunization, total days in estrus for both the BMP-15 group (21.0 days, $P = 0.05$) and GDF-9 group (21.1 days, $P = 0.03$,) were less when compared to controls (25.7 days).

4. Discussion

Ovarian function was altered in both GDF-9 and BMP-15 treatment groups. Although the GDF-9 treatment did not affect ovulation rates, altered estrous behavior, a decreased pre-ovulation follicle size and an increased incidence of abnormal follicles indicate treatment did have an effect on follicular growth. Variable antibody responses in the majority of GDF-9 vaccinated mares may indicate a limited capacity of the vaccine to elicit an immune response to GDF-9 protein used in this study. The peptide sequence used in the vaccine formulation, therefore, may not induce antibodies that readily bind epitopes on the mature GDF-9 protein of mares
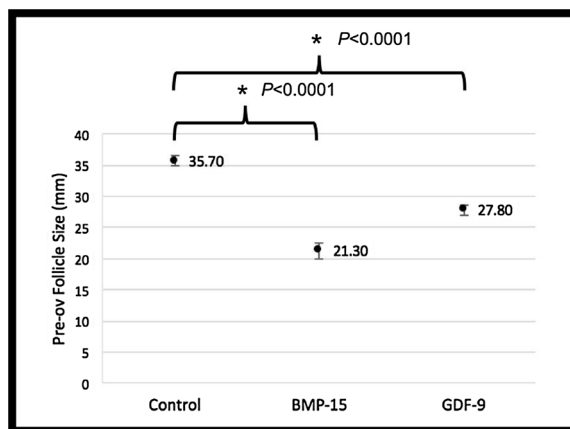
Fig. 4. Least squares mean pre-ovulation follicle size for each treatment, measured in millimeters. Significance at $P < 0.0001$ when compared to controls is denoted for each value with an asterisk and error bars indicate standard error of the mean.

(Murphy et al., 2008). The peptide sequence used in this study, however, was 100% homologous to sequences used in other studies where the reported fertility was reduced following immunization against GDF-9 in deer and sheep (Eckery et al., 2014; McNatty et al., 2007). Eckery et al. (2014) reported that deer immunized with the same GDF-9 peptide did not have altered fertility rates in the first year, but became infertile in the second and third years subsequent to the time immunizations were initiated. It is possible that a similar delay in effect will be observed in mares. This may have been due to an incomplete deactivation of GDF-9 in the first year, but complete inactivation in subsequent years. Additionally, even though there was no change in ovulation rates of GDF-9 vaccinated mares, immunization could still be successful in depleting oocyte reserves in these animals as indicated by an increased number of abnormal follicles reported. Because recruitment of follicles is an irreversible physiological function, follicles that begin to grow but there is a failure of ovulation occurring as a result of follicles becoming hemorrhagic or anovulatory still contribute to there being one less oocyte in the reserve pool of primordial follicles (Gastal et al., 1997). If GDF-9 vaccinated mares, therefore, are recruiting more follicles to grow, even though these follicles become abnormal, there would be accelerated depletion of the oocyte reserve of primordial follicles, potentially resulting in permanent sterility in the future. Further research is needed to confirm whether there is a delayed effect on fertility or an increased depletion rate of the oocyte reserve in vaccinated mares.

The BMP-15 treatment induced a consistently positive antibody response after the second immunization, indicating a classic immune response to the peptide used. Additionally, altered ovarian activity and decreased ovulation rates suggest that antibodies created in response to immunization were effective in binding and inactivating the BMP-15 protein in mares. Results of the present study for BMP-15 treatment are similar to those reported in other BMP-15 immunization studies in sheep and cattle, with an overall decrease in ovulations and some animals having a complete cessation of ovulations (Juengel, 2002; Juengel et al., 2009, 2004; McNatty et al., 2007). Four mares in this group had only one or no ovulations, whereas mares with several ovulations had a relatively greater incidence of abnormally small follicles from which there were ovulations. This is similar to reports by Juengel et al. (2009) where it was reported that some vaccinated cattle failed to have ovulations, while the animals with multiple ovulations appeared to have ovulations from smaller than average follicles. In deer immunized against BMP15, ovulation rate (as indicated by fawns/doe born) was increased over three breeding seasons even though animals had a significant immune response. It, however, is not known if there were ovulations from follicles with a smaller diameter. Immunization against BMP-15 in horses, therefore, appeared to have a similar effect on ovarian function as cattle and sheep. It may be possible that the ovulations from abnormally small follicles in mares vaccinated with BMP-15 would not have resulted in fertility had the mares been bred because the average size of follicles from which ovulations occur in horses is 35 to 50 mm in diameter (Hughes et al., 1975). Follicles smaller in diameter may not yet have the appropriate ovulatory factors or contain oocytes that are mature enough for fertilization to occur. There, however, is evidence in sheep that smaller follicles contain viable oocytes and offspring can result if ovulations occur from these smaller follicles (McNatty et al., 2017) and similar findings have been reported in the mare (Bruck et al., 1997). If ovulation from the small follicles containing these oocytes occurs prematurely in mares, fertility may not occur, therefore, immunization against BMP-15 would be an effective contraceptive even though ovulations occur in vaccinated horses. Fertility trials in vaccinated animals, however, are required to come to definitive conclusions as to treatment effectiveness. Also, it is possible that the small luteal structures observed with ultrasonic examination in the present study are a result of a spontaneous luteinization of the follicle, without ovulation occurring from the follicle and release of the oocyte. This occurs in sheep immunized with BMP-15, with luteinized structures still containing an oocyte being present in the ovaries (McNatty et al., 2007). Further investigation into the histological appearance of these structures on the ovaries of vaccinated mares, therefore, is required to determine whether corpora lutea that were detected by ultraonogaphy resulted from an ovulation occurring or from spontaneous luteinization of an intact follicle.

Relatively greater incidences of hemorrhagic and anovulatory follicles in BMP-15 vaccinated mares also indicated altered follicular maturation, indicating rates at which follicles are recruited may also be affected; however, subsequent studies are required to determine this effect. In BMP-15 vaccinated mares, 92% of ovulations occurred after the final vaccination when antibody titers were decreasing. This may be a limitation of the vaccine used in the present study until the fertilization capacity of these oocytes is
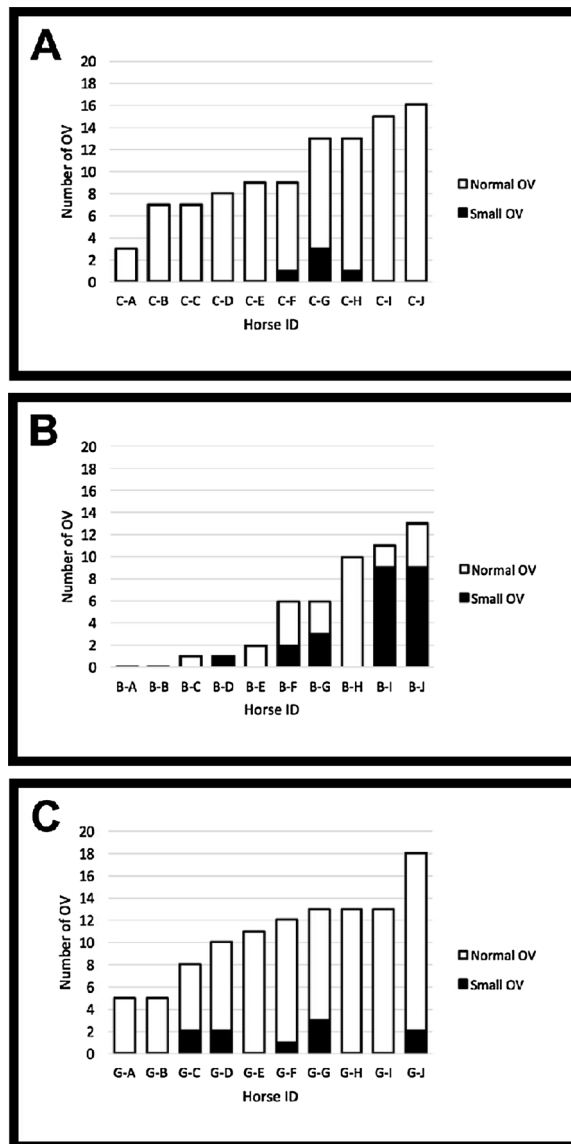
Fig. 5. Number of ovulations for each mare in each treatment categorized by either small or normal ovulations. A) Ovulation occurrence for control mares. B) Ovulation occurrence for BMP-15 vaccinated mares. C) Ovulation occurrence for GDF-9 vaccinated mares.
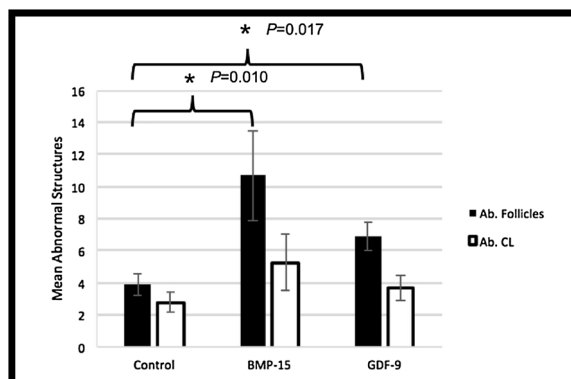

Fig. 6. Occurrence of abnormal follicles (hemorrhagic or anovulatory) and abnormal corpus lutea per treatment group. Significance of $P < 0.05$ when compared to controls is denoted by an asterisk. Error bars denote standard error of the mean.

assessed. These results suggest that there is an optimal antibody titer required to induce an anovulatory state in mares, and when antibody responses are greater than this threshold, immunization against BMP-15 will be successful as a contraceptive by preventing ovulation.

Following the second vaccination, both the GDF-9 and BMP-15 vaccinations altered estrous behavior with vaccinated mares observed in estrus fewer days than control mares. This would be expected in the BMP-15 group because this treatment also decreased ovulation rates. It, therefore, is anticipated that with fewer ovulations, mares have there will be fewer large follicles secreting estrogen, decreasing the effects of estrogen on inducing estrous behavior. Further research to determine the hormone concentrations in GDF-9 vaccinated mares may indicate altered progesterone or estrogen concentrations resulting from the greater incidence of abnormal follicles, which would explain the altered estrous behaviors in this group. The impact of altered behavior on wild horse herd dynamics as a result of contraceptive administration is a topic of consideration for the BLM (BLM, 2005). Further research must be conducted to determine the extent of decreased estrous behavior in both treatment groups; however, it should be considered that a decreased incidence of estrous behavior is normal in wild horse herds because mares most often become pregnant early in the breeding season.

5. Conclusions

Immunization against BMP-15 and GDF-9 both led to altered ovarian function in mares in the present study. Although GDF-9 vaccinated mares did not have lesser ovulation rates in the first year of vaccinations, follicular maturation and estrous behaviors were altered, with increased incidences of abnormal follicles and smaller pre-ovulation follicle size. Further research is required to determine if greater incidences of abnormal follicles leads to a decrease in follicular reserve and if ovulation rate is less in vaccinated mares in subsequent years of treatment. Immunization against BMP-15 significantly decreased ovulation rate and follicle size at ovulation in vaccinated horses, which highlights potential success of vaccination against BMP-15 as a contraceptive in mares. Investigation into the fertility of BMP-15 and GDF-9 immunized mares is required to determine if the greater incidence of abnormal follicles and abnormally small pre-ovulation follicle size contributes to a reduction in fertility or a depletion of the oocyte reserve.

Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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